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Differential Splicing of Human Androgen Receptor Pre-mRNA in X-linked Reifenstein Syndrome, because of a Deletion Involving a Putative Branch Site

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Summary

The analysis of the androgen receptor (AR) gene, mRNA, and protein in a subject with X-linked Reifenstein syndrome (partial androgen insensitivity) is reported. The presence of two mature AR transcripts in genital skin fibroblasts of the patient is established, and, by reverse transcriptase–PCR and RNase transcription analysis, the wild-type transcript and a transcript in which exon 3 sequences are absent without disruption of the translational reading frame are identified. Sequencing and hybridization analysis show a deletion of >6 kb in intron 2 of the human AR gene, starting 18 bp upstream of exon 3. The deletion includes the putative branch-point sequence (BPS) but not the acceptor splice site on the intron 2/exon 3 boundary. The deletion of the putative intron 2 BPS results in 90% inhibition of wild-type splicing. The mutant transcript encodes an AR protein lacking the second zinc finger of the DNA-binding domain. Western/immunoblotting analysis is used to show that the mutant AR protein is expressed in genital skin fibroblasts of the patient. The residual 10% wild-type transcript can be the result of the use of a cryptic BPS located 63 bp upstream of the intron 2/exon 3 boundary of the mutant AR gene. The mutated AR protein has no transcription-activating potential and does not influence the transactivating properties of the wild-type AR, as tested in cotransfection studies. It is concluded that the partial androgen-insensitivity syndrome of this patient is the consequence of the limited amount of wild-type AR protein expressed in androgen target cells, resulting from the deletion of the intron 2 putative BPS.

Introduction

The human androgen receptor (hAR) is a ligand-dependent transacting transcription factor belonging to the steroid hormone/thyroid hormone/retinoic acid receptor zinc-finger family (O'Malley 1990). The androgen receptor (AR) mediates the actions of testosterone and 5α-dihydrotestosterone in male sexual development and in the maintenance of normal male reproductive functions during adult life (Griffin and Wilson 1989).

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It is generally accepted that mutations in the AR gene are the cause of the X chromosome–linked androgen insensitivity syndrome (AIS). The nature of these mutations can range from complete or partial gene deletions to single base changes and can result in a range of defects in virilization of a 46,XY individual, ranging from male infertility or aberrant virilization (partial AIS [pAIS]) to a genetic male with a complete female habitus (complete AIS [cAIS]) (Brinkmann and Trapman 1992; Griffin 1992; Pinsky et al. 1992). The human AR (hAR) is a phosphoprotein with 910 amino acid residues (Van Laar et al. 1991). The number of amino acid residues, however, can vary, because of polymorphic glutamine and glycine stretches located in the N-terminal domain of the AR (Chang et al. 1988; Lubahn et al. 1988; Trapman et al. 1988; Tilley et al. 1989; McPhaul et al. 1991; Sleddens et al. 1991).

The N-terminal domain of the AR harbors transcrip-

tion-activating functions, the central DNA-binding domain consists of two zinc fingers, and the ligand-binding domain is located in the C-terminal part of the protein (Jenster et al. 1991). On ligand binding, the C-terminal zinc finger is assumed to be involved in dimerization, whereas the N-terminal zinc finger of the AR is able to recognize and interact with the hormone-responsive elements in a target gene, thus regulating the transcription of that gene, as has been shown for the glucocorticoid receptor (Luisi et al. 1991).

hAR mRNA presents itself as a 10.6-kb transcript, containing relatively large 5′- and 3′-UTRs (1.1 and 6.8 kb, respectively). In human prostate tissues and human foreskin fibroblasts, an additional mRNA species of 8 kb is present, generated by alternative splicing in the 3′-UTR (Lubahn et al. 1988; Faber et al. 1991).

The diversity of the AIS, both in the molecular cause and in the resulting clinical characteristics of the syndrome, provides detailed information about the structure-function relationship of the hAR protein. In the present studies the nature of the AR protein in a patient with a severe form of X-linked pAIS is presented. In this particular case, differential pre-mRNA splicing resulting from a genomic deletion involving the intron 2 branch-point sequence (BPS) is the molecular cause of the syndrome.

