

# Characterization and variation of a human inwardly-rectifying K-channel gene (KCNJ6): a putative ATP-sensitive K-channel subunit

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**Abstract** The ATP-sensitive K-channel plays a central role in insulin release from pancreatic  $\beta$ -cells. We report here the cloning of the gene (KCNJ6) encoding a putative subunit of a human ATP-sensitive K-channel expressed in brain and  $\beta$ -cells, and characterisation of its exon–intron structure. Screening of a somatic cell mapping panel and fluorescent *in situ* hybridization place the gene on chromosome 21 (21q22.1–22.2). Analysis of single-stranded conformational polymorphisms revealed the presence of two silent polymorphisms (Pro-149: CCG-CCA and Asp-328: GAC-GAT) with similar frequencies in normal and non-insulin-dependent diabetic patients.

**Key words:** ATP-sensitive K-channel; Pancreatic  $\beta$ -cell; Diabetes; BIR1; KCNJ6

## 1. Introduction

ATP-sensitive K-channels ( $K_{ATP}$  channels) are closed by an increase in the intracellular ATP concentration of the cell and thereby provide a means of linking cellular metabolism to the electrical excitability of the plasma membrane [1]. Their physiological function is best understood in the pancreatic  $\beta$ -cell where they play a key role in the regulation of insulin secretion in response to nutrients [2]. Closure of  $K_{ATP}$  channels, as a result of metabolically generated ATP, produces membrane depolarisation. This leads to activation of voltage-sensitive  $Ca^{2+}$  channels,  $Ca^{2+}$  influx and ultimately insulin release.  $K_{ATP}$  channels are also believed to also play important roles in the response to cardiac and cerebral ischaemia [1].

A complementary DNA encoding a  $K_{ATP}$  channel subunit ( $K_{ATP-1}$ ) was first cloned from rat cardiac muscle [3]. Northern blot analysis showed that although this mRNA is expressed in heart and brain it is not found in pancreatic  $\beta$ -cells. Subsequently, a related channel (BIR1) was cloned from rat insulinoma cells [5]. This subunit shows 72% homology with  $K_{ATP-1}$  and is expressed in insulinoma cells and in brain. Both of these channel subunits possess only two transmembrane segments, linked by a highly conserved P-region which is believed to form the lining of the pore itself. This molecular architecture, together with the electrophysiological properties, places  $K_{ATP-1}$  and BIR1 in the inward rectifier family of K-channels [8]. Although BIR1 lacks many of the properties of the native  $\beta$ -cell  $K_{ATP}$  channel, the high sequence homology to  $K_{ATP-1}$  suggests

it may represent a pore-forming subunit of a brain and  $\beta$ -cell  $K_{ATP}$  channel [5].

In this study, we report the cloning and genomic structure of the human BIR1 gene, identification of its chromosomal location and determination of the frequency of polymorphisms in this gene.

## 2. Materials and methods

### 2.1. Isolation of the human BIR1 genomic DNA

The human BIR1 gene was isolated from a human genomic library (Clontech and ATCC) using the full-length rat BIR1 cDNA [5] as a probe. High stringency screening of  $10^6$  plaques was carried out using 50% formamide,  $5 \times$  SSPE,  $5 \times$  Denhardt's solution, 0.5% SDS, and 100  $\mu$ g/ml denatured salmon sperm DNA at 37°C. The filters were washed with  $0.2 \times$  SSC at room temperature. Clones were purified by screening at reduced density, DNA prepared from positively-hybridizing clones, and the exons identified by restriction enzyme mapping and Southern blot analysis. Appropriate restriction fragments were then subcloned into pBluescript SK<sup>-</sup>, and nucleotide sequences determined. The positions of exon–intron boundaries were determined by comparison of the human genomic and rat cDNA sequences.

