The Variant Human Isovaleryl-CoA Dehydrogenase Gene Responsible for Type II Isovaleric Acidemia Determines an RNA Splicing Error, Leading to the Deletion of the Entire Second Coding Exon and the Production of a Truncated Precursor Protein That Interacts Poorly with Mitochondrial Import Receptors*

(Received for publication, July 3, 1991)

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Isovaleryl-CoA dehydrogenase (IVD) is a mitochondrial enzyme involved in leucine metabolism. Previous studies of fibroblasts from patients with isovaleric acidemia (IVA), an inherited defect in IVD, have revealed that IVD precursor protein produced by type II IVA cells is 3 kDa smaller than normal and is processed inefficiently to a mature form which is also 3 kDa smaller than normal. Using the polymerase chain reaction, we have identified a 90-base pair deletion encompassing bases 145–234 in type II IVD cDNA. This deletion is caused by an error in RNA splicing and predicts the in-frame deletion of 30 amino acids beginning with leucine 20 of the mature IVD. The rate of leader peptide cleavage by purified mitochondrial leader peptidases was similar for the variant and normal precursor IVDs expressed in vitro, and radiosequencing confirmed that both mature proteins contain identical amino termini. In vitro import studies showed that the efficiency of overall mitochondrial import of type II variant IVD precursor was approximately 30% of normal, as was its binding to the mitochondrial surface. Unlike its normal counterpart, the bound variant IVD precursor was readily released. These data suggest that binding of the variant protein to mitochondrial membrane receptors per se is hindered, resulting in the inefficient mitochondrial processing.

* This work was supported in part by National Institutes of Health Grant DK 17453 and March of Dimes Grant 1–1230. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: IVA, isovaleric acidemia; IVD, isovaleryl-CoA dehydrogenase; p, precursor; PCR, polymerase chain reaction; bp, base pair(s); nt, nucleotide(s); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

kDa (3). It is nuclear-coded and is synthesized in the cytosol as a 45-kDa precursor (pIVD) (4). Recently, rat and human IVD cDNAs have been cloned and sequenced (5, 6). Human pIVD cDNA encodes 423 amino acids, 1 residue shorter than rat pIVD. The amino terminus of human mature IVD is currently unknown, but, in rat mature IVD, it is histidine 31. This corresponds to histidine 30 in the human pIVD sequence.

Five classes of variant IVD protein have previously been identified in our laboratory using fibroblasts from patients with IVA, based on the presence or absence of the synthesis of immunologically detectable IVD protein and its molecular characteristics (7). We have recently reported the molecular basis of four of these variant IVA types (8). Type I cell lines contain mutant IVD alleles with heterogeneous point mutations. The type III IVD mutation results from a single deletion of T-1179 in the IVD coding region, leading to a shift in reading frame and premature termination of protein translation. The type V variant allele produces an mRNA with a completely normal coding sequence and appears to be defective in protein translation. In addition, a sixth mutant IVD allele producing no mRNA was identified. This variant allele is deficient either in RNA transcription or processing.

Fibroblasts from type II IVA patients have previously been shown to synthesize an truncated precursor IVD which was inefficiently processed to a mature form (7). In cell-labeling experiments using [35S]methionine and immunoprecipitation in the absence of a mitochondrial inhibitor, IVD was detected almost exclusively in its 43-kDa mature form in normal cells, whereas in the type II IVA cells, a variant IVD was detected predominantly in its 42-kDa precursor form and was scarcely processed to a 40-kDa mature form under the same conditions. The intracellular location of type II variant pIVD has not been determined.