Subject, Material, and Methods

The Propositus

The propositus is a member of a family in which four of nine males are affected by X-linked Reifenstein syndrome. The 46,XY index patient was diagnosed as having pAIS, with infertility, hypospadias, small penis, absence of typical male body hair pattern, and gynecomastia. Genital skin fibroblasts of the patient showed a normal specific binding capacity of 39 ± 12 fmol/mg protein, with a K_d of 0.36 ± 0.1 nM. These values are within the normal range. There was no indication of defects in the testosterone or 5α-dihydrotestosterone synthesis.

DNA and RNA Isolation and Analysis

Genomic DNA was isolated from genital skin fibroblast monolayers or from white blood cells by using standard methods (Sambrook et al. 1989). Total cellular RNA was extracted using 3 M LiCl and 6 M urea (Auffray and Rougeon 1980), followed by phenol and chloroform extractions. RNase protection analyses (Sambrook et al. 1989) were performed using 20 μg total RNA and a ^{32}P probe complementary to the cDNA fragment *Sac*I/*Stu*I ranging from the 3′ part of exon 2 to the 5′ part of exon 4 (fragment B in Kuiper et al. 1989). First-strand cDNA synthesis was performed using 4 μg total RNA, 100 ng oligonucleotide (oligo) complementary to part of exon 6 (5′-TGCTGAAGAGTAGCAGTGCT-3′), 10 units AMV reverse transcriptase (Promega), and 10 units RNasin, according to the protocol of the manufacturer. These cDNAs were amplified by PCR using the above oligo as primer, along with an oligo situated in exon 1 (5′-GACTTCACCGCACCTGATG-3′). All PCRs were done in a 100-μl reaction volume, using the Perkin Elmer thermocycler and 2.5 units *Taq* polymerase (AmpliTaq) and the appropriate reaction buffer and conditions, as described by the supplier (Cetus). A typical cycling protocol was denaturation for 1 min at 94°C, primer annealing for 2 min at 55°C, and primer extension for 1–3 min at 72°C, for 25–30 cycles. The template was either 1 μg genomic DNA or 2% of the cDNA synthesis reaction mixture. The fragments were size fractionated on 2% agarose gels, ethidium bromide stained, and directly sequenced using the dideoxy chain-termination method (Sanger et al. 1977) after purification with Qiaex (Qiagen). The yields of five PCR reactions were pooled for one direct sequencing reaction with 500 ng of sequencing primer. Oligos used for PCR and sequencing were as follows (s = sense; as = antisense; and mismatches in the sequences are indicated by lowercase letters): 1s, 5′-GTTTGGTGCCATACTCTGTCCAC-3′; 2s, 5′-TCAGGTCTATCAACTCTTG-3′; 3s, 5′-TGTCCATCTTGTCGTCTTgGcgccTGTTATGAAGCAGGGATG-3′; 4s, 5′-TCCTCCTCCTTCCTCTCTCC-3′; 1as, 5′-GACGAgAAGATGGAgAATTcTTCCTTCGG-3′; 2as, 5′-CATCCCTGCTTCATAACAggcgCcAAGACGACAAGATGGACA-3′; 3as, 5′-CTGATGGCCACGTTGCCTATGAA-3′; and 5s, 5′-AGAAGTGTCTGTTCATGTCC-3′.

Cell Culture Conditions and Transfections

Genital skin fibroblasts and HeLa cells were cultured in Eagle's minimum essential medium supplemented with nonessential amino acids and antibiotics. Fibroblasts were maintained in medium containing 10% FCS. HeLa cells were maintained in medium containing 5% full or 5% dextran charcoal–treated (DCC) FCS. HeLa cells grown to 50%–60% confluency were transfected using the calcium phosphate precipitation method (Chen and Okayama 1987) essentially as described elsewhere (Ris-Stalpers et al. 1991).