### 2.2. Chromosomal localisation

**PCR amplification.** Two PCR primers (sense: 5'-GGCCGAGTTA-GCCAGCA-3'; antisense: 5'-TGCCAGCTAGGGCAC-3') were designed to amplify a 178 bp fragment from the 3' end of the human BIR1 coding sequence and PCR carried out with a Perkin-Elmer Cetus thermocycler (GeneAmp PCR System 9600). The PCR conditions consisted of 35 cycles of 94°C for 20 s, 63°C for 40 s and 72°C for 20 s. Each 25  $\mu$ l PCR reaction contained 25 ng of DNA template, 0.2  $\mu$ mol of each PCR primer, 400  $\mu$ M dNTPs and 1 unit of Amplitaq.

**Fluorescence *in situ* hybridisation.** Total phage DNA was labelled with biotin-11-dUP by nick translation [6]. The hybridisation mixture contained 200 ng labelled probe DNA and 2.5  $\mu$ g unlabelled human Cot-1 DNA, previously denatured and annealed at 37°C for 15 min prior to hybridization. Hybridization to normal human male metaphase chromosomes was carried out at 42°C overnight. After stringent washes, the site of hybridization was detected with successive layers of fluorescein-conjugated avidin (5  $\mu$ g/ml; Vector Labs) and biotinylated with anti-avidin (5  $\mu$ g/ml; Vector Labs). Slides were mounted in Vectashield (Vector Labs) containing antifade, 1  $\mu$ g/ml propidium iodide and 1  $\mu$ g/ml 4',6'-diamidinophenylindole (DAPI) to allow concurrent G-banded analysis under UV light. Results were analysed and images captured using a Bio-Rad MRC 600 confocal laser scanning microscope.

### 2.3. PCR-SSCP analysis

Genomic DNA was obtained from white peripheral blood cells from normal humans and from 96 patients with non-insulin-dependent diabetes mellitus (NIDDM) chosen from the UK Prospective Diabetes Study [10]. PCR primers (Table 1) were chosen to amplify each of the exons and the adjacent flanking regions containing the exon–intron junctions. In order to maximize the possibility of detecting genetic variations by SSCP, the amplified DNA fragments were designed to

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Table 1  
Sequence of PCR primer pairs used to amplify the coding regions of the human BIR1 gene

Segment no.	Sense upstream primer	Antisense downstream primer	Fragment size
1	AAAGAGCCAGCCTTTCATTC	CGTCTTTCCTCACGTACCTC	207
2	CCTGCCAAGACACATCAGCC	CTATGTGGTCCATGTCTCCC	257
3	ATGATCTGGTGGTTGATCGC	CCCACCATGAATGCATTGAC	236
4	ATCCAATCTGTGTTGGGGTC	TGAAC TCCCCCTCCGAGGTC	244
5	ACATTGTGGAGGCTTCCATC	CCAGTTCCTCTTTGGGCAGC	216
6	GTCACCGCTGATCATTAGCC	ATCCCAGGATAGAACACATC	149
7	CATGGAAGCCACACTTAC	GACCAACTCAGGGGCAGCTC	258
8	ATCCCTTAGTGCCAAGAGC	AAGAGAAGGGTTTGCCAGC	211

All primer sequences are written in the 5'-to-3' orientation.

overlap with one another and to be smaller than 300 bp. SSCP was performed using the method of Kishimoto et al. [9]. Briefly, 1  $\mu$ l of the primer-pair (10 pmol each) was labelled by phosphorylation with polynucleokinase and [ $\gamma$ - $^{32}$ P]ATP. 0.5 pmol of labelled primer was used for each PCR. The amplification conditions comprised an initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min; 50°C (segments 1, 5, 6) or 55°C (segments 2, 3, 4, 7 and 8) for 1 min; 72°C for 1 min, and finally 72°C for 5 min. The PCR products were loaded onto a 5% polyacrylamide gel, with or without 10% glycerol, and subjected to electrophoresis at room temperature at a constant power of 40 W. After electrophoresis, the gel was dried and exposed to X-ray film without an intensifying screen for 12–24 h. To prepare single-stranded templates for DNA sequencing, a second PCR was performed (25 cycles) with 2  $\mu$ l of the first PCR product as a template and 100 pmol of only one of the primers. The single-stranded products of the second PCR were purified by NucleiClean (Sigma) and sequenced with a  $^{32}$ P-labelled primer. Data were analysed using  $\chi^2$ -analysis using the program Minitab.

