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ORIGINAL ARTICLE

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Extreme androgen resistance in a kindred with a novel insertion/deletion mutation in exon 5 of the androgen receptor gene

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Abstract Androgen insensitivy syndrome (AIS) is the most frequent cause of male pseudohermaphroditism resulting from target-organ resistance to androgen action. Individuals bearing the complete form of the disease (CAIS) present a female phenotype and a lack of pubic and axillary hair. In the present study, four 46,XY patients born in two generations from a kindred with a history of AIS were examined for genetic abnormalities in the androgen receptor gene (*AR*). All eight exons encoding the AR protein were individually amplified from genomic DNA followed by a mutation screening with single-strand conformation polymorphism analysis. Sequencing of the mutant *AR* revealed a novel insertion/deletion mutation in exon 5. A deletion of 7 bp is replaced by an insertion of 11 nucleotides, which represents a duplication of the adjacent downstream sequence. The mutation g.2640_2646delAGGATGC/2652_2662insTTCGCCCCTGA, results in a frameshift that introduces a premature termination signal TGA, nine codons downstream. Such a rearrangement predicts a truncation of the AR, thereby deleting a large portion of the ligand-binding domain (amino acid position 768–919). Furthermore, although this mutation breaks the translational reading frame starting from codon 760, examination of the complementary DNA suggested that it does not disturb mRNA splicing. These changes have been found in all the patients and appear to account for the observed absence of detectable androgen binding to the AR in cultured fibroblasts and for the CAIS phenotype in the kindred. This disorder represents the first insertion/deletion mutation of the AR that probably arose by a slipped-strand mispairing mechanism.

Keywords Androgen insensitivity · *AR* mutations · Insertion/deletion · Male pseudohermaphroditism · Slipped-strand mispairing

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Introduction

Androgen insensitivity syndrome (AIS) is an X-linked form of male pseudohermaphroditism caused by mutations in the androgen receptor (*AR*) gene (Lubahn et al. 1989; French et al. 1990). Impairment of AR function results in a severe virilization deficit of the external genitalia in patients with a 46,XY karyotype. The phenotype of affected individuals is variable and has been reported to range from completely female to genital ambiguity to normal male, depending on the type of mutation and its effect on AR activity (Jakubiczka et al. 1997). To date, about 250 different mutations scattered throughout the gene have been described (Chávez et al. 2001b; Gottlieb 2002). Complete AIS (CAIS) is often associated with molecular defects that may or may not affect androgen binding, including complete and partial *AR* deletions and single-base mutations that introduce nonsense codons into *AR* or disrupt the splicing of the mRNA (Quigley et al. 1995). Such abnormalities almost invariably result in a non-functional or subfunctional receptor protein, causing undervirilization defects of varying degrees (McPhaul 2002; Zenteno et al. 2002).

Here, we describe a molecular study of a kindred with CAIS in which affected subjects present an unusual

rearrangement, previously unreported in *AR*, consisting of an insertion/deletion mutation within the ligand binding domain that results in a frameshift and early stop codon.

Subjects and methods

Patients

The propositi were two siblings (aged 24 and 20 years, respectively) who were referred to our outpatient clinic because of primary amenorrhea. On clinical examination, both patients presented with a similar phenotype, characterized by female external genitalia with hypoplasia of the labia majora and minora, a short blind vagina, absence of axillary and pubic hair, and abundant breast development (Tanner 5). Cytogenetic studies revealed a 46,XY karyotype with no evidence of mosaicism in both patients. After physical and endocrinological evaluation, they were diagnosed as having CAIS (grade 7; Quigley et al. 1995). A female cousin (aged 39 years) bearing the same phenotype and her newborn niece (1 month old) presenting one left inguinal mass were included for molecular analysis. Noticeably, all patients showed a left inguinal mass corresponding to a testis, whereas ultrasonographic studies detected an intra-abdominal right testis. The pedigree of this kindred (Fig. 1A) depicts four generations, two of which include members who carry a defective AR. Studies were carried out with informed consent from the subjects or relatives (Case IV, Fig. 1A) and approval of the Ethical Committee of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán.

