Splice donor site mutations in the 3-hydroxy-3-methylglutaryl coenzyme A reductase gene cause a deficiency of the endoplasmic reticulum 3-hydroxy-3-methylglutaryl coenzyme A reductase protein in UT2 cells

William Harrison Engfelt, Kimberly R. Masuda, Vincent G. Paton, and Skaidrite K. Krisans[1]

Department of Biology, San Diego State University, San Diego, CA 92182

Abstract **UT2 cells are a mutant clone of Chinese hamster ovary (CHO) cells that are deficient in the 97 kDa endoplasmic reticulum (ER) 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase protein. The analysis of UT2 cell cDNA and genomic DNA has led to the identification of two novel point mutations in intronic sequences of the ER HMG-CoA reductase gene. One mutation identified at the +1 position (G → A) of the 5′ splice site of exon 11–12 junction was shown to cause exon 11 skipping which resulted in the insertion of premature stop codons. We also identified a second mutation at the +5 position (G → A) of the 5′ splice site in the intron spanning exons 13 and 14. Furthermore, the data indicate that the two mutations in the reductase gene are present on the same allele. As demonstrated by reverse transcription-polymerase chain reaction (RT-PCR) of UT2 cell mRNA, the mutations produce aberrant spliced messages. If the aberrant messages were translated, truncated proteins of 44 kDa or 66 kDa would be predicted. More importantly, these truncated proteins would be expected not to have catalytic activity. In addition, we have also recently demonstrated that the UT2 cells express a 90 kDa HMG-CoA reductase protein that is localized exclusively in peroxisomes, and is up-regulated when the cells are grown in the absence of added mevalonate. Thus, the mutations identified in the ER reductase gene in UT2 cells indicate that neither a 97 kDa nor a 90 kDa reductase protein can be produced from this gene.—**Engfelt, W. H., K. R. Masuda, V. G. Paton, and S. K. Krisans. **Splice donor site mutations in the 3-hydroxy-3-methylglutaryl coenzyme A reductase gene cause a deficiency of the endoplasmic reticulum 3-hydroxy-3-methylglutaryl coenzyme A reductase protein in UT2 cells. *J. Lipid. Res.* 1998.** 39: **2182–2191.**

Supplementary key words cholesterol • endoplasmic reticulum • peroxisomes • 90 kDa peroxisomal reductase • Chinese hamster ovary cells

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase catalyzes the rate-limiting step of cholesterol biosynthesis (1). Several mutant cell lines deficient in HMG-CoA reductase have been described (2). One such cell line, designated UT2, is a mutant clone of Chinese hamster ovary (CHO) cells that requires mevalonate for growth that cannot be met by supplemental cholesterol (3). This cell line has been stable for over 12 years and has a calculated spontaneous reversion rate of less than 1.5×10^{-7} (3). A deficiency in the 97 kDa ER HMG-CoA reductase protein is believed to be the only abnormality in the UT2 cell line, due to the observations that the rate of cholesterol biosynthesis from mevalonate is normal and that the two enzymes of the cholesterol biosynthetic pathway preceding HMG-CoA reductase, i.e., acetoacetyl-CoA thiolase and HMG-CoA synthase, are normal or slightly elevated in these cells. Although UT2 cells are utilized as mevalonate auxotrophs, there is some detectable HMG-CoA reductase activity found in these cells. This activity is bona fide as it is completely eliminated when a competitive HMG-CoA reductase inhibitor, such as compactin, is added to the assay mixture. The interpretation of these results concluded that the UT2 cells may have a mutation in the structural gene for reductase that results in the production of an abnormal protein with residual activity, but insufficient to be detected by immunoblotting, or alternatively, that another isozyme of HMG-CoA reductase is present (3).

We have recently demonstrated that UT2 cells maintained in media supplemented with fetal calf serum (FCS), yet lacking mevalonate, are able to survive and divide (4). These cells exhibited a marked increase in HMG-CoA reductase activity compared to that measured in UT2 cells cultured in the presence of mevalonate. The UT2 cells grown in the absence of mevalonate are designated UT2* While the UT2* cells do not express a 97 kDa HMG-CoA reductase protein, we have demonstrated that these cells ex-

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ER, endoplasmic reticulum; CHO, Chinese hamster ovary; FCS, fetal calf serum; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; RT, reverse transcription; PAGE, polyacrylamide gel electrophoresis; RFLP, restriction fragment length polymorphism.

[1] To whom correspondence should be addressed.

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press a 90 kDa HMG-CoA reductase protein, which is localized exclusively in peroxisomes (4). The localization of the 90 kDa reductase protein to peroxisomes was demonstrated by a number of different methods: *i*) analytical subcellular fractionation and measurement of enzyme activities; *ii*) immunoblotting for HMG-CoA reductase in the isolated fractions with a monospecific antibody against HMG-CoA reductase; *iii*) immunofluoresence microscopy; and *iv*) immunoelectron microscopy. The wild type CHO cells contain two HMG-CoA reductase proteins, the well-characterized 97 kDa protein, localized in the ER, and a 90 kDa protein localized in peroxisomes (4).

Our interpretation of these results is that all wild-type cells contain two forms of HMG-CoA reductase, as previously demonstrated (4–6). The UT2 cells lack the ER HMG-CoA reductase, and contain suppressed levels of the 90 kDa peroxisomal reductase due to growth of the cells in mevalonate-containing media. However, when mevalonate is removed, the peroxisomal reductase is up-regulated. When these cells are placed back in media containing mevalonate, the peroxisomal reductase activity levels again decrease. Thus, this is a physiological regulation that is reversible.

In this study we have determined that the deficiency of the 97 kDa ER HMG-CoA reductase protein in UT2 cells is due to the presence of two mutations within the gene. The UT2 cells contain a mutation in the 5′ splice junction (+1 position) of the intron located between exon 11 and exon 12. A second similar mutation was found in the 5′ splice junction (+5 position) between exons 13 and 14. Both of these mutations cause aberrant splicing events that ultimately result in premature stop codons present in the mature mRNA. The presence of the premature stop codons in the cDNA, if translated, would produce nonfunctional, truncated proteins with molecular masses no larger than 66 kDa. Furthermore, these proteins would be deficient in the reductase catalytic domain (7). We also demonstrate that the UT2 and UT2* cells have the same point mutations.