Targeting of nuclear-encoded mitochondrial proteins to the proper location and subsequent maturation of the precursor forms are achieved by a sequence of complex biologic processes of current interest. These processes include directing precursors to the mitochondrion (9, 10), binding of the precursors to a putative receptor(s) on the mitochondrial surface (11, 12), import into the inside of the mitochondria (13), cleavage of the leader peptide to produce mature subunits (14–16), and assembling the subunits into a native polymeric form. Recently, the role of chaperonins in the maintenance of an unfolded, import-competent precursor protein in the cytoplasm, and in the intramitochondrial assembly of the native enzyme within mitochondria, has been demonstrated in yeast (17, 18). The study of the nature of the type II variant
IVD defect offers the opportunity to explore the specific steps in the sequence of these naturally occurring cellular events.

EXPERIMENTAL PROCEDURES

RESULTS

Amplification and Sequencing of Normal and Type II IVD cDNA—Initial amplification of the entire coding region of pIVD cDNA was carried out in two sections as shown in Fig. 1A, lines a and b in the Miniprint. The amplification of pIVD cDNA from YH747, an apparent type II variant IVD homozygote cell line, yielded a 5' fragment approximately 100 bp shorter than control, while the 3' fragment was of normal size (Fig. 2A). The same smaller 5' fragment was observed in numerous experiments using three independently synthesized cDNA samples from YH747, ensuring that the size difference seen was reproducible. Each amplified PCR fragment was then reamplified to produce single-stranded DNA and sequenced directly in its entirety in both directions using IVD-specific oligonucleotide primers. Sequencing of the 5' fragment from YH747 cDNA revealed the deletion of 90 nucleotides beginning with position 145 and ending with nucleotide 234 (Fig. 2C). This result was confirmed using PCR fragments amplified from two independent samples of mutant fibroblast cDNA. The 90-bp deletion allows for maintenance of the normal translational reading frame and leads to the predicted loss of 30 amino acids beginning with leucine 49 of pIVD (leucine 20 of the mature protein); however, the leader peptide is unaffected (Fig. 3).

In vitro transcription/translation and mitochondrial import of normal and type II variant IVD. Control and type II variant precursor IVD cDNAs were transcribed and translated in vitro as described under "Experimental Procedures," and each preparation was divided into 2 aliquots. After an aliquot of each translation product was incubated with freshly isolated rat liver mitochondria to test for import competence, both aliquots of the reaction mixture were analyzed by SDS-PAGE and autoradiography. Without incubation with mitochondria, the sizes of normal (lane 1) and type II variant (lane 3) IVD precursors are 45 and 42 kDa, respectively. After incubation with mitochondria, normal and type II pIVDs are processed to a respective mature form of 43 and 40 kDa (lanes 2 and 4, respectively). Note that the distance between the precursor and the mature IVD bands is markedly greater in type II variant than in normal.
poorly processed, with the bulk remaining as precursor (Fig. 6, lane 4), reproducing the observation made in vitro.

In Vitro Proteolysis of Normal and Variant pIVD by Purified Mitochondrial Matrix Leader Peptide Peptidases—One possible reason for inefficient conversion of the type II variant pIVD to its mature form is resistance to mitochondrial leader peptide peptidases. Hence, we studied susceptibility of normal and variant pIVDs to the two described peptidases, mitochondrial processing peptidase and mitochondrial intermediate peptidase, using the purified preparations (14). Both normal and variant pIVD were proteolytically cleaved by mitochondrial processing peptidase at similar efficiency: under the experimental conditions, 12.3% and 11.7% of normal and variant pIVDs, respectively, were converted to their mature counterpart (Fig. 7, Miniprint). Mitochondrial intermediate peptidase had no effect on either normal or variant pIVD, either by itself or in conjunction with mitochondrial processing peptidase. It should be noted that the difference in the mobility between the precursor and mature protein bands on SDS-PAGE appeared greater in type II IVD than in normal IVD, both in this experiment (Fig. 7, Miniprint section) and in the previous one (Fig. 6).