### 3. Results

#### 3.1. Isolation and characterization of the human BIR1 gene

Four clones containing the human BIR1 gene were identified by high stringency hybridization. Restriction mapping of these clones demonstrated that three (BIR1 nos. 6, 8 and 23) were probably identical and one (BIR1 no. 10) was distinct. More detailed mapping was performed on clones nos. 8 and 10. Fig. 1 shows the nucleotide sequences of the coding regions and the adjacent introns obtained from these two clones. BIR1 no. 10 contained the sequences encoding a putative initiation methionine, the pore region and both of the flanking transmembrane region but did not contain the intracellular C-terminal domain. The coding region of BIR1 no. 8 was contiguous with that of BIR1 no. 10 and contained in addition the C-terminal region and the 3' untranslated region. There was no sequence overlap between the introns of BIR1 no. 8 and 10, indicating that the distance between the two exons must be more than 10 kilobases. Although we obtained the entire coding region of the human BIR1 channel gene we were unable to isolate the 5' untranslated region from the genomic library, suggesting that there may be reduced homology between the 5' untranslated regions of the rat and human genes. Comparison of amino acid sequences of the rat and human BIR1 channels within the coding region reveals that only 5 out of 407 residues (1.2%) are different (all lying within BIR1 no. 10, Fig. 1B), and that the

amino acids around the two transmembrane and the pore regions are completely conserved across these species (Fig. 1).

#### 3.2. Chromosomal localisation

Screening of the NIGMS human/rodent somatic cell mapping panel no. 2 amplified a single band of the predicted size (178 bp) from human genomic DNA and the hybrid containing chromosome 21, but not from any other hybrids or from rodent genomic DNA (Fig. 2A). The identity of this product was confirmed by a TaqI restriction site. Thus the BIR1 gene resides on chromosome 21. A more detailed localisation was carried out using fluorescence in situ hybridization using the clone BIR1 no. 10 as a probe. Approximately 20 metaphases were analysed at the 550 band level. As shown in Fig. 2B, the results confirm the assignment of the BIR1 gene to chromosome 21, placing it at the interface between bands q22.1 and q22.2 (21q22.1–22.2).

#### 3.3. Identification of genetic variations detected by SSCP

Eleven normal individuals were screened for polymorphisms in the BIR1 gene by single-stranded conformational polymorphism analysis (SSCP) under two different conditions (presence or absence of 10% glycerol). As the entire protein coding region of BIR1 is contained within the two exons identified, only these exons were studied. Two polymorphic regions were detected (Fig. 3), one in each exon. Direct sequence nucleotide analysis (Fig. 3B) demonstrated that these are silent mutations: Pro-149 CCG-CCA and Asp-328 GAC-GAT. We also identified the same polymorphisms in 96 NIDDM patients at a similar frequency to that found in normals (Pro-149,  $P = 0.30$ ; Asp-328,  $P = 0.60$ ; normal vs. diabetic subjects). The allelic frequency was CCG = 0.77 and CCA = 0.23 in the case of Pro-149, and GAC = 0.34 and CAT = 0.66 in the case of Asp-328, including both normal and diabetic individuals. There was no linkage disequilibrium between these polymorphisms ( $P = 0.86$ ).