Mutation analysis

Coding sequence abnormalities in the *AR* gene were assessed by exon-specific polymerase chain reaction (PCR), single-stranded conformation polymorphism (SSCP) analysis, and direct sequencing analysis. Genomic DNA was isolated from peripheral blood leukocytes of all patients and normal control males by standard methods (John et al. 1991). Exon DNA amplifications were carried out with primers and PCR conditions described previously (Chávez et al. 2001a). SSCP analysis was performed according to the method of Orita et al. (1989) with minor modifications previously described (Chávez et al. 2001b). Mutant and control PCR fragments were sequenced in both directions by using the Thermosequenase ([α-^{33}P] ddNTP) terminator cycle sequencing kit (Amersham Life Science, Cleveland, Ohio, USA), following the manufacturer's recommendations, or by using the ABI PRISM dye terminator cycle sequencing core kit (PE Applied Biosystems, Warrington, UK) and the automated sequencing system 373 (Applied Biosystem, Foster City, Calif., USA). For cDNA analysis, total cellular RNA extracted from cultured fibroblasts was reverse-transcribed into cDNA with the SuperScript First-Strand synthesis system for reverse transcription PCR (RT-PCR; Invitrogen Life Technologies, Carlsbad, Calif., USA). Aliquots of cDNA were amplified by PCR with a set of primers designed from exon 4 (sense, 5'-GCCTCAATGAACTGGGAGAGAGACAG-3') and exon 6 (antisense, 5'-AACTCTTGAGAGAGGTGCCTCATTCG-3'). Subsequently, both mutant and control DNA fragments were purified and sequenced as described previously.

Cell culture conditions

Forearm skin-derived fibroblasts were cultured in MEM (Gibco-BRL, Grand Island, N.Y., USA) containing 10% newborn calf serum. Fibroblasts were grown to confluence at 37°C in 5% CO_2. Androgen-binding activity was measured in whole cells (monolayers) as previously described (Ulloa-Aguirre et al. 1988).

Western blotting and immunostaining

Cellular extracts from normal and mutant fibroblasts were immunoprecipitated by using the anti-human AR (N-20) polyclonal antibody (Santa Cruz Biotechnology, USA), which detects an epitope in the AR N-terminus, at 4°C for 18 h. After addition of goat anti-rabbit γ-globulin, aliquots of the precipitates were electrophoresed on 10 % polyacrylamide gels and processed for immunostaining as previously described (Larrea et al. 1993). In brief, membranes (PolyScreen PVDF; NEN LifeSci Products, Boston, Mass., USA) were fixed, blocked, and incubated overnight at room temperature in a 1:100 final dilution of AR (N-20), i.e., SC-816 polyclonal antibody in 0.01 mol/l TRIS-buffered saline pH 7.4, containing 0.1% Tween-20 and 3.0% powdered nonfat milk. Immunocomplexes were visualized by autoradiography after incubation in ^{125}I-labelled protein A (200,000 cpm/ml). The excess radioactivity was removed by several washes in TRIS-buffered saline, and the filter was dried and autoradiographed.

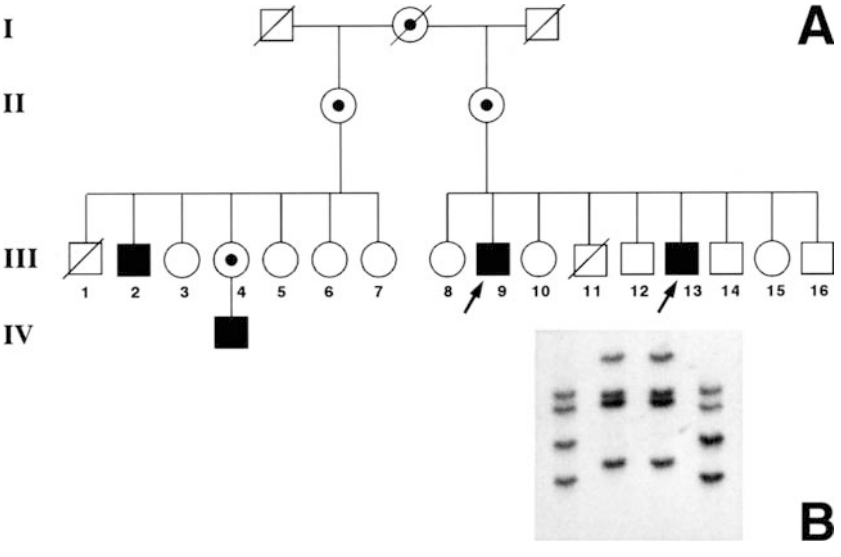

Fig. 1 **A** Pedigree of the kindred with X-linked CAIS (*circles filled with a dot* heterozygous carrier females, *filled squares* hemizygous affected males, *arrows* propositi). **B** SSCP mutational screening showing the DNA of the propositi in the *middle lanes* flanked by two normal DNA controls (*left lane, right lane*). The exon 5 ^{32}P-labeled PCR products were electrophoresed on neutral 5.4% polyacrylamide gels containing glycerol