AR Expression Vectors

The original hAR cDNA expression vector pSV.AR_0 (Brinkmann et al. 1989) was slightly modified with re-

spect to the restriction-enzyme sites, rendering the *Eco*RI site in the open reading frame unique (BHEX-AR0; a gift from L. Pinsky). The unique *Kpn*I site and the unique *Eco*RI site were used to generate an expression plasmid with an in-frame deletion of exon 3 (BHEX-ARΔ3), by exchanging the 898-bp *Kpn*I-*Eco*RI fragment of the wild-type expression plasmid with the 781-bp *Kpn*I-*Eco*RI fragment lacking the exon 3 sequence obtained by amplification of cDNA of the patient. The deletion mutant was sequenced to verify the correct reading frame and to exclude misincorporation of nucleotides by *Taq* polymerase.

Computerized Axial Tomography (CAT) Assays

HeLa cells were cotransfected with AR expression plasmids and the pG29GtkCAT reporter plasmid (Schule et al. 1988). Twenty-four hours after transfection, cells were incubated for another 24 h, with or without 1 nM of the synthetic androgen methyltrienolone (R1881) or 10 nM dexamethasone, before the CAT activity in the cell lysates was measured essentially as described by Seed and Sheen (1988). Data were corrected for the amount of protein in the cell lysates, and the transfection efficiency was determined by using the CAT activity induced by dexamethasone via the endogenous glucocorticoid receptor.

Immunoblot Analysis

Immunoprecipitation and western blot analysis of the AR protein obtained either from $\sim 5 \times 10^6$ genital skin fibroblasts or from transiently transfected HeLa cells were performed as described elsewhere (Ris-Stalpers et al. 1991). The AR protein on immunoblot was visualized by chemiluminescence (Veldscholte et al. 1992).

Results

Identification of the Genomic Deletion Involving the Intron 2 Branch Site of the hAR Gene

The initial amplification of all hAR coding sequences from genomic DNA of the patient was successful for all coding sequences of the gene except for exon 3, which encodes the second DNA-binding zinc finger. By using several primer combinations corresponding to intron sequences upstream and downstream of exon 3 and exon 3 sequence itself, the presence of a deletion of part of intron 2 and possibly part of exon 3 was established (fig. 1).

To narrow the boundaries of the deletion, the intron sequences upstream of exon 3 from genomic DNA of

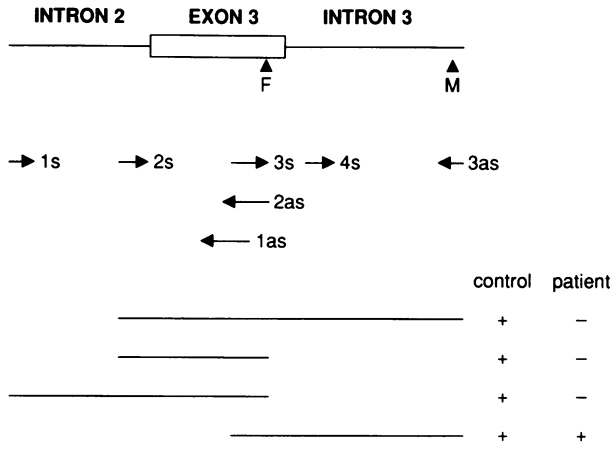

Figure 1 Amplification of exon 3 of the hAR gene and flanking intron sequences: schematic illustration of exon 3 and flanking sequences. Oligos for PCR (s and as), as well as location of restriction sites, are indicated. F = *Fok*I; and M = *Msc*I. The (dis)ability of specific fragments, to be amplified by PCR, from genomic DNA of either the patient or a control sample is shown.