### 4. Discussion

The predicted amino acid sequence for the rat and human BIR1 channel are highly homologous with 402 out of 407 residues (98.8%) being identical. There are 121 differences at the nucleotide level within the coding region (90.9% identity). The

Fig. 1. (A,B) Nucleotide and predicted amino acid sequence for two exons (A,B) of the human BIR1 gene which span the coding region and the adjacent exons: Upper case letters = exon, lower case, intron. (A) BIR1 no. 8. \*\*\* = Putative initiation codon; arrow = polymorphic nucleotide. (B) BIR1 no. 10. The corresponding rat amino acid sequence is included below for comparison: dots represent common residues. \* = termination codon; arrow = polymorphic nucleotide.

ttcaccagccatacaaaagtgtgccgttcattgtcctcaaagagccagcctttcattcagtgtttctttctttct

\*\*\* 14  
ccaaaaccaccag/CTAACGTCCTGGAGGGGACTCCATGGATCAGGACGTCGAAAGCCAGTGGCCATTCACCCAGCCA  
N V L E G D S M D Q D V E S P V A I H Q P

40  
AAGTTGCCTAAGCAGGCCAGGGATGACCTGCCAAGACACATCAGCCGAGATCGGACCAAAGGAAAATCCAGAGGTAC  
K L P K Q A R D D L P R H I S R D R T K R K I Q R Y

41 66  
GTGAGGAAAGACGCGAAAGTCAATGTTTCATCAGCGCAACGTGAGGGAGACCTATCGCTACCTGACCGATATCTTCACC  
V R K D G K C N V H H G N V R E T Y R Y L T D I F T

67 92  
ACATTAGTGGACCTGAAGTGGAGATCAACCTATTGATTTTTGTTCATGGTTTACACAGTACCTGGCTCTTTTTTTGGA  
T L V D L K W R F N L L I F V M V Y T V T W L F F G

93 118  
ATGATCTGGTGGTTGATCGCATAACATACGGGGAGACATGGACCACATAGAGGACCCCTCTGGACTCCTGTGTTTACC  
M I W W L I A Y I R G D M D H I E D S P W T P C V T

119 144  
AACCTCAACGGGTTTCGTCTCTGCTTTTTTATTCTCAATAGAGACAGAAACCACCATTGGTTATGGCTACCGGGTCAATC  
N L N G F V S A F L F S I E T E T T I G Y G Y R V I

145 170  
ACAGATAAATGCCCGGAGGAAATTATCTCTCTTAATCCAATCTGTGTGGGGTCCATGTCAATGCATTTCATCGGTG  
T D K C P E G I I L L L I Q S V L G S I V N A F M V

171 196  
GGATGCATGTTTGTAAAAATCTCTCAACCCAAGAAGAGGGCAGAGACCCTGGTCTTTTCCACCCATGCAGTGCATCTCC  
G C M F V K I S Q P K K R A E T L V F S T H A V I S

197 222  
ATGCGGGATGGGAAACTGTGCCTGATGTTCCGGGTAGGGACCTTAGGAATCCACATGTGGAGGCTTCCATCAGA  
M R D G K L C L M F R V G D L R N S H I V E A S I R

223 248  
GCCAAGTTGATCAAATCCAACAGACCTCGGAGGGGAGTTCATCCCGTTGAACCAGACGGATATCAACGTAGGGTAT  
A K L I K S K Q T S E G E F I P L N Q T D I N V G Y

249 274  
TACACGGGGATGACCGTCTGTTTCTGGTGTCAACCGTGCATTTAGCCATGAAATTAACCAACAGAGTCTTTCTGG  
Y T G D D R L F L V S P L I I S H E I N Q Q S P F W

275 299  
GAGATCTCCAAAGCCAGCTGCCAAAGAGAACTGGAATTTGGTTCATCTAGAAAGGAATGGTGAAGCCACAG/g  
E I S K A Q L P K E E L E I V V I L E G M V E A T

taagatgtgttctatcctgggatggctgtgggatagatgctcattttaaactggacctgatgttcttatctattacaa

gatctagaataactgaacctcaatattgacaacatcccctt **A**

..ggctctctgtgactccttgccatggaagcccacacttacatagctaaagcctccattcttctctctgcag/GG  
G

301 326  
ATGACATGCCAAGCTCGAAGCTCCTACATCACCAGTGAGATCCTGTGGGGTTACCGGTTACACCTGTCTGACCCCTG  
M T C Q A R S S Y I T S E I L W G Y R F T P V L T L

. . . . . V . . . . . H . . . . .