Results

Mutation detection

Molecular analysis of the *AR* by PCR-SSCP (Fig. 1B) and DNA sequencing (Fig. 2) revealed the presence of a mutation in exon 5 leading to a gross rearrangement of the coding region. This mutation is characterized by a 7-bp deletion (g.2640_2646del AGGATGC) in combination with an 11-bp insertion, duplicating nucleotides 2652 to 2662 (ins TTCGCCCCTGA) of the 3' region of the exon. This insertion/deletion yields a frameshift at codon 760 and two premature termination signals (Fig. 2). Identical results were obtained when the mutant PCR fragments were analyzed by the automatic sequencing system (data not shown).

Analysis of *AR*-mRNA by RT-PCR

Amplification of the cDNA from mRNA by RT-PCR and its subsequent sequencing analysis confirmed the presence of the mutation and also indicated that this rearrangement does not affect normal splicing at the exon 5/intron 5 boundary (Fig. 3). cDNA from the controls yielded an expected fragment of 273 bp, whereas those from the patients yielded a similar band with a calculated size of 277 bp that spanned part of exon 4, the entire exon 5, and a portion of exon 6. Examination of this cDNA showed a structural change in the coding sequence begining at codon 760, which introduces a stop codon (TGA) downstream, at nucleotide 2639 (Fig. 3).

AR-binding and Western blotting

Androgen-binding activity of the mutant receptor was studied in a whole-cell monolayer assay. Examination of cultured skin fibroblasts indicated that the defect was characterized by the absence of [^{3}H]-dihydrotestosterone (DHT) binding. In all cases, specific [^{3}H]-DHT uptake was practically undetectable when compared with that of normal controls (Fig. 4). These results suggested that either no AR was synthesized in these cells or that an altered AR protein was produced because of an affect on mRNA translation. As seen in Fig. 4, immunoblot analysis of fibroblast extracts probed with an anti-AR polyclonal antibody showed no specific signal, suggesting an impairment in AR protein synthesis or a highly unstable truncated AR protein.

Discussion

In this report, we have presented evidence that four members from a family with CAIS carry an unusual type of mutation that alters the *AR* open-reading frame. Sequence analysis of both genomic and cDNA has revealed a deletion/insertion mutation in the 3' region of exon 5, an AR segment that is considered essential for receptor stability and for maintaining a stable association of the receptor with the ligand (McPhaul et al.