Amino-terminal Sequence Analysis of Mature Normal and Type II IVD—Although the variant pIVD sequence contains the known cleavage signal for the mitochondrial leader peptide peptidases, because of the apparent larger size difference between the precursor and mature variant IVDs, it was prudent to test whether the normal and variant pIVDs were cleaved at the same site. Normal and variant mature IVDs labeled with [3H]leucine were prepared and sequenced as described under “Experimental Procedures.” The release of [3H]leucine occurred in cycles 3, 4, and 13 from both normal and variant IVD (Fig. 8, Miniprint), indicating that the leader sequence of both normal and variant pIVDs was cleaved between alanine 29 and histidine 30. This is in agreement with the predicted cleavage site and with the mature amino terminus identified in the rat enzyme, and it definitively identifies histidine 30 as the amino terminus in human IVD.

In order to evaluate the presence of the amino acids encoded by the deleted exon, five additional cycles of radiosequencing were performed on the type II variant IVD. In normal IVD, the 20-position should be leucine, and it represents the first amino acid sequence.

Mitochondrial Import of Normal and Type II Variant pIVD—The above data preclude the possibility that the inefficient overall processing of type II variant pIVD is the result of hindrance of leader peptide cleavage, thus implicating mitochondrial uptake as being deficient. When the import reaction was stopped after varying lengths of time and separated into a mitochondrial pellet and supernatant fractions, the mature form of normal IVD could be detected in a significant amount in the pellet within 10 min, with a gradual increase in accumulation over the subsequent 20 min (Fig. 9). The precursor was also detected in all pellets in similar amounts, suggesting its location at the mitochondrial surface. However, the mature variant IVD band was barely detectable at 10 min, and, although it increased with time, the amount of mature IVD was much smaller at 30 min in the variant than in the control. The proportion of the mutant enzyme processed to the mature form at each time point was much smaller than for normal IVD.

To study the intramitochondrial location of the precursor and mature IVD proteins in the pellet, in vitro translation product was first incubated with isolated rat liver mitochondria at 27 °C. The mitochondrial pellet was then treated with 5 μg of trypsin per mg of protein on ice for 10 min and analyzed by SDS-PAGE and autoradiography. A, SDS-PAGE analysis of the reaction mixtures. Lanes 1–4 and 5–8 show the total mitochondria-associated, radioactive IVD proteins detected without and with trypsin treatment, respectively. Note that the distance between the precursor and the mature IVD bands is markedly greater in type II variant than in normal. B, time course of the amount of normal and type II variant pIVD that is processed. The mature IVD bands from the trypsin-treated samples, shown in A, were excised form the gel, and their radioactivity was counted by liquid scintillation and compared with the counts contained in the precursor band at 0 min without trypsin digestion. The time course of the percentage of the trypsin-resistant counts is plotted.

FIG. 9. Fractionation of normal and type II variant pIVDs following mitochondrial import. [35S]Methionine-labeled normal and variant pIVDs, produced via in vitro transcription/translation, were incubated with freshly prepared rat liver mitochondria at 27 °C in order to allow the import process to proceed, separated into supernatant and pellet fractions, and analyzed by SDS-PAGE. Lane 1 shows the import reaction mixtures at 0 min. Lanes 2–4 and 5–7 show the supernatant and pellet fractions, respectively. The length of incubation is indicated at the top of each lane.