327 352  
GAGGACGGGTTCTACGAAGTTGACTACAACAGCTTCCATGAGACCTATGAGACCAGCACCCCATCCCTTAGTGCCAAA  
E D G F Y E V D Y N S F H E T Y E T S T P S L S A K

352 378  
GAGCTGGCCGAGTTAGCCAGCAGGGCAGAGCTGCCCTGAGTTGGTCTGTATCCAGCAAACCTCAACCAACATGCAGAA  
E L A E L A S R A E L P L S W S V S S K L N Q H A E

. . . . . N . . . . .

379 404  
CTGGAGACTGAAGAGGAAGAAAAGAACCTCGAAGAGCAAACAGAAAGAAATGGTGTATGTGGCAAACCTGGAGAAATGAA  
L E T E E E E K N L E E Q T E R N G D V A N L E N E

. . . . . P . . . . . L . . . . .

205 407  
TCCAAAGTTTGTGCGCTAGCTGGGCAAACCCCTCTCTTCTCCCCCAACACAATCTTCTCTGCTCTCATCTCTCTT  
S K V \* **B**

. . . . . TCTTTTT

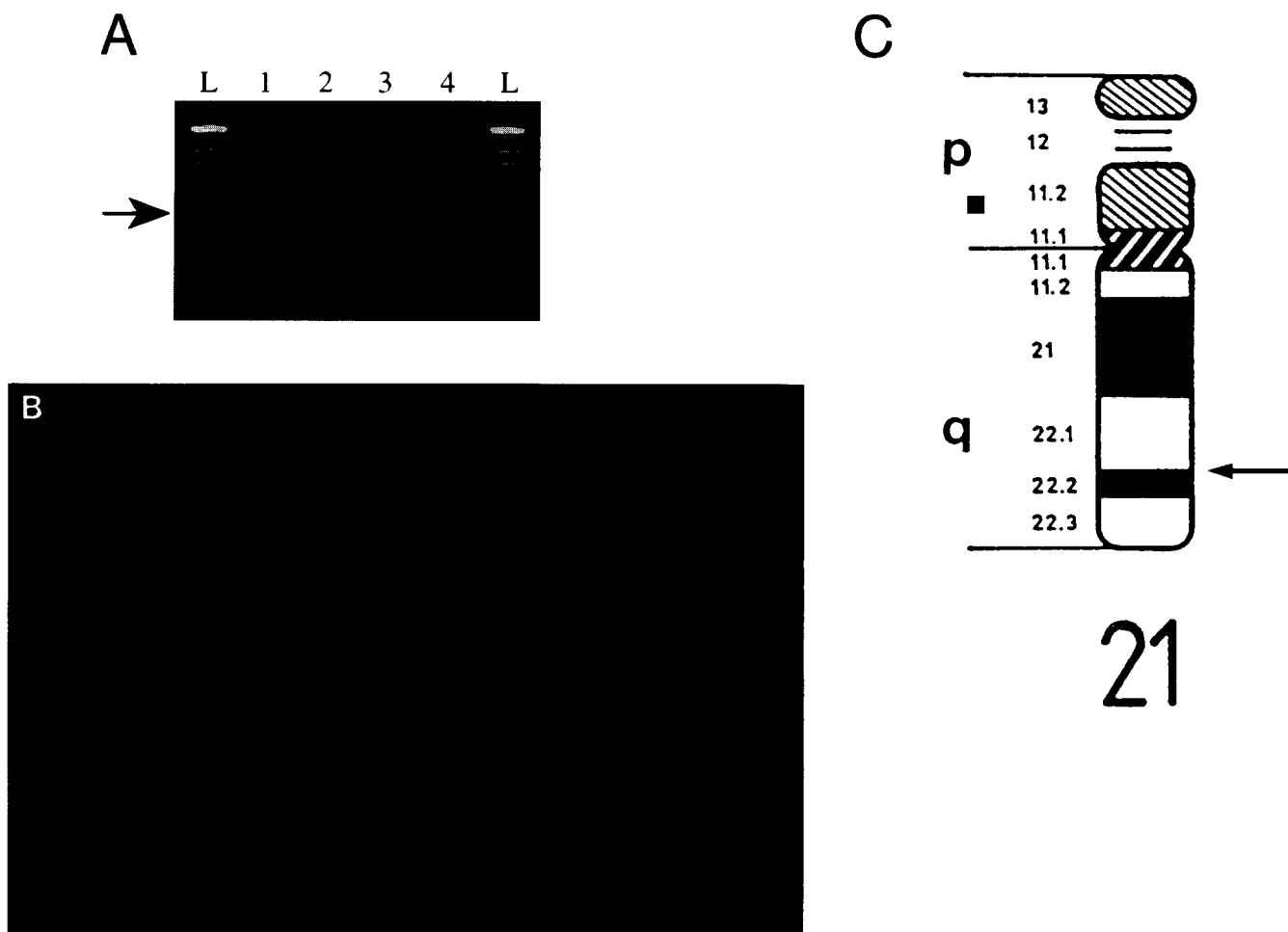


Fig. 2. (A) PCR amplification of human BIR1 from chromosome 21. L = DNA size markers; 1 = hybrid containing chromosome 21; 2 = human genomic DNA; 3 = hamster/mouse genomic DNA; 4 = no DNA control. The expected 178 bp band is indicated by the arrow. (B) Fluorescence in situ hybridization of BIR1 to normal human metaphase. The fluorescent signal appears green and the propidium iodide stained chromosomes appear red. (C) Ideogram of chromosome 21 showing location of BIR1.

coding region of the human BIR1 gene is contained within 2 exons. However, we cannot exclude the possibility that further exons exist, 5' of the putative initiator methionine. Indeed, potential coding sequences 5' of the equivalent methionine in the rat BIR1 gene have been identified (unpublished observations): it is not known if these additional codons are actually translated, but expression studies using the form of the rat cDNA equivalent to the human sequence described here did result in functional channel activity [5]. We were not successful in obtaining the 5' untranslated region of the human BIR1 gene using a rat probe presumably due to significant sequence variability between the human and rat genes in this region.