the index patient were amplified by inverse PCR (Ochman et al. 1988). Genomic DNA of the pAIS subject was digested with *Msc*I, ligated, and redigested with *Fok*I (fig. 1). The linearized DNA was amplified using oligos 1as and 4s, and the resulting 500-bp fragment was sequenced using oligo 4s. Subsequent sequence analysis identified intron 3 sequence, the *Msc*I site, followed by an unknown sequence, 18-bp intron 2 sequence including the splice acceptor site, and part of exon 3. Comparison with the wild-type genomic sequence revealed the presence of the complete exon 3 coding sequence and 18-bp wild-type intron 2 sequence upstream of exon 3 (including the splice acceptor site at the intron 2/exon 3 junction) and an additional 262 bp of unknown intron 2 sequence (fig. 2). This was confirmed by PCR directly on genomic DNA by using primer 2as situated in exon 3 and a primer (5s) situated in the unknown intron 2 sequence, located ~200 bp upstream of the intron 2/exon 3 boundary, followed by direct sequencing of this amplification product. We also used this amplification product to probe digests of two genomic phages, in order to determine the size of the deletion.

In an earlier study, both the structural organization of the hAR gene and the intron sizes were determined (Kuiper et al. 1989). No genomic clones spanning the complete intron 2 region could be identified, and the size of intron 2 was determined to be ⩾15 kb. One of the phages described in that study (designated "8.2")

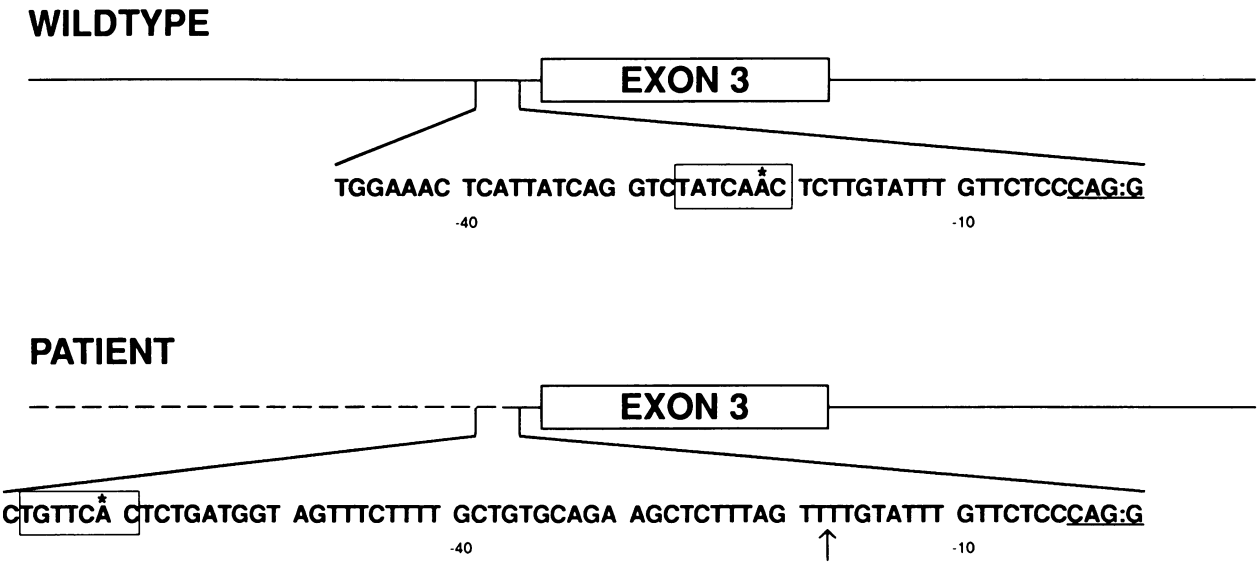

Figure 2 Partial mapping of the deletion in intron 2, using inverse PCR: direct-sequencing results of the PCR product generated using oligos 1as and 4s after digesting genomic DNA of the Reifenstein patient with *Msc*I, ligation, and subsequent recutting of the ligated DNA by *Fok*I. The position where the sequence deviates from the wild-type sequence is indicated by an arrow, and the splice acceptor site is underlined. The wild-type putative branch site and the putative cryptic branch site, in the wild-type and mutant AR gene, respectively, are boxed, and the branch nucleotide is indicated by an asterisk.