FIG. 10. Time course of mitochondrial import of normal and variant pIVDs. After [35S]methionine-labeled normal and variant pIVDs, produced via in vitro transcription/translation, were incubated with freshly prepared rat liver mitochondria at 27 °C for 10–30 min, mitochondrial pellet was isolated and treated with 5 μg (normal IVD) or 20 μg (variant IVD) of trypsin per mg of protein on ice for 10 min and analyzed by SDS-PAGE and autoradiography. A, SDS-PAGE analysis of the reaction mixtures. Lanes 1–4 and 5–8 show the total mitochondria-associated, radioactive IVD proteins detected without and with trypsin treatment, respectively. Note that the distance between the precursor and the mature IVD bands is markedly greater in type II variant than in normal. B, time course of the amount of normal and type II variant pIVD that is processed. The mature IVD bands from the trypsin-treated samples, shown in A, were excised form the gel, and their radioactivity was counted by liquid scintillation and compared with the counts contained in the precursor band at 0 min without trypsin digestion. The time course of the percentage of the trypsin-resistant counts is plotted.
The rate of import/processing was quantitated by excising from the gel the precursor IVD band from the zero time point of the import reaction (Fig. 10A, lanes 1 and 8) and counting the incorporated [35S]methionine by liquid scintillation. This was then compared with radiolabel incorporated into the trypsin-resistant mature bands at each time point. Approximately 35% of the normal IVD precursor was processed within 10 min, increasing to 40% by 30 min (Fig. 10B). In contrast, variant IVD precursor was processed much more slowly, at about one-third of the rate for normal IVD at each time point. A difference of similar magnitude between normal and the variant was consistently found in three separate experiments.

**Binding of Normal and Variant pIVDs to the Mitochondrial Surface**—In order to identify the step in the processing pathway causing the ineffective mitochondrial uptake of the type II variant pIVD, we first evaluated the efficiency of binding to the mitochondrial membrane. Cell-free translation product was incubated with rat liver mitochondria at 0 °C for 0, 10, 20, or 30 min. Under these conditions, attachment of the precursor protein to mitochondrial membrane transport receptors occurs, but import does not proceed (4). The incubation mixture was separated into supernatant and pellet fractions which were analyzed by SDS-PAGE and autoradiography (Fig. 11). The labeled precursor band from the supernatant and pellet fractions was excised from the gel and was quantitated by liquid scintillation. Three independent sets of experiments were performed to confirm the consistency of the results as summarized in Table I. In each experiment, all steps including transcription, translation, and isolation of fresh mitochondria were performed identically. The amount of variant pIVD synthesized by in vitro transcription/translation was 1.5–2.3 times greater than that of normal pIVD in all three experiments, although the same amount of template, precursor, and reagent were used. In the binding experiments, the same volume of normal or variant translation mixture was added to the binding reaction mixture. Thus, the amount of variant pIVD added in the reaction mixture was larger than that of normal pIVD. Binding of both normal and variant pIVD reached near plateau within the first 10 min and gradually increased thereafter. In spite of the greater amount of variant pIVD added to the same amount of mitochondria as compared with normal pIVD, the amount of bound variant pIVD was 1.5–2.4 times less than that of bound normal pIVD at all time points in all three experiments. When the data are expressed as percentages, 48–62% of total normal pIVD was bound to mitochondria at 30 min, whereas only 14–20% of total variant pIVD added was recovered in the mitochondrial pellet at the same time point. The percentage of bound pIVD at each point is consistent for each pIVD protein regardless of the marked difference in the amount of added pIVDs, with the variant representing only 22–41% of normal control at all time points in three experiments.

**Dissociation of Bound Precursors**—In the study of internalization, we first tried to wash the mitochondrial pellet with fresh buffer to remove unbound precursors before raising the temperature to 27 °C. After one wash, no drastic reduction was observed in the amount of the mitochondria-bound normal and variant pIVDs. After washing twice, the amount of mitochondria-bound normal and variant pIVDs were both decreased, but a significant amount of normal pIVD remained on the mitochondrial surface. In contrast, a much smaller amount of variant pIVD remained bound after two washes at 0 °C (not shown). This suggested that the bound variant pIVD more readily dissociates from the receptor on the mitochondrial surface than its normal counterpart.