EXPERIMENTAL PROCEDURES

Materials and general procedures

Biochemicals were purchased from Sigma. Electrophoresis supplies, AG1-X8-200-400 mesh formate resin, Zeta Probe GT membrane (used for Northern analysis) and Trans-Blot Transfer Medium (used for Western analysis) were purchased from Bio-Rad, except for Protogel, which was purchased from National Diagnostics (Atlanta, GA). Cell culture media and FCS were purchased from Life Technologies, Inc. Lipoprotein-deficient media was obtained from PerImmune. 3-Hydroxy-3-methylglutaryl coenzyme A, dl-3[glutaryl-3-^{14}C] and (RS)-[5-^{3}H]mevalonic acid were purchased from DuPont NEN. Hybridization conditions for Northern and Southern analyses were performed as described (8), except that hybridization was performed at 42°C. DNA probes were labeled with α^{32}P-dCTP (NEN) using the Nick Translation Kit (Boehringer Mannheim).

Phagemid cDNA library construction

UT2* poly(A)$^+$ RNA was used to construct an oligo dT-primed, bidirectional phagemid cDNA library as per the Copy Kit cDNA synthesis kit (Invitrogen). The library was probed using a radiolabeled full length HMG-CoA reductase cDNA (9).

PCR and clone isolations

cDNA was synthesized from 500 ng of poly A RNA using Superscript Choice System for cDNA Synthesis™ kit (Gibco BRL). Oligonucleotide primers were synthesized by Gibco BRL or SDSU Microchemical Core Facility (San Diego, CA): primer A (5-ATG GAC ATT GAA CAA GTG GTT ACC CTG AGC-3), primer C (5-TTT TGA TTC TGG AAC CAG AAA ATC TCA GCC-3), primer D (5-GGA GCC TGG GGT GAG CCA AGA TAG AAA AGG-3), primer E (5-CCA AGT GTG AGT GCA CAC CCT CGC CCT CAC-3), primer F (5-CCA AGT GTG AGT GCA CAC CCT CGC CCT CAT-3), primer G (5-GAT CCA GGG ACC GAG TGG CTA CA-3), primer H (5-GCT TCT CGC TCC TTC TCA CA-3), primer I (5-GCC ATG GGG ATG AAC ATG A-3), primer J (5-TCA GTT CAA TAC TGT CAG GCT GC-3), primer K (5-AAG AAG GCT CTG GAA GCT TTG TGG AGA GG-3), primer L (5-GAT GGG AGC TTG CTG TGA GAA TGT G-3), primer M (5-CTG CTA GTG CTA TCG AAG GCG TCC T-3), primer N (5-GTT ACC ACT AAC TGC CAG AAT CTG C-3). Thermocycling conditions from genomic DNA and cDNA are as indicated in figure legends. All polymerase chain reactions (PCR) were performed as previously described (8). Amplified DNA was then analyzed on agarose gels and documented on a Stratagene Eagle Eye. All PCR products were subcloned using the TA Cloning kit (Invitrogen). Nucleotide sequence data of each PCR product was compiled from the automated sequencing (SDSU Microchemical Core Facility, San Diego) of three independent clones, in duplicate. PCR products analyzed with restriction enzymes were first precipitated with ethanol, then digested with 10 units of enzyme for 16 h.

Tissue culture

UT2 cells were obtained from Dr. J. Goldstein. The UT2 cells were maintained in 1:1 Dulbecco's modified Eagle's media:F12, supplemented with 0.2 mm mevalonate, 5% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate (designated Media 1), in a 37°C incubator with 5% CO_2. UT2 cells maintained in the presence of FCS but in the absence of mevalonate are designated UT2*. CHO cells were maintained and harvested in the same medium as UT2 cells except mevalonate was not added to the media. Twenty-four h prior to experimental procedures, all cell culture media were changed to media containing 5% lipoprotein-deficient serum.

Immunoblotting and immunoprecipitations of HMG-CoA reductase

Cell extracts, sodium dodecyl sulfate (SDS)-gel electrophoresis, immunoblotting, and immunoprecipitations were performed as previously described (4). A polyclonal ER HMG-CoA reductase antibody made against the rat 55–60 kDa C-terminal portion of the protein was used for the Western blots, and the polyclonal HMG-CoA reductase anti-peptide G (residues Arg^{224} through Leu^{242}), and anti-peptide H (residues Thr^{284} through Glu^{302}) were used for the immunoprecipitations (4). Membranes were processed using the ECL kit (Amersham) according to the supplied protocol, and exposed on X-ray film (Kodak). Films were then analyzed using a densitometer (Molecular Dynamics).

Northern blot analysis

Total RNA was extracted from 80% confluent cultured cells as described (8). Twenty four h prior to harvesting cells, media were changed from Media 1 ± mevalonate supplemented with

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5% FCS, to Media 1 ± mevalonate supplemented with 5% LPDS. Poly(A)$^+$ mRNA was isolated using Collaborative Biochemical type 3 primer dT cellulose according to the supplied protocol. Northern blots were performed using standard molecular biology protocols. Formaldehyde–agarose gels used for Northern analysis were electrophoresed in a Gibco BRL Horizon® 20•25 apparatus run at 65 volts for 18 h.

Genomic Southern blot analysis and isolation of an HMG-CoA reductase pseudogene

Genomic DNA was purified from confluent cells grown in culture using the QIAamp Tissue Kit (Qiagen). DNA (12 μg) was digested with 50 units of the indicated restriction enzymes (New England Biolabs) for 16 h. Digested DNA was electrophoresed in a 0.8% agarose gel and processed as described (8). The DNA used to make exon 2 probe was obtained by first performing PCR using primer 1 (5-GTA AGC GCA GTT CCT TCC-3) and primer 2 (5-GCC TGT CAA TTC TTT GTC-3) on rat liver cDNA, then gel purifying a 195 bp BamHI, DdeI, and NaeI DNA fragment. The reductase 3′UTR probe was obtained by PCR using primer 3UTR1 (5-AGC CTG ACA GTA TTG AAC TG-3) and primer 3UTR2 (5-TAA GAT TCA ACA ACT CTG CTG-3) with full-length reductase plasmid vector, pRED227 (9) as template. Similarly, exon 11 and exon 12 probes were isolated using primer ex11for (5-GAG GTT ATA AAA CCA TTA GTG-3), primer ex11rev (5-CTC GGC ACT CTC CAG TAT CTG-3) to obtain a 173 bp exon 11 product; primer 12p (5-TGC AAA GTT CCT TAG CGA TG-3) and primer 12q (5-GGG AAT AAT TAT AAT CTC TG-3) to obtain a 187 bp exon 12 product.