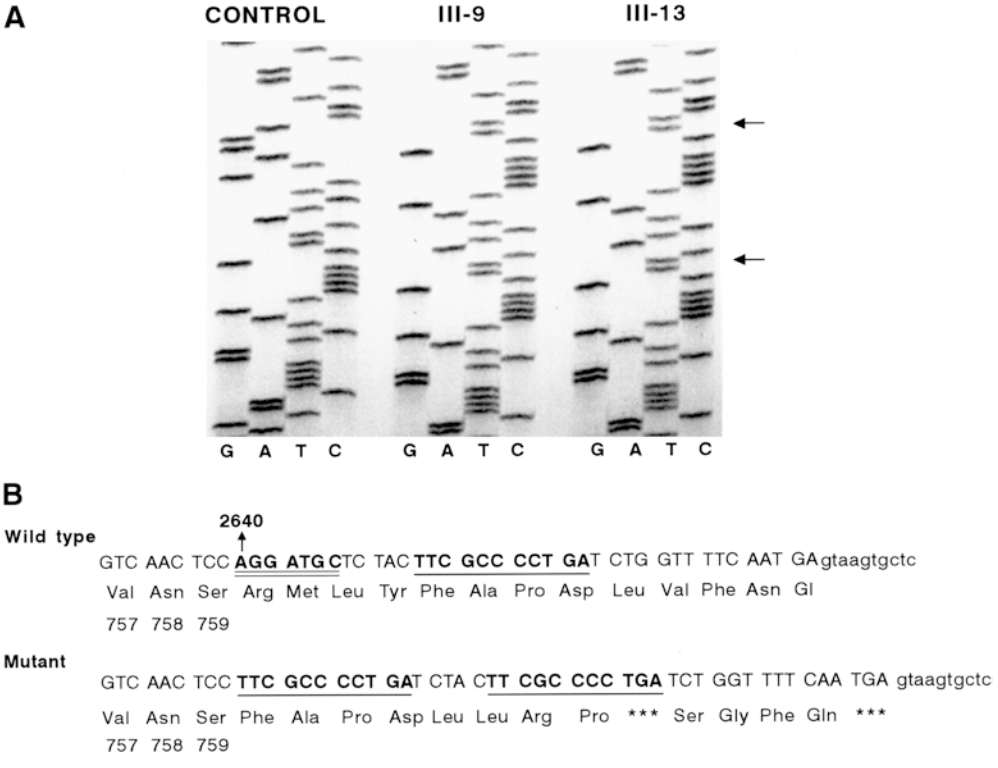

Fig. 2A, B Partial sequence of *AR* exon 5 showing a short deletion/insertion mutation in two patients with CAIS. **A** Sequences of a normal control and the mutation from patients III-9 and III-13 are depicted. **B** The 7-bp wild-type sequence AGGATGC (*double underlined*) is deleted and replaced by the 11-bp sequence TTCGCCCCTGA (*underlined*). Mutant DNA contains two identical 11-bp sequences TTCGCCCCTCA separated by only 5 bp, suggesting that the mutation arose by a slipped-strand mispairing mechanism. Structural mutation of the AR gene results in a TGA stop codon (*bottom*)

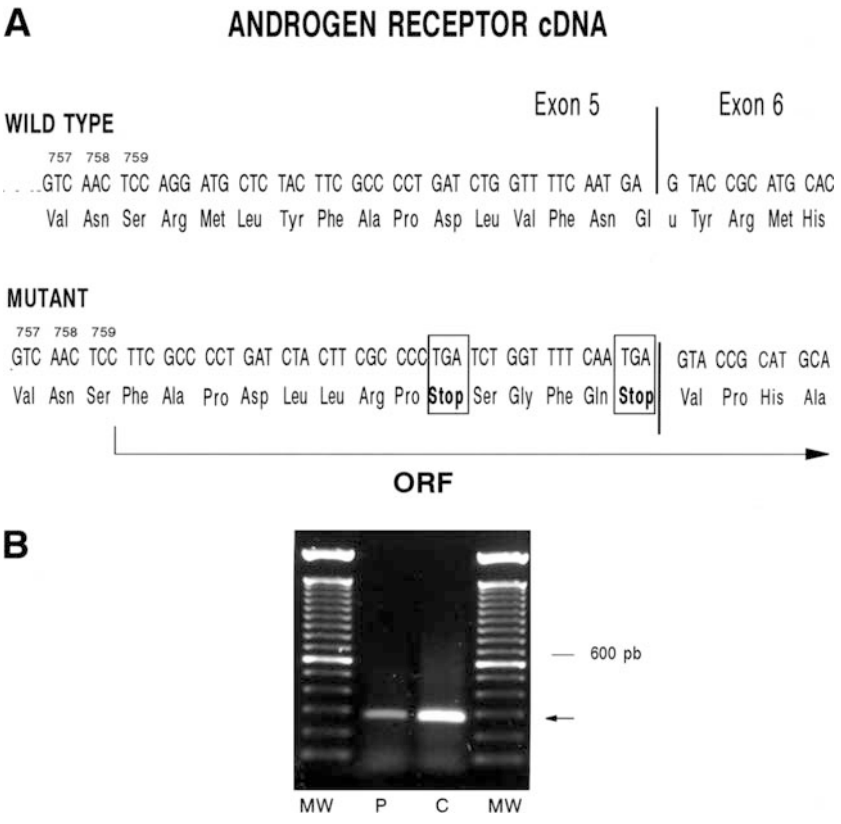

Fig. 3A, B Sequence comparison of nucleotides and amino acids of the mutant and wild type *AR* cDNA and size comparison of the cDNA amplified by RT-PCR. **A** Sequencing analyses of the cDNA indicate that the insertion/deletion mutation in exon 5 does not affect splicing but does affect the open reading frame (ORF) starting from residue Arg760. **B** Amplification of the cDNA segment covering exons 4–6 yielded similar bands (*arrow*) with a calculated size of 273 bp and 277 bp for the control (*C*) and patient (*P*), respectively (*MW* molecular weight markers)