contains exon 2 and 8-kb downstream intron 2 sequence. Hybridization of digested 8.2 phage DNA by the probe described above resulted in a positive hybridizing signal, indicating that the deletion originates in this area. A phage (designated "9.2"; Kuiper et al. 1989) containing exon 3 and 6-kb upstream intron 2 sequence was also digested. Hybridization in this case resulted in a positive band, corresponding to a genomic fragment containing exon 3 itself. No hybridization was seen with the 6-kb upstream sequence of exon 3. Since these two phages do not overlap, it was concluded that the part deleted from intron 2 is ≥6 kb. When a sense oligo in exon 2 and an antisense oligo in exon 3 were used, no positive PCR signal could be obtained from the mutated AR gene, indicating that the size of the remaining intron 2 sequence is >1,500 bp (data not shown).

Effect of the Intron Deletion on RNA Splicing

RNA was isolated from genital skin fibroblasts of the patient and from control genital skin fibroblasts. First-strand cDNA synthesis was performed using an AR-specific oligo located in exon 6 of the hAR gene. Subsequent PCR amplification using this oligo and a sense oligo situated in exon 1 resulted in two amplification products—contrary to control RNA, which showed only one amplification product (fig. 3). Sequencing of both amplification products showed the minor product to be the wild-type transcript and the major product to be a transcript from which the complete exon 3 sequence was deleted.

An RNase protection assay using a ^{32}P-labeled *Sac*I(exon 2)-*Stu*I(exon 4) fragment was performed, to establish the relative amounts of both transcripts. Densitometric analysis of the autoradiograms showed a ratio of 8% wild-type transcript to 92% exon 3–deleted transcript (in three separate experiments the ratios of wild-type transcript:exon 3–deleted transcript were 8:92, 13:87, and 4:96) (figs. 4 and 5).

Binding Characteristics and Immunoblot Analysis of the Mutant AR Protein

The complete deletion of exon 3 from the hAR mRNA transcript is in-frame and will, on translation, result in a normal ligand-binding protein with a deletion of 39 amino acids from the DNA-binding domain of the hAR. Both the wild-type and the mutant AR protein are expected to bind ligand normally. Genital skin fibroblasts of the patient showed a specific binding capacity for androgens that was within the normal range (see Subject, Material, and Methods).

Immunoprecipitation of the AR from genital skin fibroblasts cultured from a biopsy of the pAIS patient

with the AR-specific monoclonal antibody F39.4.1 was followed by SDS-PAGE and immunoblotting using the polyclonal antibody Sp061. Visualization was done by chemiluminescence. This procedure showed only a mutant protein with an apparent molecular mass of 100 kD in genital skin fibroblasts of the patient. The translation of the 10% wild-type transcript was below the detection limit. Similar to the wild-type AR protein, the mutant AR protein migrated as a doublet, with the majority of protein in the band with the highest apparent molecular mass (Jenster et al. 1991) (fig. 6). The mutant AR doublet, however, was much less spaced, compared with the wild-type AR doublet.

Functional Activity of the Mutant AR Protein in HeLa Cells

In order to analyze the functional activity of the mutant AR, an hAR expression plasmid was generated with an in-frame deletion of exon 3 (BHEX-ARΔ3). The BHEX-AR0 (wild-type expression vector) and BHEX-ARΔ3 constructs were transiently transfected in HeLa cells with the p29GREtk-CAT reporter gene. Two ratios (1:1 and 1:8) of the wild-type and the Δ3 construct were transfected. Only the wild-type construct showed transactivating potential in the presence of the synthetic androgen R1881. The ARΔ3 construct had no transactivating properties with or without ligand and did not influence the transactivating properties of the wild-type expression vector when cotransfected with equal amounts of AR0 or with eight times in excess of it (table 1). The lack of transactivating potential of the mutant AR was not due to underexpression of the construct in HeLa cells, as was assessed by western/immunoblotting analysis (data not shown).