The 3 kb, XbaI-digested genomic DNA fragment that hybridized to each of the exon 11 and 12 probes, as visualized by Southern analysis, was isolated by screening a genomic library enriched for 3 kb, XbaI genomic DNA fragments. Briefly, UT2* genomic DNA was digested with XbaI, then run on an 0.8% agarose gel. A region of the gel corresponding to 3 kb (±300 bp) was excised, and the genomic DNA contained within was purified using the Gene Clean kit (Bio101). The purified DNA was then treated with T4 DNA polymerase (New England Biolabs) to create blunt ends, according to the supplied protocol. The blunt-ended, genomic DNA was then ligated to BstXI/EcoRI linkers (Invitrogen), bidirectionally subcloned into pCDNA2.1, and transformed into *E. coli* TOP10F′ (Invitrogen). The resulting library was screened with a cDNA probe corresponding to HMG-CoA reductase exon 11 region. Two independent clones, 3 kb in size, were isolated and sequenced. Both strands of each clone were sequenced via automated DNA sequencing (Scripps Sequencing Facility, San Diego, CA).

RESULTS

Screening of a UT2* cell cDNA library

A cDNA library was constructed from poly(A)$^+$ mRNA isolated from UT2* cells and screened at relatively low stringency, using a full-length ER HMG-CoA reductase cDNA probe. Out of 1.5×10^6 colonies screened, 12 positive clones were isolated and sequenced. All of the positive clones were determined to be abnormal ER HMG-CoA reductase cDNAs (data not shown). A thorough analysis of the sequence data from each clone revealed a normal coding sequence up to exon 10, a total deletion of exon 11, and in addition, insertion of downstream intronic sequences between exons 13 and 14.

Sequencing of genomic DNA products

We speculated that a mutation in a splice site junction could explain the exon 11 splicing abnormalities observed in the UT2* cell transcripts. Efficient splicing is dependent upon consensus sequences at the intron/exon boundaries. Invariably, the 5′ donor splice site dinucleotide GT and the 3′ acceptor splice site AG are necessary for accurate splicing (10–13). Mutations in the invariant dinucleotides prevent normal splicing and have been shown to lead to exon skipping (14). To determine whether a splice site mutation was present in an intronic region neighboring exon 11, we PCR-amplified genomic HMG-CoA reductase DNA from exon 10 to exon 12. Amplification was performed on genomic DNA isolated from CHO, UT2, and UT2* cells. Only one band, corresponding to a predicted 2.78 kb product, was resolved in the samples from all three cell lines (data not shown). The 2.78 kb amplification products were subcloned and sequenced. The sequence data from the CHO cell product was identical to the known genomic HMG-CoA reductase, whereas, some of the clones from the UT2 and UT2* cells contained a transition mutation at the donor 5′ splice site of the intron spanning exon 11 and exon 12. Specifically, the invariant +1 nucleotide of intron 11 was altered from a G to an A. This single base pair transition in the invariant GT is a likely cause for the skipping of exon 11. A schematic representation of the splicing event detected in

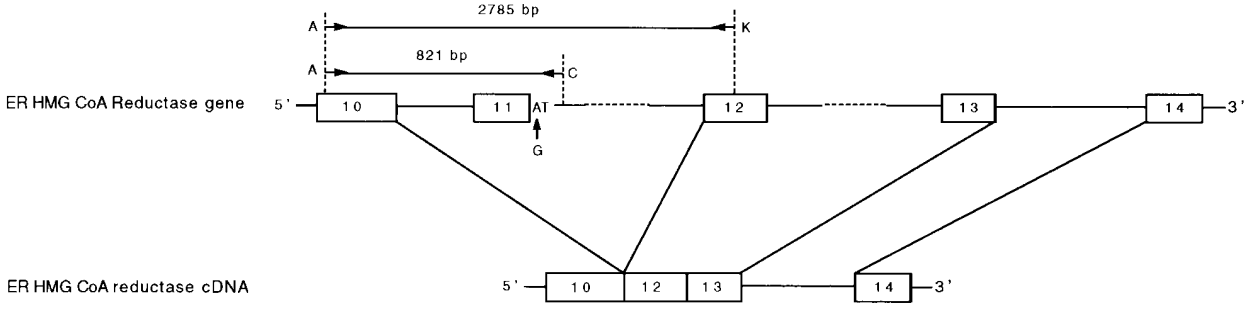

Fig. 1. Schematic representation of proposed splicing events in UT2 and UT2* cell genomic DNA. The positions of primers A, K, and C are shown. Primers A and K amplify a 2785 bp genomic DNA region, and primers A and C amplify an 821 bp region. The transition mutation found at the invariant 5′ splice site is designated by an arrow. The proposed splicing events are shown depicting the skipping of exon 11 with the inclusion of intronic sequence between exons 13 and 14 of the ER HMG-CoA reductase cDNA.

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the ER HMG-CoA reductase gene from UT2 and UT2* cells is illustrated in **Fig. 1**.

Restriction fragment length polymorphism (RFLP) analysis of the exon 11 genomic region

To confirm the mutation found via nucleotide sequencing, RFLP analysis was performed on an 821 bp genomic PCR product which spanned intron 11. The normal reductase allele in CHO cells contains an HphI restriction enzyme site at the 5′ splice site of exon 11. The G to A transition found in UT2 and UT2* cells at the exon 11 splice site causes the loss of this HphI restriction site. Therefore, using the HphI enzyme, we over-digested an 821 bp PCR product obtained from CHO, UT2, and UT2* cell genomic DNA. The restriction pattern from CHO cell DNA was of the predicted sizes (i.e., 296 bp, 231 bp, 205 bp, and 89 bp), demonstrating the presence of the HphI cleavage sequence at the intron 11 splice site (**Fig. 2**). However, analysis of the digestion products from the UT2 and UT2* cells revealed a 501 bp band, indicating that the 821 bp genomic DNA sequences from these two cells lines do not have the HphI recognition site at the 5′ splice site of exon 11. If the UT2 and UT2* genomic PCR product contained only the mutated allele we would expect to observe only the 89 bp, 231 bp, and the 501 bp fragments. However, the 205 bp and 296 bp fragments were also observed, which can be explained by the presence of a second allele containing the wild-type sequence in this region. Thus, RFLP analysis confirms the presence of a G to A transition at position +1of the 5′ splice site of the intron spanning exon 11 and exon 12 in UT2 and UT2* cells, and suggests also the presence of a second allele containing a normal sequence in this region in these cells.

