Molecular cloning and DNA sequence analysis of preproinsulin genes in the NON mouse, an animal model of human non-obese, non-insulin-dependent diabetes mellitus

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ABSTRACT

Two insulin genes of the NON mouse, an animal model of human non-obese, non-insulin-dependent diabetes mellitus, were isolated and characterized to examine the hypothesis that these genes are structurally different from those of normal mice. The NON mouse was found to have two non-allelic insulin genes, as does the normal mouse, and no structural differences were found between the normal and NON mouse in the nucleotide sequence of the insulin gene, including that of the 5'-transcriptional regulatory region, and in the deduced amino acid sequence. There was, however, an additional 113 bp sequence and seven point mutations in a further 5'-flanking region, and three point mutations in the 3'-flanking region of the insulin II gene. We conclude that reduced expression of insulin genes in the NON mouse is not due to the structural change in the known transcriptional regulatory region, although the effect on insulin II gene expression of an additional sequence upstream of the 5'-flanking region, as the negative regulatory factor, remains to be elucidated.

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INTRODUCTION

The NON mouse is a subline separated from the Jcl-ICR mouse, as is the NOD mouse. The NOD mouse has been known as an animal model for insulin-dependent diabetes mellitus, whilst the NON mouse shows no glycosuria, but slight hyperglycaemia on fasting (Makino, Kunimoto, Muraoka et al. 1980; Tochino, 1986; Eisenbarth, 1988). In previous studies we have characterized the NON mouse as follows: (1) glucose intolerance without showing overt diabetes, (2) hypoinsulinaemia (Ohgaku, Morioka, Sawa et al. 1988) and (3) no pathological findings, including autoimmune changes in pancreatic islets and the thyroid gland (S. Ohgaku, H. Morioka, & S. Yano, unpublished data). Thus we have proposed that the NON mouse is an animal model suitable for studying human non-obese, non-insulin-dependent diabetes mellitus. Furthermore, we have demonstrated lower insulin contents and remarkably reduced contents of preproinsulin mRNA in the pancreas of the NON mouse. Analysis of restriction fragment length polymorphism of the insulin gene in NON, NOD, and Jcl-ICR mice revealed an extra band with weak intensity only in the NON mouse after BamHI digestion (Ohgaku et al. 1988). From these results we have proposed a hypothesis that the NON mouse has a defect which induces glucose intolerance by reduced expression of the insulin gene before translation, and that the defect is caused by structural changes in the insulin gene especially in its transcriptional regulatory region. In the present study we isolated two non-allelic insulin genes from the NON mouse and determined their nucleotide sequences to verify this hypothesis.

MATERIALS AND METHODS

Mice

NON mice were provided from Shionogi Research Laboratories, Osaka, Japan.
Materials

N-Lauroyl sarcosine, sodium salt, was obtained from Sigma Chemical Co., St Louis, MO, U.S.A. Proteinase K was from E. Merck, Darmstadt, F.R.G. [α-³²P]dCTP (111 TBq/mmole) was purchased from ICN Chemicals, Irvine, CA, U.S.A., and [α-³²P]dATP (24·1 TBq/mmole) from Amersham International plc, Amersham, Bucks, U.K. Deoxyribonuclease I was from Pharmacia LKB Biotechnology AB, Uppsala, Sweden. MboI and BstXI were from New England Biolabs, Beverly, MA, U.S.A. Other restriction endonucleases and DNA modifying enzymes were from NIPPON GENE, Toyama, Japan. Rat preproinsulin I cDNA was a generous gift from Dr H. Okamoto, Department of Biochemistry, Tohoku University School of Medicine, Japan, who cloned and analysed its sequence (S. Takasawa, H. Yamamoto, & H. Okamoto, unpublished data).

Extraction of high-molecular-weight DNA

High-molecular-weight DNA was isolated from the mouse liver as described elsewhere (Cox, Damjanov, Abanobi & Sarma, 1973) with slight modifications. After the mice were killed by decapitation under ether anaesthesia, their livers were immediately excised and rinsed in 0·9% (w/v) NaCl. About 1 g of the tissue was put into nylon mesh (EIKEN, Tokyo, Japan), immersed in 7 ml ice-cold 0·5 M EDTA (pH 8·0) and squashed gently with the blunt end of a spatula on ice. Remaining connective tissue in the mesh was removed. Liver cells were suspended in 10 ml 0·5 M EDTA (pH 8·0) in a 50 ml Falcon polypropylene tube, and lysed by addition of 0·1 ml proteinase K (10 mg/ml) and 0·25 ml 20% (w/v) sodium lauryl sarcosinate. After overnight incubation at 50 °C, DNA was extracted three times with phenol/chloroform/isoamylalcohol (25:24:1, by vol.) and subjected to dialysis until light absorption of the dialysate was less than 0·05 at 270 nm.