Our results show that the human BIR1 gene is located on chromosome 21, towards the distal end of the long arm: we are not aware of any human disease which is associated with this region. We found two common polymorphisms in the human BIR1 gene. Both of these are single base substitutions, and do not result in coding sequence alterations. Although we have not yet found linkage between the BIR1 gene and a human disease, linkage may yet be found in the 5', 3' or gene control sequences which we have not yet examined. The  $K_{ATP}$  channel plays an

important role in the regulation of insulin secretion from the pancreatic  $\beta$ -cell, and functional mutations in this channel may be expected to lead to defects in insulin secretion. If BIR1 indeed comprises the pore-forming subunit of the  $\beta$ -cell  $K_{ATP}$  channel, with other subunits conferring additional functional properties, then mutations in these ancillary subunits may be associated with NIDDM.

Our data provide an essential basis for subsequent screening of diabetic patients to determine if mutations in BIR1 are involved in the aetiology of maturity-onset diabetes of the young or non-insulin-dependent diabetes. In addition, mutations of the  $K_{ATP}$  channel might be involved in other pathological conditions. For instance, BIR1 is expressed in brain, and  $K_{ATP}$  channels are believed to be important in the cerebral response to ischaemia and hyperglycaemia [1,4,7]. It may therefore be appropriate to determine whether individuals who have had a stroke possess mutations in BIR1.