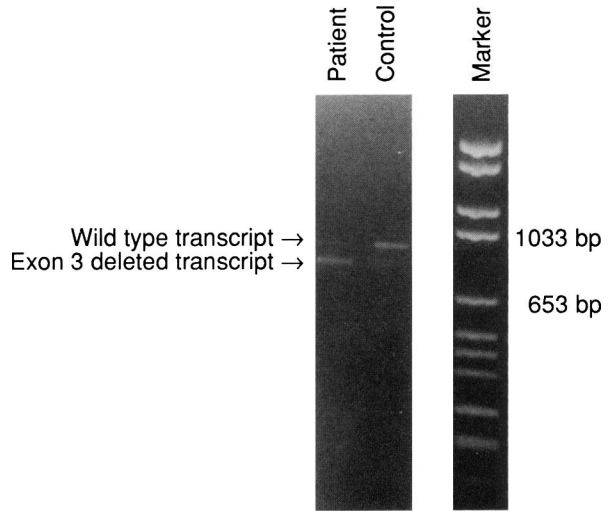

Figure 3 Alternative splicing of the AR pre-mRNA, as a result of a partial intron 2 deletion of the hAR gene: RT-PCR on RNA isolated from genital skin fibroblasts of the patient and from a control genital skin fibroblast strain. First-strand synthesis and PCR amplification using an exon 6–exon 1 primer combination shows a majority of mutant transcript and a minority of wild-type transcript in RNA from the patient.

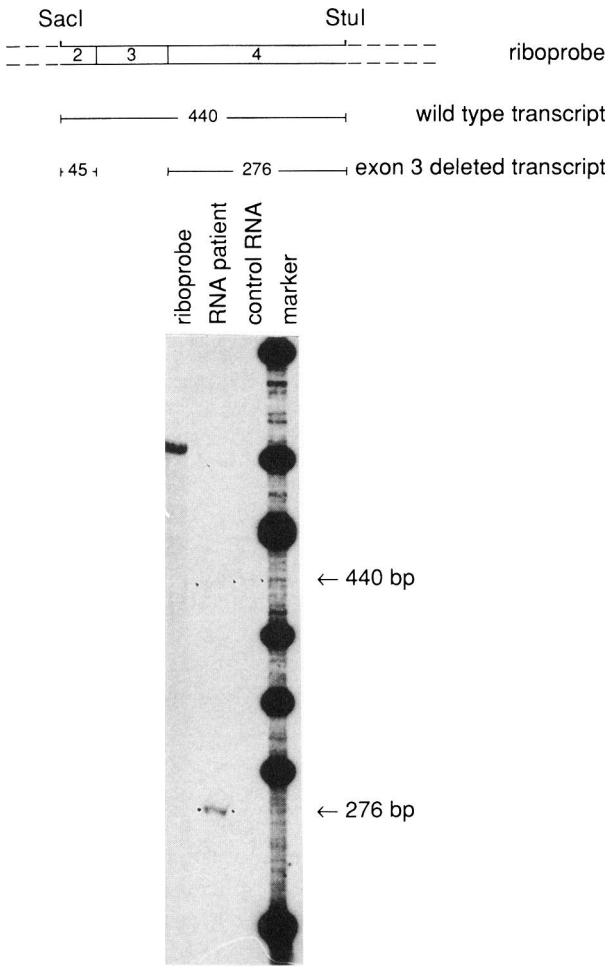

Figure 4 RNase protection assay: expression of both the wild-type AR mRNA and a transcript lacking exon 3 sequences in genital skin fibroblasts of the index patient. A schematic representation of the RNase protection analysis, including the used riboprobe, is given. The marker used is pBR322 *Hin*fIx*Eco*RI.