Construction of the genomic library of the NON mouse

After partial digestion of high-molecular-weight DNA with MboI, 20 kb DNA fragments were isolated by sucrose gradient ultracentrifugation, ligated with λDASH/BamHI arms (Stratagene, San Diego, CA, U.S.A.) and assembled into phage using an in-vitro packaging kit (Amersham) and a standard protocol (Maniatis, 1982).

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Isolation and subcloning of the genes encoding insulin I and II from the genomic library of the NON mouse

Using rat preproinsulin I cDNA labelled with ³²P by nick translation as a probe, approximately 6 × 10⁵ plaque-forming units (pfu) phases of the amplified NON liver genomic library were plated on a lawn of Escherichia coli 803 supF, and screened by plaque hybridization as described by Benton & Davis (1977) with slight modifications as follows. Nitrocellulose replicas were incubated at 45 °C overnight with prehybridizing solution containing 50% (v/v) deionized formamide, 50 mM Tris–HCl (pH 8·0), 1 M NaCl, 0·1% (w/v) sodium dodecysulphate (SDS), 5 mM EDTA, 4 mM sodium phosphate, 5 × Denhardt’s solution (1 × Denhardt’s is 0·02% (w/v) polyvinylpyrrolidone, 0·02% (w/v) ficoll and 0·2% (w/v) bovine serum albumin) and 200 µg sonicated, heat-denatured salmon testis DNA/ml. They were then hybridized with the probe labelled with ³²P in the above solution at 45 °C for 24 h. After hybridization, nitrocellulose filters were washed at 45 °C in a solution containing 10 mM Tris–HCl (pH 8·0), 25 mM NaCl, 0·1% SDS, 1 mM EDTA, 1 mM sodium phosphate and 1 × Denhardt’s solution for a total of 6 h, with several changes of the washing solution. The positive plaques were picked up and subjected to secondary and tertiary screening, and phage DNA was prepared by glycerol step-gradient centrifugation (Maniatis, 1982). For restriction mapping and subcloning, prepared phage DNA was digested with EcoRI and BamHI, separated by agarose gel electrophoresis and transferred to a nitrocellulose filter. The filter was hybridized to rat preproinsulin I cDNA probe as described above. DNA fragments thus examined and containing the putative preproinsulin gene were subcloned into Bluescript KS(+) plasmid (Stratagene). Plasmid DNA of the subclone was propagated in E. coli MC1061 and isolated by alkaline lysis and polyethylene glycol precipitation methods (Brush, Dodgson, Choi et al. 1985).

Construction of deletion mutants and nucleotide sequence determination

After the preparation of plasmid subclones with DNAs in both orientations, deletion mutants were prepared according to the method of Henikoff (1984). The mutant subclones which contained appropriate deleted DNAs were selected and plasmids again prepared by alkaline lysis and polyethylene glycol precipitation.

DNA sequencing was accomplished by the dideoxy chain-termination method (Sanger, Nicklen & Coulson, 1977) using synthetic primers to T7
promoter, alkali-denatured plasmid DNAs and \([\alpha-^{35}S]\)dATP. We have used 7-deaza-dGTP (Boehringer Mannheim GmbH, Mannheim, F.R.G.) instead of dGTP in the sequencing to avoid secondary structure problems (Mizusawa, Nishimura & Seela, 1986).

