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MAPPING OF THE GENES AROUND MEN2A LOCUS USING PULSED-FIELD GEL ELECTROPHORESIS

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Summary The gene for multiple endocrine neoplasia type 2A (MEN 2A) is closely linked to RBP3 (retinol-binding protein 3, interstitial, probe IRBP.H4) and the DNA marker D10S15 (probe pMCK2), which have been assigned to the proximal long arm of chromosome 10 by linkage analysis both in Caucasian and Japanese populations. We have constructed a rare-cutting restriction map around the RBP3 and D10S15 loci by pulsed-field gel electrophoresis (PFGE). The RBP3 and D10S15 loci appeared to be within a single 160 kb *Mlu*I fragment. In 5 patients with MEN 2A, gene rearrangements, such as a gross deletion, were not found in the 880 kb *Nru*I fragment which covered the closest region to the MEN-2A locus from the RBP3 and D10S15 loci.

Key Words multiple endocrine neoplasia type 2A (MEN 2A), pulsed-field gel electrophoresis

INTRODUCTION

Multiple endocrine neoplasia type 2A (MEN 2A) is an autosomal dominant disease characterized by association of medullary thyroid carcinoma, pheochromocytoma and hyperplasia of parathyroid glands. The gene has been mapped to the pericentromeric region of chromosome 10 by the familial linkage studies between the MEN2A and the RBP3 (retinol-binding protein 3, interstitial) loci (Mathew *et al.*, 1987; Simpson *et al.*, 1987; Yamamoto *et al.*, 1989).

Updated lod score data suggested very tight linkage between the MEN2A locus and the markers, IRBP.H4 (RBP3) and pMCK2 (D10S15): a maximum lod score (Z_{max})=18.10 at theta=0.024 for RBP3 and Z_{max}=19.48 at theta=0.011

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for D10S15 (Nakamura *et al.*, 1989), or $Z_{max}=13.1$ at theta=0.045 for RBP3 and $Z_{max}=17.3$ at theta=0.054 for D10S15 (Wu *et al.*, 1990). Nakamura reported that there is no definite crossover between RBP3 and D10S15 (Nakamura *et al.*, 1989), while Wu observed two obligate crossovers between two loci (Wu *et al.*, 1990). Although these three markers are linked together, it is difficult to estimate the accurate distance among them.

We therefore have constructed the rare-cutting restriction map in the MEN2A region using pulsed-field gel electrophoresis (PFGE) which is a powerful technique for analysis of large genomic regions.

MATERIALS AND METHODS

To prepare high molecular weight DNA for PFGE, lymphocytes isolated from fresh whole blood or lymphoblastoid cells transformed by Epstein-Barr virus are suspended in low melting point agarose (Smith *et al.*, 1986). The blocks containing 3×10^6 cells per 100 μl are treated for 48 hr at 50°C with buffer of 0.5 M EDTA and 2 mg/ml proteinase K. After washing the blocks in 1×TE buffer (10 mM Tris, 1 mM EDTA), digestion by rare-cutting restriction enzymes was performed overnight. Then, the blocks were placed in a 1.0–1.5% agarose gel. The pulsed-field gel system used is a cross-field gel electrophoresis produced by ATTO Corporation (Tokyo). The system alternates the field by rotating the gel. To obtain optimal resolution of DNA fragments between 50 to 1,000 kb, gels were run for 24–72 hr at 100–200 V, with a constant switching interval from 20 to 300 sec. After electrophoresis, the ethidium bromide-stained gels were exposed to short-wave UV light and treated with 0.25 M HCl (twice for 15 min) (Maniatis *et al.*, 1982). The DNA probes used were the cloned cDNA for RBP3 (retinol-binding protein 3, interstitial), IRBP.H4 (Liou *et al.*, 1987), and an anonymous genomic clone pMCK2, which defines the D10S15 locus (Nakamura *et al.*, 1988). The DNA probes were radioactively labeled by random priming to a specific activity of 10^9 dpm/μg (Feinberg and Vogelstein, 1983). The gels were denatured and transferred to nylon membranes (Hybond, Amersham) according to manufacturer's instructions and hybridized as previously described (Nishisho *et al.*, 1986). After autoradiography, probes were removed from the filter by washing in 0.4 N NaOH at 42°C for 30 min and in buffer containing 0.2 M Tris-HCl, 0.1×SSC and 0.5% SDS for 30 min.