Discussion

AR disorders in the AIS are well documented. There is no indication for a hot spot for mutations; rather, there is a whole range of mutations, varying from (par-

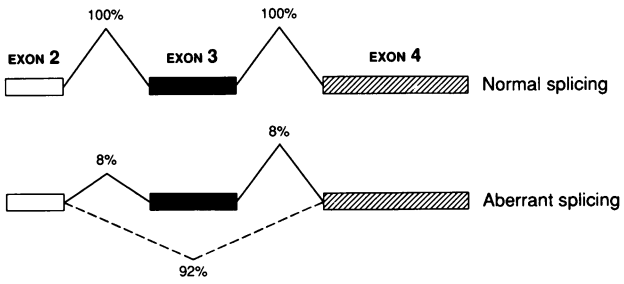

Figure 5 Schematic illustration of exons 2–4 of the hAR gene and of the alternative splicing event resulting from the partial intron 2 deletion. The solid lines represent wild-type splicing events, and the dashed line represents the aberrant splicing event. The ratio of the two splicing events, as determined by densitometric determination of the intensity of the bands shown in fig. 4, is indicated.

tial) gene deletions to single base changes, which result in the introduction of either a premature stop codon or an amino acid change.

In the present study a unique, >6-kb deletion in intron 2 of the AR gene, which does not involve any protein-coding sequences or splice acceptor/donor sequences, is reported. The deletion leaves 18 bp upstream of exon 3 intact. The intronic deletion has serious consequences for the splicing of the AR pre-mRNA, because of the deletion of the putative BPS.

The first event in pre-mRNA splicing is the cleavage at the 5′ splice site. Subsequently a loop or lariat structure is formed where an adenosine residue (the branch nucleotide) is linked to the 5′ end of the intervening

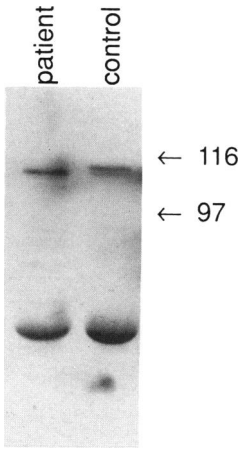

Figure 6 SDS-PAGE analysis of hAR protein immunoprecipitated from genital skin fibroblasts of the patient and of control fibroblasts. The position of marker proteins is indicated.

Table 1

Transcriptional Activity of the ARΔ3 Mutant

BHEX-AR0:BHEX-ARΔ3[a]	CAT Activity[b] (%) −R1881	+R1881
1:0	<1	100 (*n*=7)
0:1	<1	<1 (*n*=7)
1:1	<1	142 ± 37 (*n*=5)
1:8	<1	118 ± 8 (*n*=2)

NOTE.—HeLa cells were transiently cotransfected with different amounts (0.5–5 μg/ml precipitate) of the expression plasmids BHEX-AR0 and BHEX-ARΔ3 and the reporter plasmid pG29GtkCAT. CAT activity was measured in cell lysates after culturing for 24 h in the absence (−R1881) or presence (+R1881) of 1 nM R1881.

[a] Ratio of the amounts of expression plasmids used.

[b] Presented as a percentage relative to that of the wild-type AR expression plasmid, which was set at 100% for each individual experiment. The number of experiments and the SD of the mean are indicated.

sequence. Following the lariat formation, cleavage at the 3′ splice site takes place, resulting in the excision of the intron as a lariat and in the concomitant ligation of both exons.

The site of branch formation is typically located 20–50 nt upstream of the 3′ splice site, which is always the first AG sequence downstream of the BPS. The consensus sequence for the mammalian BPS and acceptor splice site is T/C,T/C,T/C,G/A,A,T/C. . .$(T/C)_{11}$. . .N,C/T,A,G:G (Reed and Maniatis 1985; Padgett et al. 1986; also see references therein). In eukaryotes, base pairing occurs between the BPS and the small nuclear ribonucleoprotein particle U2snRNP, indicating the direct involvement of the BPS in the assembly of the spliceosome (Wu and Manley 1991). The BPS in intron 2 of the AR gene fits the consensus sequence closely, and the branch nucleotide is on position −22, within the range of 20–50 nt upstream of the acceptor splice site. The AG:G used as splice acceptor site is the first AG:G downstream of the putative BPS. The intron 2 deletion described in the present study ends 18 bp proximal to the 3′ end of the intron and completely abolishes the putative BPS.