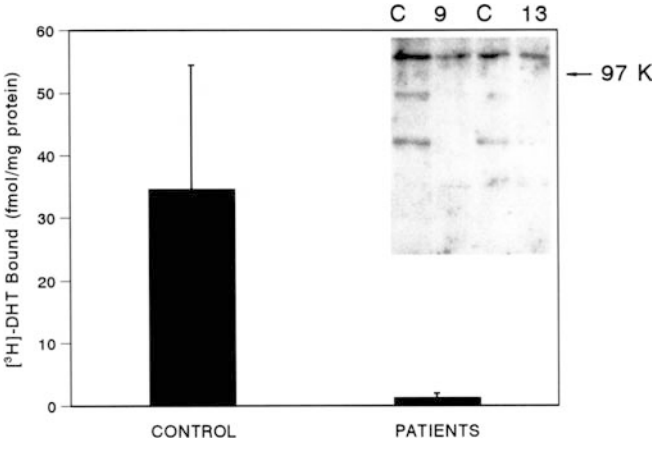

Fig. 4 Specific androgen binding and Western blot analyses from cultured skin fibroblasts. The normal range of binding is between 15 fmol and 55 fmol [^{3}H]DHT/mg protein. Mean values of 20 normal samples and those from patients (III-2, III-9, and III-13) are shown. Extracts of normal (*C*) and mutant fibroblasts (*9* III-9, *13* III-13) were subjected to SDS/PAGE, transferred to nylon membranes, and probed with anti-AR (N20):SC-816 polyclonal antibody (*inset*). The migration pattern of phosphorylase B (*97 K*) is indicated

1993). In this particular case, the insertion appears to have arisen by duplication of a contiguous sequence (Fig. 2). This mutation putatively results in a frameshift and a new stop codon in the translated mRNA. Such changes are probably responsible for the absence of AR functional activity and therefore for the extreme phenotypic appearance observed in these patients. Similar structural disorders have been published for other genes causing human genetic diseases, where deletions and/or duplications of short sequences similarly lead to the synthesis of truncated proteins (Cooper and Krawczak 1991; Krawczak and Cooper 1991). Examples of genes that have been found to contain these types of mutations are *PCCB* (Tahara et al. 1990), *IDUA* (Moskowitz et al. 1993), *WT1* (Huff et al. 1995), *FAA* (Levran et al. 1997), *HBA2* (Oron-Karni et al. 1997), and *BRCA1* (Hardouin et al. 2000). More recently, a similar rearrangement to ours was found in *CYP27B1* from a patient with 1α-hydroxylase deficiency (Wang et al. 2002).

In all the preceding cases, these deletions/insertions could be explained by a slipped-strand mispairing mechanism initially proposed by Streisinger et al. (1966), in which one DNA strand of a repeat may be misaligned by chance with the downstream repeat of the complementary strand (Levran et al. 1997). This process has also been invoked to explain putative coupled deletion/duplication events (Flanagan et al. 1984). Furthermore, this slippage misalignment mechanism has been proposed as an ubiquitous mechanism of mutagenesis and as being responsible for a significant proportion of mutations in mammalian cells (Kimura et al. 1994).

The 11-base insertion with a 7-base deletion found in our patients is unique, because it contains a duplication of the sequence TTCGCCCCTGA, separated by five bases. The mechanism by which this genic lesion occurred is not

entirely clear, in particular because no direct or tandem repeats could be identified adjacent to the molecular defect. However, a careful examination of the DNA sequence involved has revealed some features previously reported to be associated with gene rearrangements. Thus, the tetranucleotide sequence CCTG (CAGG), present in the proximity of a high percentage of human gene deletions (Oron-Karni et al. 1997), is present at both sides of the rearrangement. This tetranucleotide has been observed at deletion hot spots of globin, *FAA* and *WT1* genes, further implicating this motif in the genesis of DNA deletions (Krawczak and Cooper 1991; Huff et al. 1995; Levran et al. 1997). In addition, the TTC repeat described in the Chinese hamster *APRT* gene as a hot spot for spontaneous deletions (Krawczak and Cooper 1991; Smith and Adair 1996) has also been identified near and within the mutant sequence.