RESULTS

The long-range restriction map for normal DNA samples was established using the restriction nucleases *Not*I, *Mlu*I, *Nru*I and *Sal*I. Figure 1 shows the autoradiographs obtained after the same PFGE filter was hybridized with the RBP3 and D10S15 probes. In this filter, IRBP.H4 detected two *Not*I fragments of 400

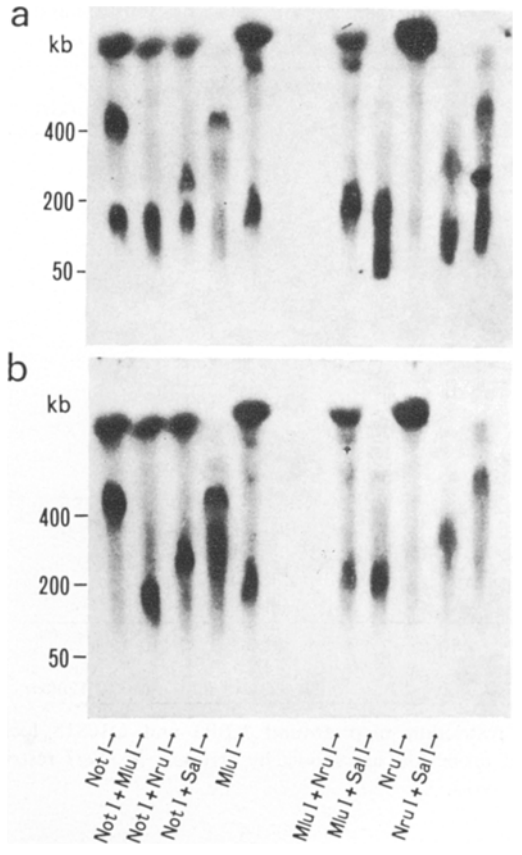

Fig. 1. PFGE analysis of human genomic DNA hybridized with IRBP.H4 (a) and subsequently with D10S15 (b). Digested enzymes were shown under the each lane. The sizes of DNA are shown on the left side of each autoradiograph. The 80 kb *Not*I+*Sal*I (a) and the 220 kb *Sal*I fragment (b) were faint in this filter.

and 100 kb, while pMCK2 detected two *Not*I fragments of 400 and 300 kb. IRBP.H4 and pMCK2 showed the same 160 kb *Mlu*I fragment. Moreover, both probes detected the same 880 kb *Nru*I fragment. IRBP.H4 detected a 80 kb *Sal*I fragment, and pMCK2 a 220 kb *Sal*I fragment, which was the faint band in this filter. More than one band of hybridization were detected in the cases of *Not*I, *Mlu*I, and *Sal*I digestions. These fragments might have been produced by partial digestion, variable methylation, or restriction fragment length polymorphisms (RFLPs). Partial digestion was excluded by experiments in which decreasing the amount of enzymes gave rise to the same fragments. The fragments with the same size were observed in the unrelated individuals, so these fragments probably do not result from RFLPs. The most reasonable explanation is that variable methylation might cause these fragments. A summary of the smallest

Table 1. Summary of restriction fragment sizes identified by IRBP.H4 and pMCK2. In all cases, the smallest fragments were shown.