The mammalian BPS shares sequence homology with the yeast "TACTAAC" BPS, which is critical in yeast; only small deviations from the consensus sequence in yeast are tolerated (Langford et al. 1984). It has been reported that the deletion of mammalian BPS either

only slows down the splicing reaction or does not influence the pre-mRNA splicing efficiency at all, as was shown for the rabbit β-globin IVS 2, where no specific internal intron sequences are required for correct and efficient splicing, but only a minimal intron length of 80 nt (Wieringa et al. 1984). However, in this case it cannot be totally excluded that a cryptic branch site has been utilized.

The results presented in this study strongly indicate that the deletion of the BPS is very deleterious for pre-mRNA splicing. The deletion of the putative BPS in intron 2 of the hAR gene inhibits 90% of wild-type splicing, by skipping the acceptor splice site on the intron 2/exon 3 boundary, which results in a transcript with an in-frame deletion of exon 3 sequence. That the BPS in this case is not indispensable for proper pre-mRNA splicing is evident, since ~10% wild-type transcript is still present, as shown by reverse transcriptase-PCR (RT-PCR) and RNAse protection. Whether the wild-type splicing event is the result of the use of a cryptic BPS is not clear. A possible BPS at position −63 in the mutant AR intron 2 sequence, which partly fits the consensus sequence, could function as such. There are, however, four other AG dinucleotides between the putative cryptic BPS and the regular splice acceptor site. RT-PCR demonstrated that they are not used as such. If this cryptic BPS is involved in the 10% wild-type splicing, then it is clearly a weak BPS probably due to the deviation from the consensus sequence on the first base upstream of the branch nucleotide.

Aberrant splicing events are a relatively rare phenomenon in AIS. Elsewhere we have reported a G→T mutation on position 1 in the splice donor site of intron 4 in the AR gene of a completely androgen-insensitive individual, which completely abolished wild-type splicing (Ris-Stalpers et al. 1990).

Mutations in the second DNA-binding zinc finger of members of the steroid hormone receptor family have been described elsewhere (Hughes et al. 1988; Sone et al. 1990; Chang et al. 1991; Marcelli et al. 1991; Klocker et al. 1992; Zoppi et al. 1992). They result in either partial or complete hormone insensitivity. It is therefore not unexpected that the in-frame deletion of the second DNA-binding zinc finger of the hAR gene, due to a genomic deletion including exon 3 sequences, results in cAIS. The protein in vitro is unable to activate transcription of an androgen-reporter gene, as has been shown both by Quigley et al. (1992) and in the present study. Specific androgen binding in genital skin fibroblasts of the Reifenstein patient is within the normal range and does not corroborate the supranormal concentration found by Quigley et al. (1992) in a cAIS patient with an exon 3 deletion because of failure of autologous down-regulation.

The occurrence of a mutant AR protein in combination with the wild-type protein in an androgen-insensitive patient has not been described before. The partial syndrome of the patient described here must be the result of an insufficient amount of functional AR protein, since the mutant protein itself is inactive. Cotransfection studies showed that the mutant protein has no dominant negative effect on the wild-type protein, as has been described for a mutant estrogen receptor. Mutant estrogen receptors lacking the second DNA-binding zinc finger inhibit estrogen-dependent transcription activation in a dominant negative fashion when cotransfected with the wild-type estrogen receptor and a reporter plasmid. This inhibitory effect probably occurs through protein-protein interactions (Wang and Miksicek 1991).

A synergistic effect on transcription-activating potential has been described for a transcription factor SP1 mutant lacking all of the DNA-binding zinc fingers in combination with the wild-type transcription factor (Courey et al. 1989; Pascal and Tjian 1991). The glutamine-rich activation domains involved in this superactivation are also present in the hAR, but we have found no evidence of superactivation of the mutant AR protein lacking the second DNA-binding zinc finger.

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