RESULTS

We constructed a \(1 \times 10^6\) pfu NON mouse genomic library, which was sufficient to isolate a particular single-copy sequence of genomic DNA. After amplification of the library, about \(6 \times 10^5\) pfu were screened with rat preproinsulin I cDNA as a probe. After a third screening, five positive clones were obtained. These were analysed by Southern analysis, and two independent clones chosen. In one clone, \(\lambda Nins-I\), the probe hybridized with an EcoRI fragment of 1-4 kbp, whilst in the other, \(\lambda Nins-II\), the probe hybridized with a BamHI fragment of 2-5 kbp. From comparison of restriction maps of the BALB/c mouse preproinsulin genes (Wentworth,}

![Restriction maps and sequencing strategies of the subclones (a) pNins-I and (b) pNins-II, encoding the NON mouse preproinsulin I and II genes respectively. Nucleotide numbers are given above each map. The deduced restriction endonuclease sites are shown, and exons are indicated by solid boxes. Horizontal arrows indicate the sequencing strategy.](image-url)
Schaefer, Villa-Komaroff & Chirgwin, 1986) we considered that λNins-I and λNins-II clones carried putative NON mouse preproinsulin I and II genes respectively.

For further restriction mapping and sequencing analysis, we constructed the plasmid subclone pNins-I, with a 1.4 kbp EcoRI fragment from λNins-I, and we also constructed pNins-II, with a 2.5 kbp BamHI fragment from λNins-II. The restriction maps and sequencing strategies for the NON mouse pNins-I and -II subclones are shown in Fig. 1.

Figure 2 shows the nucleotide and deduced amino acid sequences of pNins-I encoding the putative NON mouse preproinsulin I gene. After comparison with the preproinsulin I gene of the BALB/c mouse, we suggested that the putative transcription initiation site was present at A in position 648. The 5'-flanking region (positions 1–647), which includes the TATA box (position 624), CAAT box (position 579), enhancer core sequence (position 339), 'Nir box'-like sequence and 'Far box'-like sequence (positions 542 and 416 respectively) as previously reported (Karlsson, Edlund, Moss et al. 1987), was identical to that of the BALB/c mouse preproinsulin I gene. The other regions: exon 1 (positions 648–693), exon 2 (812–1209) including the polyadenylation signal (position 1190), intron (694–811) and the 3'-flanking region, were also identical to

![Nucleotide sequence](image)

**Enhancer core sequence**

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attaggctcaataacatataagaattttctgaccttatatatctatgtcaggtatatattttggtttgttggttaatgtgaagaag
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**Far box like sequence**

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gaattttctgaccttatatatccatgacagactaggtagatatattttggtttgttggttaatgtgaagaag
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**Nir box like sequence**

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cacagggtaactttggaacagcctgtcacagcttgggaactcgagttattttggtttgttggttaatgtgaagaag
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**CAAT box**

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taatagttttctgaccttatatatccatgacagactaggtagatatattttggtttgttggttaatgtgaagaag
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**TATA box**

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gaattttctgaccttatatatccatgacagactaggtagatatattttggtttgttggttaatgtgaagaag
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**Figure 2.** Nucleotide and deduced amino acid sequence of the NON mouse preproinsulin I gene. Capital letters indicate exons and lower-case letters are used for introns and 5'- and 3'-flanking sequences. The number of nucleotide residues is indicated at the end of each line. The deduced amino acid sequence is given below the nucleotide sequence. The stop codon is indicated by three asterisks. The sequences of putative promoter, enhancers and the poly(A) signal are underlined.

**Figure 3.** Nucleotide and deduced amino acid sequence of the NON mouse preproinsulin II gene. Capital letters indicate exons and lower-case letters are used for introns and 5'- and 3'-flanking sequences. The numbering of nucleotide residues is indicated at the end of each line. The deduced amino acid sequence is given below the nucleotide sequence. The stop codon is indicated by three asterisks. Nucleotide differences found in the BALB/c sequence are displayed beneath the NON mouse sequence and indicated by arrows. Deletions are indicated by dashes. The sequences of putative promoter, enhancers and the poly(A) signal are underlined.

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those of the BALB/c mouse preproinsulin I gene. The deduced amino acid sequence of NON mouse preproinsulin I was identical to that of BALB/c mouse preproinsulin I.