	IRBP.H4 (kb)	pMCK2
*Not*I	100	300
*Mlu*I	160	160
*Nru*I	880	880
*Sal*I	80	220
*Not*I+*Mlu*I	80	80
*Not*I+*Nru*I	100	240
*Not*I+*Sal*I	70	180
*Mlu*I+*Nru*I	160	160
*Mlu*I+*Sal*I	50	120
*Nru*I+*Sal*I	80	220

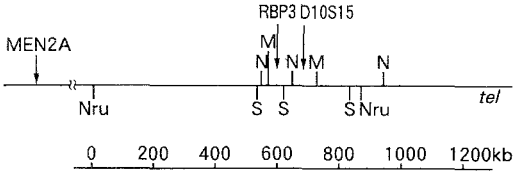

Fig. 2. Long-range restriction map around RBP3 and D10S15 loci. The positions of MEN2A and probe loci are shown by arrows. N, *Not*I restriction site; M, *Mlu*I; S, *Sal*I; Nru, *Nru*I.

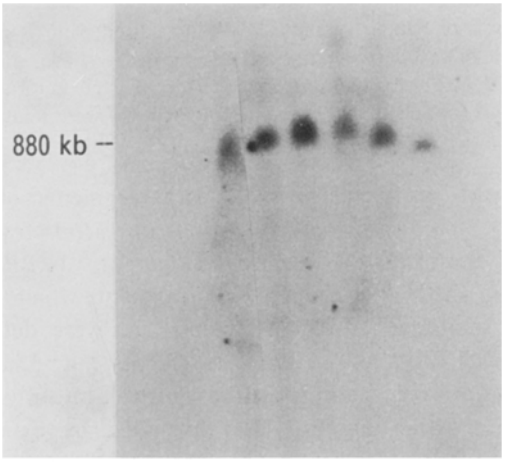

Fig. 3. PFGE analysis of MEN 2A patients. *Nru*I-digested DNAs from the MEN 2A patients (lanes 2–6) and control (lane 1) were hybridized with the probe IRBP.H4.

restriction fragments of single and double digestion of these enzymes is presented in Table 1. We have constructed a rare-cutting restriction map around the RBP3 and D10S15 loci (Fig. 2). Both the RBP3 and D10S15 loci are localized within a single 160 kb *Mlu*I fragment. Restriction sites for *Not*I, *Mlu*I, and *Sal*I cluster in a small region close to the RBP3 locus. The map orientation in Fig. 2 was deduced from the linkage data showing RBP3 is probably closer to MEN2A than is D10S15 (Wu *et al.*, 1990). DNA samples from a normal individual and 5 patients with MEN 2A have been digested with *Nru*I and hybridized with IRBP.H4, because the 880 kb *Nru*I fragment might cover the region closest to the MEN2A locus. However, all of them showed only one 880 kb band without altered bands (Fig. 3).

DISCUSSION

Once the linkage map around the gene for a genetic disease has been constructed, the next important step is to refine the physical map around this genetic locus and to analyze DNA from patients in order to find the genetic rearrangement in this region by the application of long-range restriction techniques, what is called PFGE. PFGE has proven very useful in the molecular genetic analysis of Duchenne muscular dystrophy and cystic fibrosis. Some patients with Duchenne muscular dystrophy or hemophilia A have shown deletion in the region of the responsible gene as shown by PFGE (Kunkel *et al.*, 1986; Youssoufian *et al.*, 1987). The physical map which we have made around the RBP3 and D10S15 loci by PFGE demonstrates that the molecular distance between RBP3 and D10S15 is within 160 kb. This result is consistent with the familial linkage data which showed that there were no obligate recombinations observed between them (Nakamura *et al.*, 1988) or only two recombination between them (Wu *et al.*, 1990). The possibility that the MEN2A locus would be located within a few megabases from RBP3 and D10S15 encouraged us to analyze DNA from five patients with MEN 2A using the DNA probe RBP3 after digestion by *Nru*I, because the 880 kb *Nru*I fragment covered the region nearest to the MEN2A locus from the RBP3 and D10S15 loci. However, we could not detect any altered fragments that would have suggested a deletion or insertion in them. We are going to analyze DNA from other patients with MEN 2A and are trying to find other enzymes which cover a longer fragment extending toward MEN2A from the RBP3/D10S15 locus.

In this paper, we constructed the physical map only around the RBP3 locus, but in the near future, we will be able to construct a long range map containing the MEN2A locus by using new markers.

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