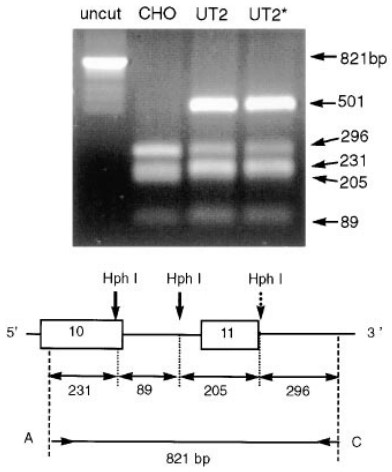

Fig. 2. RFLP analysis of genomic DNA isolated from CHO, UT2, and UT2* cells. PCR amplification with 10 pmol primer A and primer C was performed using 200 ng of genomic DNA as template. DNA underwent 34 rounds of amplification with the following conditions; denature = 94°C for 15 sec; anneal = 67°C for 20 sec; extend = 72°C for 30 sec. PCR products were overdigested for 16 h with 10 units of Hph I restriction enzyme, then run on a 1.2% agarose gel, stained with ethidium bromide. Lower, schematic representation of the PCR amplification and positions of the HphI restriction sites.

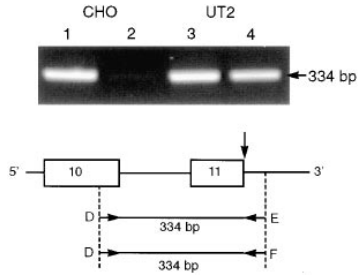

Fig. 3. Allelic amplification of CHO, UT2, and UT2* cell genomic DNA. PCR amplification was performed with 10 pmol of primer D and primer E using CHO cell genomic DNA (lane 1) or UT2 cell genomic DNA (lane 3) as template; or PCR was performed using 10 pmol of primer D and primer F using CHO cell genomic DNA (lane 2) or UT2 cell genomic DNA (lane 4). Antisense primers E and F differ only in the final 3' nucleotide, where primer E terminates with deoxycytidine while primer F terminates with deoxythymidine. Two-step PCR was performed for 34 rounds with the following conditions; denature = 94°C for 15 sec; extension/annealing = 72°C for 30 sec. After amplification, PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide. Lower, schematic representation of oligonucleotide primer positions with respect to the exon 10 to exon 11 genomic DNA region of HMG-CoA reductase.

Detection of two alleles

To clearly demonstrate the presence of two different alleles in UT2 cells, PCR primers were designed that specifically recognized either the normal allele or the mutant allele. PCR primers specific for the mutant allele amplified a product from UT2 cell genomic DNA (**Fig. 3**, lane **4**) but not from CHO genomic DNA (Fig. 3, lane 2). Furthermore, primers designed to amplify the normal allele gave a product from CHO and UT2 cell genomic DNA (Fig. 3, lanes 1 and 3), demonstrating the presence of a normal allele in CHO cells and a second allele in UT2 cells, retaining the wild-type sequence in this region. The presence of a second allele in UT2 and UT2* cells has also been confirmed by sequencing the 2.78 kb genomic PCR products (data not shown).

The UT2 and UT2* cells displayed a wild type 2.78 kb genomic PCR product as well as one containing the G to A transition at position +1 of the 5′ splice site of the intron spanning exon 11 and exon 12.

RT-PCR of CHO and UT2 cell mRNA

To analyze the HMG-CoA reductase cDNA of CHO and UT2 cells, CHO and UT2 cell mRNA was amplified in three parts by RT-PCR using primers shown in **Fig. 4**. Amplification of the entire coding region (exon 2 to exon 20) from CHO cells produced only one expected 2.71 kb product, whereas amplification of cDNA from UT2 cells revealed three distinct products, 2.75 kb, 2.66 kb, and 2.58 kb, none of which corresponded to 2.71 kb (Fig. 4, panel A). To delineate the differences between the CHO and UT2 cell cDNA, a region from exon 10 to exon 16 was amplified. Again, as illustrated in Fig. 4, panel B, three PCR products were amplified from UT2 cell cDNA while only one product was amplified from CHO cells. All three UT2 cell cDNA

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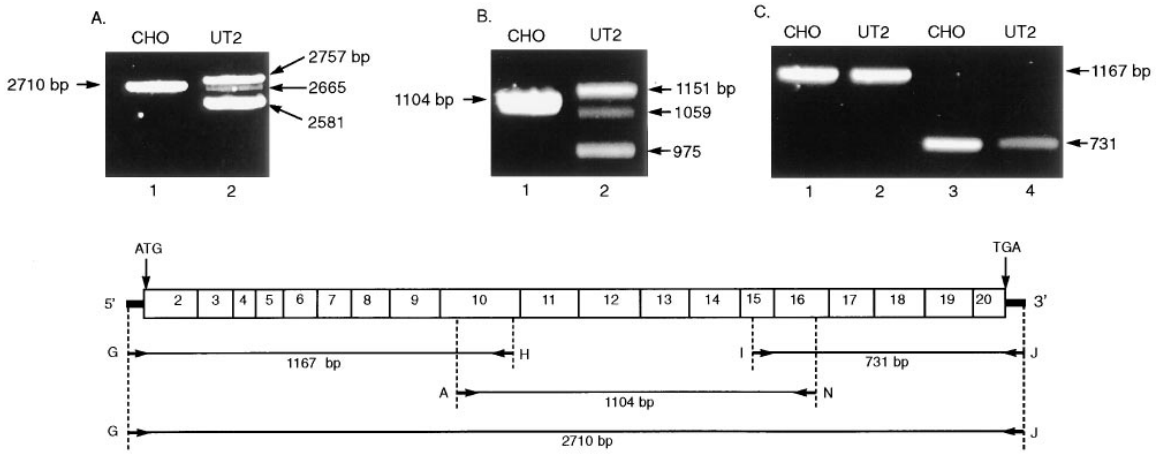

Fig. 4. RT PCR analysis of CHO and UT2 cell transcripts. RT PCR was performed on mRNA isolated from CHO and UT2 cells. The following primer combinations were used: primers G and J (panel A) ; primers A and N (panel B) ; primers G and H (panel C, lanes 1–2); primers I and J (panel C, lanes 3–4). PCR was performed for 34 rounds with the following conditions; denature = 94°C for 15 sec; extension = 72°C for 90 sec; annealing = 62°C for 20 sec. After amplification, PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide. Lower, schematic representation of oligonucleotide primer positions with respect to the coding region of the HMG-CoA reductase cDNA (exons 2 through 20).

products, 1151 bp, 1059 bp, and 975 bp, spanning exon 10 to exon 16 differ from the expected normal product of 1104 bp (Fig. 4, panel B). In contrast, PCR primers designed to amplify the region from exon 2 to exon 10 and the region from exon 15 to exon 20 each display only the expected PCR product of 1.16 kb and 731 bp from CHO and UT2 cells, respectively (Fig. 4, panel C). These PCR products of 1.16 kb and 731 bp from UT2 cells were subcloned and sequenced in their entirety. The sequences of both products corresponded to the known CHO reductase sequence and revealed no mutations in the sequence spanning exon 2 to exon 10, and in the sequence spanning exon 15 to exon 20. The same results were obtained from UT2* cell cDNA.