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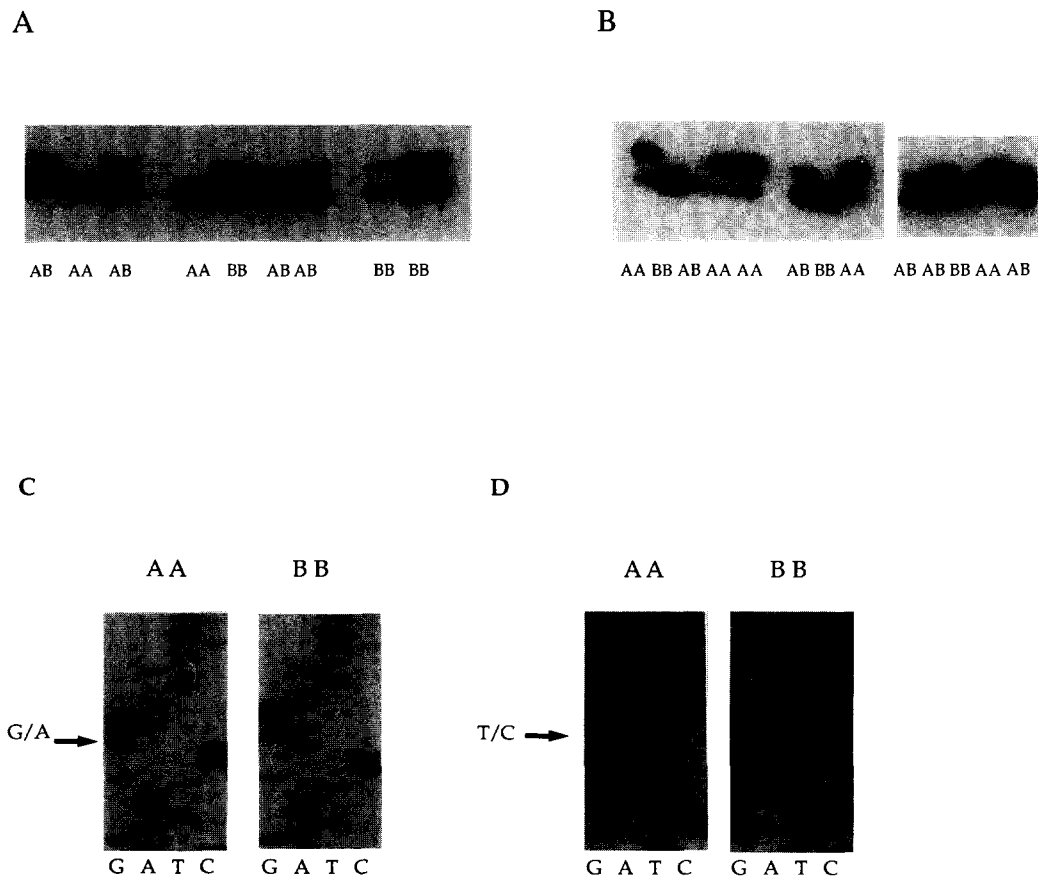


Fig. 3. (A,B) SSCP analysis showing mobility shifts of single-stranded DNA fragments due to single base substitutions in Pro-149 (A) and Asp-328 (B). Samples were amplified by PCR with the corresponding primers (segment 3 and 7) and subjected to electrophoresis in neutral polyacrylamide without glycerol at room temperature. Patients marked AA are homozygous for the major variant of the polymorphism, BB are homozygous for the minor variant and AB are heterozygotes. (C) Sequencing of PCR fragments in individuals homozygous for variant A (left) and B (right) of Pro-149. The mutation is silent: A = CCG, B = CCA. D. Sequencing of PCR fragments in individuals homozygous for variant A (left) and B (right) of Asp-328. The mutation is silent: A =  $\overline{\text{GAT}}$ , B =  $\overline{\text{GAC}}$ .

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