Based on these and other observations (Levinson and Gutman 1987; Tahara et al. 1990; Oner et al. 1991; Tatsumi et al. 1995), we viewed a slippage-misalignment as the probable mechanism in generating this mutation. As mentioned above, it has long been recognized that small deletions and also small insertions can arise from slippage and mispairing of two homologous DNA sequences during DNA replication. This may result either in the removal and/or repetition of the DNA between these sequences (Plaseska et al. 1991). Figure 5 depicts the proposed mechanism for the formation of this rearrangement. Examination of the mutant sequence has led us to consider that the deletion and duplication may have occurred in a sequential fashion. Initially, the deletion of seven nucleotides (AGGATGC) may have arisen from slippage induced by the CAGG tetranucleotide (step 2). After extension (step 3) and as a consequence of an incorrect mispairing, the resulting strands probably formed a mismatch bubble, where the sequence TTCGCCCCTGA was mismatched (step 4). Further DNA elongation may have led to the duplication of 11 nucleotides (step 5). The final result of these structural disorders is a modified sequence that affects the reading frame starting from codon 760. Thus, the gene mutation described here (g.2640_2646del7/2652_2662ins11) would encode for an inactive AR, primarily because of its inability to bind testosterone or DHT, a prerequisite for transcriptional activation. As a consequence of such a genetic defect, target genes in affected patients may be unresponsive, even to high doses of androgens, which necessarily leads to androgen resistance and the development of CAIS. To our knowledge, this is the first case of familial CAIS attributable to an insertion/deletion mutation in *AR*.

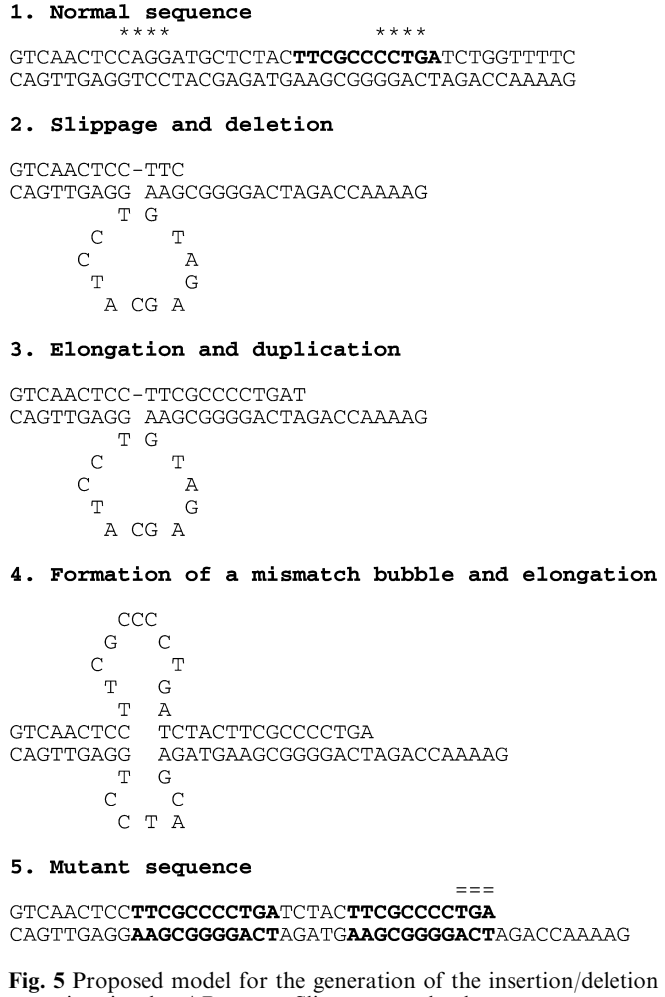

Fig. 5 Proposed model for the generation of the insertion/deletion mutation in the AR gene. Slippage at the hot spot consensus sequence CAGG/CCTG (*asterisks*) results in the deletion of the sequence AGGATGC. After elongation, misalignment of the resulting strand induces the formation of a bubble. Further DNA elongation results in the duplication of 11 nucleotides. The duplicated sequences are shown in *bold*

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