We then also subcloned and sequenced the three UT2 cell cDNA products in their entirety spanning exon 10 to exon 16 that were observed in Fig. 4, panel B. **Figure 5** summarizes the sequencing results. The 975 bp amplified product is missing exon 11 and contains the first 47 bp of the intron between exon 13 and exon 14. Similarly, the 1059 bp product was missing exon 11; however, a full-length intron of 131 bp (intron 13) was found to have been inserted. The largest product, 1151 bp, unexpectedly contained exon 11 and included the same 47 bp intronic insertion found in the 975 bp clone. The results show that transcripts missing exon 11 must terminate translation at amino acid 402 due to a novel stop codon. However, the 1151 bp product containing exon 11 plus the 47 bp insert resulted in a frame shift, and if translated, would terminate at amino acid 602 due to the presence of a stop codon. Furthermore, a transition mutation of a G to an A was present at position +5 in the conserved 5′ donor splice site at the exon 13–14 junction in all of the three clones.

RFLP analysis of the exon 13 genomic region

To confirm that a transition mutation exists at the 5′ splice site of the exon 13–14 junction, RFLP analysis was performed on genomic PCR products from CHO, UT2, and UT2* cells. A 446 bp region of DNA was PCR amplified and digested with the restriction enzyme, Tsp509I. If a transition mutation of a G to an A is present at position +5 of this 5′ splice site, then incubation with Tsp509I will cut the 448 bp product into a 286 bp fragment and a 160 bp fragment. As shown in **Fig. 6**, the digestion products from UT2 and UT2* cells indicate the presence of a nor-

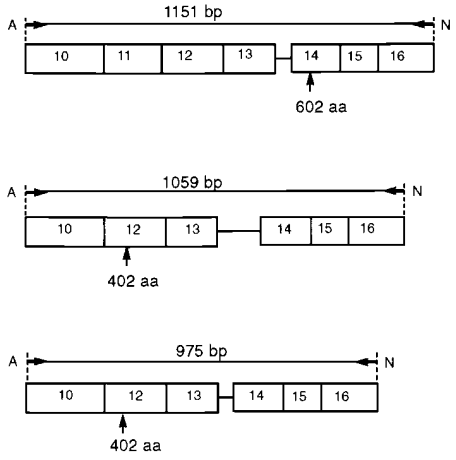

Fig. 5. Schematic representation of exon 10 through exon 16 sequenced PCR products from UT2 cells. Diagrammatic representation of three PCR products amplified using primers A and N (Fig. 4, panel B). Each of the three PCR products was subcloned and sequenced. All three contained a G to A transition at the +5 position of the 5′ splice donor site. The 1151 bp product contained the 47 bp of intron 13 causing a premature stop codon at amino acid 602; the 1059 bp product was missing exon 11 and contained the entire intron 13 region, causing a premature stop codon at amino acid 402; and the 975 bp product was also missing exon 11, and contained the 47 bp of intron 13, causing a premature stop codon at amino acid 402.

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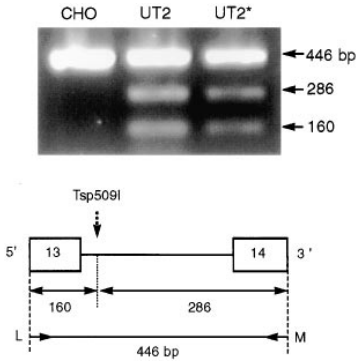

Fig. 6. RFLP analysis of genomic DNA isolated from CHO, UT2, and UT2* cells. PCR amplification was performed with 10 pmol primer L and primer M using 200 ng of genomic DNA as template. DNA underwent 34 rounds of amplification with the following conditions: denature = 94°C for 15 sec; extend = 72°C for 30 sec; anneal = 65°C for 20 sec. PCR products were overdigested for 16 h with 10 units of Tsp509I restriction enzyme. Digests were electrophoresed on a 1.2% agarose gel, stained with ethidium bromide. Lower, schematic representation of the original PCR product and the position of the Tsp509I restriction site.

mal sequence and a mutant allele, while CHO cells contain only the normal allele. Additionally, the sequencing of cDNA clones obtained from screening a UT2* cell cDNA library confirmed the existence of this splice site mutation (data not shown). Thus, these data show that the UT2 and UT2* cells contain two novel transition mutations, one present at the +1 position in the donor splice site in between exons 11 and 12, and another at the +5 position in the donor splice site in between exons 13 and 14. Furthermore, because the point mutation in the intron spanning exon 13 and 14 is observed to be coupled to the skipping of exon 11, it is likely that these two point mutations are linked to the same allele.

Immunoblot analysis

To determine whether the aberrant spliced UT2 and UT2* cell transcripts detected by RT-PCR are able to encode for 44 or 66 kDa HMG-CoA reductase proteins, immunoblot analysis was performed on CHO, UT2, and UT2* whole cell extracts. As expected, a band of 97 kDa and a band of 90 kDa were visualized in the lane corresponding to proteins isolated from CHO cells (**Fig. 7**, panel A). Whole cell extracts from UT2* cells depicted a single band of 90 kDa, previously shown to be localized to the peroxisome (4). The 90 kDa band in UT2 whole cell extracts is barely observed, but as shown previously, can be clearly demonstrated in isolated peroxisomes from UT2 cells (4). If the abnormal transcripts found in UT2 and UT2* cells were successfully translated, truncated proteins of approximately 44 kDa or 66 kDa would be predicted. However, no such products were visualized on the Western blot. In addition, we also used two HMG-CoA reductase antibodies directed against known epitopes present in the abnormal transcripts (anti-peptide G and anti-peptide H reductase antibodies) to immunoprecipitate proteins from [^{35}]S-methionine-labeled CHO and UT2* cell lysates. The anti-peptide G reductase antibody is made against amino acids Arg^{224} through Leu^{242}, and the anti-peptide H reductase antibody is made against amino acids Thr^{284} through Glu^{302}, present in the ER reductase protein. Figure 7, panel B, illustrates the data using anti-peptide H reductase antibodies. The antibody immunoprecipitated the 97 and 90 kDa proteins from [^{35}]S-methionine-labeled CHO cell lysates and the 90 kDa protein from [^{35}]S-methionine labeled UT2* cell lysates. In addition, these proteins were specifically precipitated as they were competed for by an excess of the corresponding free peptide. No smaller size proteins were detected. Similar results were obtained by use of anti-peptide G reductase antibody (data not shown). The faint higher molecular weight bands observed in Fig. 7A are nonspecific. These bands are not immunoprecipitated nor competed out with specific antigen. These data suggest that the abnormal reductase transcripts identified by RT-PCR from UT2 and UT2* cells do not produce truncated proteins that are detectable by immunoblots or immunoprecipitation analysis using antibodies made against