The structure of pNins-II, encoding the putative NON mouse preproinsulin II gene, is shown in Fig. 3. The putative transcription initiation site was present at A in position 1061. The 5'-transcriptional regulatory region (from transcription initiation site to about 350 bp upstream) was identical to that of the BALB/c mouse preproinsulin II gene. Further upstream, however, near the BamHI subcloning site, seven point mutations were found: five insertions at positions 156, 237, 264, 361 and 374, one substitution at position 315 and one deletion between positions 124 and 125. Also, from positions 1 to 113 (1061 to 948 bp upstream of the transcriptional initiation site), the NON mouse preproinsulin II gene had an additional 113 bp sequence which was absent in the BALB/c mouse gene. Sequences for exon 1 (positions 1061–1106), exon 2 (1225–1427), exon 3 (1916–2115) including the polyadenylation signal (position 2096), intron 1 (positions 1107–1224) and intron 2 (1428–1915) were all identical to those of the BALB/c mouse preproinsulin II gene. Three point mutations were found in the 3'-flanking region: one insertion at position 2495, one substitution at position 2365 and one deletion between positions 2391 and 2392. The deduced amino acid sequence of NON mouse preproinsulin II was identical to that of BALB/c mouse preproinsulin II.

DISCUSSION

The present study has shown that the NON mouse has two non-allelic preproinsulin genes, I and II, as does the normal BALB/c mouse, and that sequences for exons and introns of both preproinsulin genes in the NON mouse are identical to those of the BALB/c mouse. Thus we conclude that the NON mouse does not have a defect in the exons and introns of its preproinsulin genes, and that NON mouse preproinsulin mRNAs do not encode abnormal insulin proteins, as reported in several cases of human diabetes (Haneda, Chan, Kwok et al. 1983; Kwok, Steiner, Rubenstein & Tager, 1983; Nanjo, Sanke, Miyano et al. 1986). Furthermore, this study has confirmed our preliminary observations that the NON mouse insulin I and II proteins are identical to those of the normal mouse when analysed by reversed-phase high-performance liquid chromatography (data not shown).

The present study has also shown that nucleotide sequences located within about 350 bp upstream of the 5'-flanking regions of both preproinsulin genes are not structurally different in NON and BALB/c mice. We suggest that these regions are transcriptional regulatory regions of both preproinsulin genes, which are needed for tissue-specific expression of the insulin gene in the β cells of pancreatic islets, as reported in other mammalian preproinsulin genes (Walker, Edlund, Boulet & Rutter, 1983; Hanahan 1985). In these regions we found putative structures for promoters, which include the TATA box and CAAT box, and enhancers, which include the enhancer core sequence and putative insulin gene-specific enhancer sequences: Nii box and Far box. Recently, it has been demonstrated that artificial mutations in some of these promoters and enhancers reduce insulin gene expression (Karls& en et al. 1987; Crowe & Tsai, 1989; Hwung, Crowe, Peyton et al. 1989). Because nucleotide sequences for these promoters and enhancers in both preproinsulin genes of the NON mouse are identical to those of the BALB/c mouse, we suggest that reduced expression of the preproinsulin genes is not due to the structural change in these elements. Also, since the NON mouse has no internal BamHI site in its preproinsulin I and II genes, the 1.7 kbp extra band with weak intensity observed in the NON mouse would appear to be a false positive.

Several mutations are found in a further 5'-flanking region and in the 3'-flanking region. In particular, there is an additional 113 bp sequence upstream of the 5'-flanking region which has not been reported in the BALB/c mouse. This sequence may be similar to the polymorphic region reported in the human preproinsulin gene (Bell, Karam & Rutter, 1981; Bell, Selby & Rutter, 1982) and in the rat preproinsulin I gene (Winter, Beppu, MacLaren et al. 1987). The relationship between the length of the polymorphic region and the occurrence of diabetes in man has been discussed previously (Rotwein, Chyn, Chirgwin et al. 1981; Bell, Horita & Karam, 1984). It has also been reported that this polymorphic region is not essential for insulin gene expression (Hanahan, 1985) and does not have a functional role in insulin secretion (Permu&t, Rotwein, Andreone et al. 1985). The possibility that this polymorphic region regulates insulin gene expression, however, has not been entirely excluded in man (Takeda, Ishii, Seino et al. 1989). In the rat it has been demonstrated that a negative transcriptional regulatory ('silencer') element is present between 2.0 and 4.0 kbp upstream of the preproinsulin I sequence (Laimins, Holmgren-Koenig & Khoury, 1986). Thus it is possible that an additional sequence upstream of the 5'-flanking region of the NON mouse preproinsulin II gene has negative regulatory activity in transcription of the NON mouse preproinsulin II gene. Expression studies using the additional sequences are needed to elucidate this possibility.

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REFERENCES