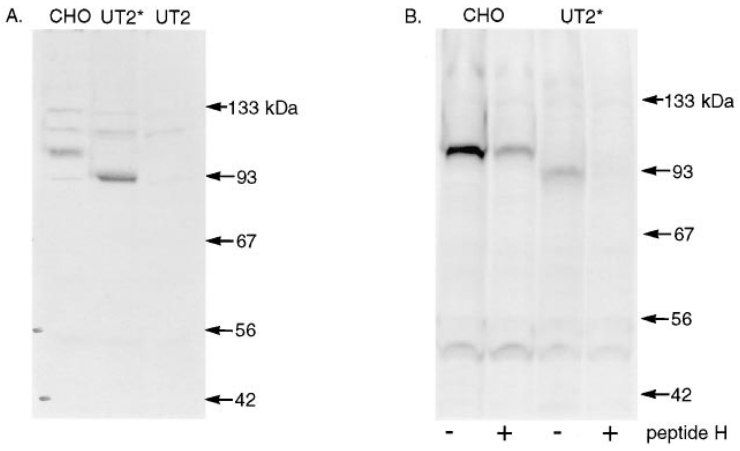

Fig. 7. HMG-CoA reductase immunoblot and immunoprecipitation analysis of cell extracts from CHO, UT2, and UT2* cells. Cell extracts were prepared as described in the Materials and Methods section. Panel A, 200 μg of protein from each cell extract was electrophoresed on a 7.5%, 12.5 cm SDS-polyacrylamide gel. The proteins in the gel were transferred to nitrocellulose, and the membrane was blotted using polyclonal anti-reductase IgG made against the 55–60 kDa catalytic domain. Panel B, CHO and UT2* cell extracts were incubated with [^{35}S]methionine for 3 h, labeled proteins were extracted and incubated with the anti-peptide H HMG-CoA reductase antibody overnight, and immunoprecipitants were isolated on Protein A-Sepharose beads. The anti-peptide H HMG-CoA reductase antibody was preincubated with a 100-fold excess of the corresponding peptide for 30 min prior to immunoprecipitation. Gels were processed as described above, and the ^{35}S-labeled proteins were detected with a Molecular Dynamics PhosphorImager.

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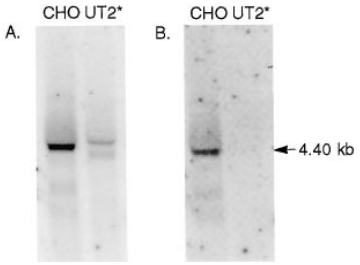

Fig. 8. Hybridization of ^{32}P-labeled reductase cDNA to poly(A)$^+$ RNA from CHO and UT2* cells. Poly (A)$^+$ RNA was isolated from CHO and UT2* cells. Samples of poly (A)$^+$ RNA (10 μg) were resolved on 0.8% agarose gel and transferred to a nylon membrane. The membrane was hybridized with a probe corresponding to the full-length HMG-CoA reductase cDNA (panel A) or with a probe corresponding to exon 11 of HMG-CoA reductase (panel B).

known epitopes present in the abnormal reductase cDNA transcripts.

Northern analysis

The splice site mutations demonstrated in the HMG-CoA reductase gene in UT2 and UT2* cells would not be expected to produce an mRNA containing exon 11. However, by RT-PCR analysis we identified an mRNA containing exon 11. To determine the relative abundance of this message, Northern blot analysis was performed on CHO and UT2* cell poly(A)$^+$ RNA. The mRNA from HMG-CoA reductase in mammalian cells consists of at least two messages, 4.4 and 4.7 kb, due to multiple polyadenylation signals (9, 15). When the Northern membrane was hybridized with a probe consisting of full-length reductase, bands in the 4.4–4.7 kb range were visualized in the lane from CHO cells (**Fig. 8**, panel A). Slightly smaller sized bands were detected in the lane from UT2* cells. When the blot was hybridized with a probe corresponding to exon 11, the same 4.4 and 4.7 kb bands were visualized in the lane from CHO cells; however, there was no message detectable in the lane from UT2* cells. We conclude that the message containing exon 11 amplified by RT-PCR in UT2* cells was produced by rare splicing events, and that the lower size messages observed in the lane from UT2* cells, panel A, are a direct result of exon 11 deletion. The conclusion that messages containing exon 11 are rare is also supported by the observation that of all 12 clones isolated by screening a UT2* cell cDNA library with the full length reductase probe, all were shown to be missing exon 11 (data not shown).

Genomic Southern

To verify that there are no gross deletions of genomic sequences near the 5′ UTR of HMG-CoA reductase or in the 3′ UTR of HMG-CoA reductase, genomic Southern analysis was performed using genomic DNA prepared from CHO, UT2, and UT2* cells. When the membrane was probed with a cDNA region corresponding to exon 2, a single band was visualized in each lane (**Fig. 9**). Similar results were obtained when the 3′UTR probe was used, except that two bands were observed in lane 1 due to the presence of a XbaI restriction site in this probe. Genomic Southern blots of CHO and UT2 cells using the 3′UTR HMG-CoA reductase probe have been previously reported with the same results (3). Additionally, the Southern membrane was hybridized with a probe corresponding to exon 18, which also revealed a single band in each lane (data not shown). As the pattern of hybridizing bands was identical amongst all the three cell lines, this indicates that no gross deletions are present in the reductase gene in UT2 and UT2* cells within the areas probed.

When the blot was probed with exon 11 or exon 12, two bands were visualized in each lane, with all three cell lines sharing an identical pattern of hybridizing bands (Fig. 9). However, when the blots were re-washed at high stringency, generally only one band remained (as illustrated in Fig. 9, for exon 12). The data for the exon 11 blot re-washed at high stringency are not shown. In order to determine the sequence of this cross-hybridizing band, we screened a genomic library enriched for the 3 kb, XbaI genomic DNA fragments. This 3 kb fragment was shown to decrease dramatically in intensity after re-washing the

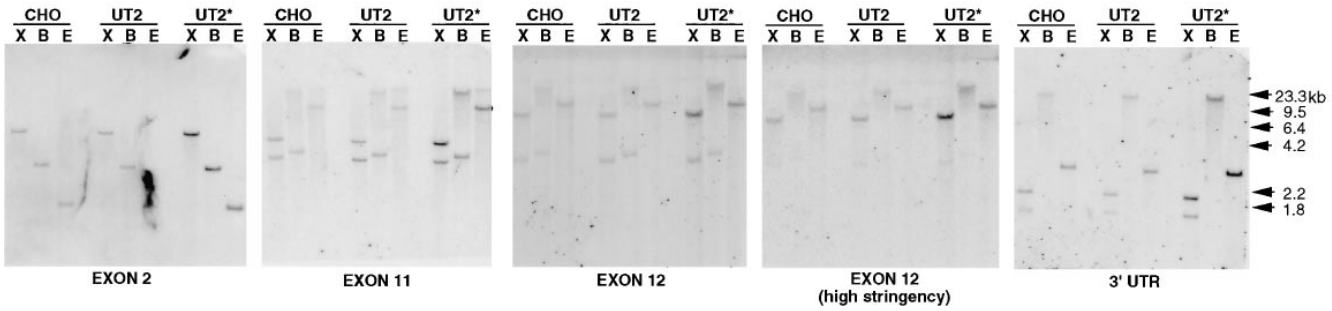

Fig. 9. Genomic Southern blot analysis of CHO, UT2 and UT2* cells. Genomic DNA from CHO, UT2, and UT2* cells was digested with XbaI (lane 1), BamHI (lane 2), and EcoRI (lane 3) and the digests (12 ug of DNA/lane) were electrophoresed in a 0.9% agarose gel for 5 h at 85 V. The DNA was then transferred to Bio-Rad's Zeta-Probe blotting membrane and hybridized with four different cDNA inserts corresponding to exons 2, 11, 12, and 3′ UTR of hamster HMG-CoA reductase. Probe sequences were verified by sequencing. Probes were nick-translated with ^{32}PαdCTP and hybridized overnight at 42°C. Hybridization buffer consisted of 50% formamide, 7% SDS, 0.12 m Na_2HPO_4 and 0.25 m NaCl. Washings for all probes were at 55°C with 0.5 × SSC solution. For exon 12 washing was also done at 72°C (high stringency). Blots were exposed with a Molecular Dynamics Phosphorimager.

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exon 11 and exon 12 blots at high stringency. Two positive clones were isolated and sequenced (data not shown). The nucleotide sequence data revealed a 600 bp region having 65% homology to the known ER HMG-CoA reductase (determined by using Sequencher software). Also, we identified two putative open reading frames (ORFs) of 138 and 60 amino acids. The potential ORF of 138 amino acids had a particularly high homology (88% identity) to the known ER hamster HMG-CoA reductase gene, which included exon 12. This was an interesting observation as the exon 12 region of HMG-CoA reductase is highly conserved among many species (16). Thus, in order to determine whether this novel clone could generate a transcription product, we designed PCR oligonucleotide primers that would selectively amplify the region of the isolated clone that shared homology with HMG-CoA reductase. The primers were used to PCR amplify cDNA from CHO and UT2* cells. There was no product obtained from this amplification from the cDNA from either cell line, but a product of the predicted size was amplified when either CHO, UT2, or UT2* cell genomic DNA was used as a template (data not shown). Because we could not demonstrate a transcription product corresponding to this novel clone, we have concluded that the isolated genomic DNA fragment must be a pseudogene containing diverged sequences of exon 11 and exon 12. This would explain the two band hybridization pattern obtained in the genomic Southern analysis using exons 11 and 12 as probes, as the pseudogene shared sequence homology with these exons. The GenBank accession number of this sequence is AF-049346.

DISCUSSION

The analysis of UT2 and UT2* cell cDNA and genomic DNA has led to the identification of two novel point mutations in the intronic sequences of the ER HMG-CoA reductase gene. The mutation at +1 position (GA) of the 5′ splice site of the exon 11–12 junction was shown to cause exon skipping which results in the insertion of premature stop codons. In all mammalian genes, the GT dinucleotide is 100% conserved at the 5′ splice junction (17) Specifically, the guanine at the +1 position is associated with spliceosome complex formation (18). There are many documented cases in which a mutation at this position causes aberrant splicing that results in skipping of upstream exons (19). In fact, a very similar mutation was recently reported in the cholesteryl ester transfer protein (CETP) gene which leads to a deficiency of the CETP (20). The mutation in the CETP gene in the 5′ splice junction resulted in exon 10 skipping (20). We also identified a second mutation at the +5 position of the 5′ splice site in the intron spanning exon 13 and exon 14. This mutation can cause the activation of a cryptic splice site, GG/GTAAG, which results in the insertion of 47 bp, between exon 13 and exon 14. Similar mutations at this +5 position have been shown to cause abnormal splicing events, such as a G → A mutation in intron 8 of the proα1 collagen gene (21), or a G → A mutation in intron 4 of the vitamin D receptor gene (22). As demonstrated by RT-PCR analysis of UT2 cell mRNA and sequencing of the 12 cDNAs isolated by screening a UT2* cell cDNA library, the point mutation in the intron spanning exon 13 and 14 is observed to be coupled to the skipping of exon 11; therefore, it is likely that these two point mutations are linked to the same allele. Thus, a reversion would be a very rare event, and this finding could explain the low calculated spontaneous reversion rate in these cells (3). The identified splice site mutations in HMG-CoA reductase and the isolated HMG-CoA reductase cDNAs are summarized in **Fig. 10**.

The splice site mutations demonstrated in the HMG-CoA reductase gene in UT2 and UT2* cells would not be expected to produce an mRNA containing exon 11. However, by RT-PCR analysis we identified an mRNA containing exon 11. Therefore, to determine the relative abundance of this message, Northern blot analysis was performed on CHO and UT2* cell poly(A)$^+$ RNA utilizing a full length reductase probe and a probe corresponding to exon 11. When the blot was hybridized with the exon 11 probe, no reductase message was detected in the lane containing the mRNA from UT2* cells. From these data, we conclude that the message containing exon 11, detected by RT-PCR in UT2* cells, was produced by rare splicing events, and that the lower size messages observed in the lane from UT2* cells, when probed with the full length reductase probe, are a direct result of exon 11 deletion. The conclusion that messages containing exon 11 are rare is also supported by the observation that of all 12 clones isolated by screening a UT2* cell cDNA library with the full length reductase probe, all were shown to be missing exon 11 (data not shown).

The data presented suggest that the mutations at the exon 11–12 splice site and the exon 13–14 splice site are responsible for the defect of the 97 kDa HMG-CoA reductase in UT2 and UT2* cells. The mutations cause aberrant spliced messages containing intronic regions. If the aberrant messages were translated, truncated proteins of 44 kDa or 66 kDa would be predicted. More importantly, these truncated proteins would not have catalytic activity due to the loss of the three amino acids required for activity located in exons 13, 17, and 19 (7). No such proteins were visualized on reductase immunoblots or by immunoprecipitation of UT2* cell lysates utilizing reductase antibodies directed against epitopes known to be present in the truncated proteins. This observation might be explained by the following: *i*) translation of the aberrant transcripts is unsuccessful, *ii*) the translation products from these aberrant transcripts are extremely unstable, or *iii*) the translation products are of such low abundance as to be undetectable by the reductase antibodies.

We also detected the presence of a second HMG-CoA reductase allele in the UT2 and UT2* cells. Sequencing of a 2.78 kb genomic HMG-CoA reductase DNA PCR product (spanning exon 10 to exon 12) and PCR analysis of a 446 bp genomic HMG-CoA reductase DNA product (spanning exon 13 to exon 14) indicated the presence of

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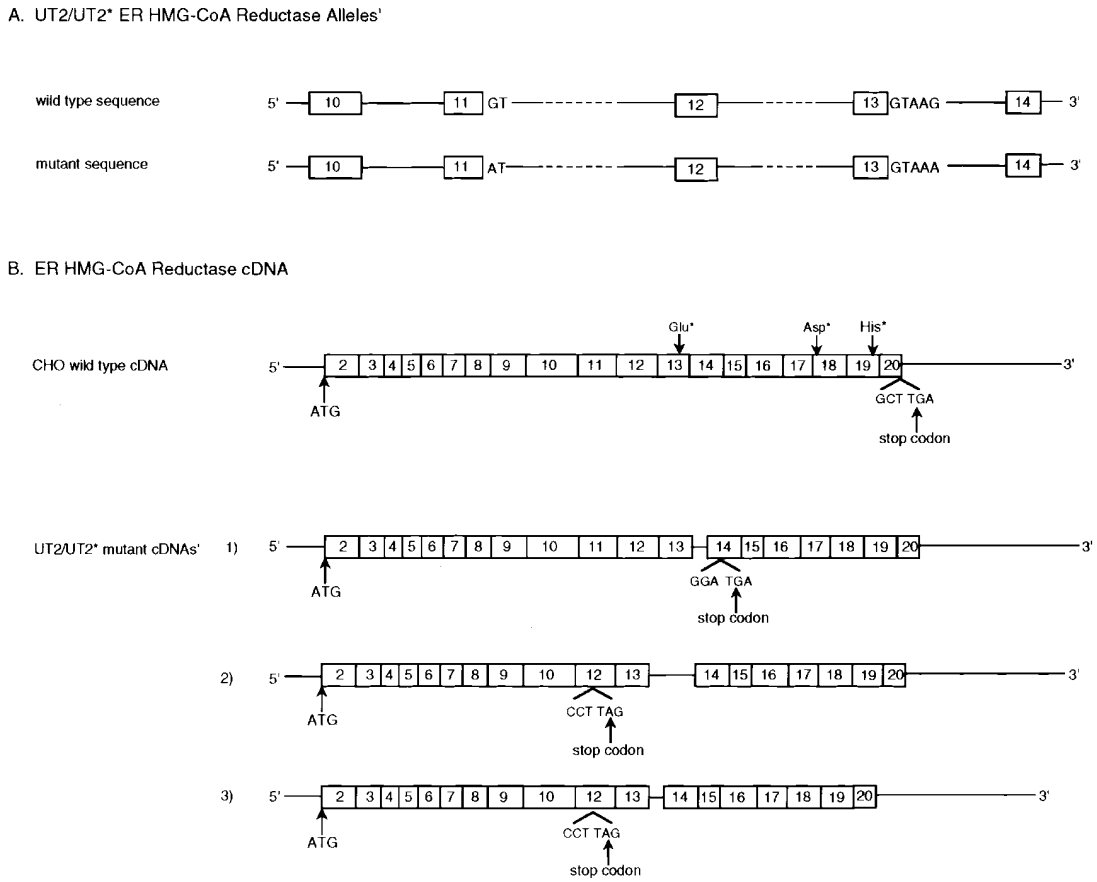

Fig. 10. Summary of ER HMG-CoA reductase genomic mutations identified in UT2/UT2* cells and the identified UT2/UT2* cell mutant cDNAs. Panel A: The ER HMG-CoA reductase in UT2/UT2* cells contains a transition mutation (G to A) at the conserved +1 position of the 5′ donor splice site spanning exons 11 and 12. A second transition mutation (G to A) was identified at the +5 position of the 5′ donor splice site spanning exons 13 and 14. In addition, a wild-type sequence was also isolated from these cells. Panel B: The wild-type sequence of ER HMG-CoA reductase from CHO cells is illustrated showing the locations of the three amino acids (Glu^{558}, Asp^{766}, His^{865}, indicated by *) which are essential for catalysis. From UT2/UT2* cells we have identified three different mutant cDNAs. Transcript 1) terminates translation at amino acid 602, due to a frame shift introduced by the addition of 47 bp of intronic sequence, and if translated would produce a 66 kDa protein. Transcript 2) terminates translation at amino acid 402, due to a frame shift introduced by the deletion of exon 11, and if translated would produce a 44 kDa protein. Transcript 3) also contains a stop codon at amino acid 402, due to a frame shift introduced by the deletion of exon 11, and if translated would be expected to produce a 44 kDa protein. All of the translation products would be predicted to be functionally inactive due to the absence of the catalytic amino acids.

a second allele with normal sequence in these areas. Based on the data that all of the observed mRNAs are consistent with the identified mutations in the "mutated allele" and there are no other discernible transcripts produced from the ER reductase genes, as determined by RT-PCR of UT2* cell mRNA or screening of cDNA libraries, we believe the second allele is not transcribed. In fact, in CHO cells the existence of one active and one silent gene at many autosomal loci is well documented (23).

The UT2 and UT2* cells produce the same aberrant spliced HMG-CoA reductase transcripts unable to code for a 97 or 90 kDa reductase protein. As we have never obtained alternatively spliced messages capable of coding for a 90 kDa protein by use of RT-PCR analysis of CHO or UT2/UT2* cell ER reductase mRNA, or by screening of UT2* cell cDNA libraries with the full-length reductase probe, these data suggest that the 90 kDa HMG-CoA reductase found in CHO and UT2/UT2* cells may be a product of a novel gene.

A similar situation was found in mammalian cells for acetyl CoA carboxylase (ACC). It was long inferred that the multiple forms of ACC detected by SDS-PAGE or immunoblots were the products of limited proteolysis or the result of differential splicing mechanisms (24). However, recently it has been determined that ACC1 and ACC2 are the products of two separate genes (25, 26).

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