We explored this phenomenon further by incubating normal and mutant pIVD with mitochondria at 0 °C, then adding 7-fold excess of either unlabeled normal or type II mutant pIVD translation mixture. This mixture was incubated for an additional 30 min at 0 °C, and aliquots were analyzed and quantitated for mitochondria-bound pIVDs as above. Under these conditions, labeled normal pIVD dissociated slightly from the mitochondrial membrane, but remained stably bound thereafter (Fig. 12, A and B). In contrast, approximately half of the variant pIVD was released from the mitochondrial surface (Fig. 12B). Similar results were obtained when cold excess variant pIVD rather than normal pIVD was used in these experiments.

### TABLE I

<table>
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<th>Normal/variant</th>
<th>Total pIVD added at 0 min</th>
<th>pIVD in the pellet</th>
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<td>Variant pIVD</td>
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<td>866</td>
</tr>
<tr>
<td>Variant pIVD</td>
<td>3485</td>
<td>613</td>
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</tbody>
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*ND, not determined.

![Fig. 11](image)
Defective Mitochondrial Binding of Type II Variant IVD

![Graph showing release of bound pIVDs from the mitochondrial surface](image)

**Fig. 12. Release of the bound pIVDs from the mitochondrial surface at 0 °C.** [125] Methionine-labeled normal and variant pIVDs produced via in vitro transcription/translation were incubated with freshly prepared mitochondria at 0 °C for 30 min. After a 7-fold excess of unlabeled translation product was added, the reaction mixture was further incubated at 0 °C for an additional 10–30 min. Mitochondrial pellets were then isolated and analyzed by SDS-PAGE and autoradiography. A. SDS-PAGE analysis of the incubation mixture. Lanes 1 and 2 show the bound pIVDs prior to and immediately following the addition of unlabeled translation product, respectively. Lanes 3–5 show pIVD that remains bound to the mitochondrial surface after 10, 20, and 30 min, respectively, of incubation after the addition of excess unlabeled translation product. B, time course of the amount of bound pIVD. The labeled pIVD bands, shown in A, lanes 2–5, were excised from the gel, and their radioactivity was counted by liquid scintillation and compared with the radioactivity in total pIVD band in lane 1. Time course of the percentage of precursor remaining bound is plotted.

The region of the IVD gene, indicating a post-transcriptional origin of the deletion. The deleted sequence precisely corresponds to the entire coding exon 2 sequence. Aberrant splicing of IVD mRNA, with direct joining of exon 1 to exon 3, is the most likely explanation for these findings. Reports of splicing abnormalities as a cause of human genetic disease are becoming more frequent. Such events have been shown to occur as a result of alteration of the splicing signals at the intron/exon junctions (for examples, see Refs. 23–28). Alternatively, mutations at a more distant site, often within an intron, can lead to the generation of a new, more efficient splicing signal that preempts the normal one (for examples, see Refs. 24 and 29–31). In the case of YH747, the consensus splicing signals (32, 33) for removal of intron 2 are intact. Thus, the cause of the aberrant splicing of type II IVD mRNA remains unknown.

At the levels of protein and cDNA, only a single type II variant IVD species was detected in YH747, giving the impression that this cell line was homozygous for a single type II variant IVD allele. However, at the gene level, the presence of two distinct alleles was indicated by the presence of a silent polymorphism at nucleotide 723 in the PCR product of the 107-bp section of exon 7. Since only the type II mRNA species with C-723 was found in the cytoplasm of the mutant fibroblasts, the second abnormal allele in this cell line must either be transcriptionally inactive or encode an RNA that is unstable and never reaches the cytoplasm. We have recently reported a similar variant IVD allele in a compound mutant cell line that also contained a point mutation (8). The analysis of variant IVD cDNA from YH834 confirmed that this cell line was indeed a compound mutant for types I and II. Although the 90-bp deletion in type II cDNA in YH834 is identical with that in YH747, the residue at position 723 is C in the YH834 allele, not T as in YH747. This finding suggests the possibility that this same mutation has independently occurred more than once in the IVD gene. Alternatively, the mutation itself may have occurred only once, with the polymorphism at position 723 representing an evolutionarily later event.

In the expression and processing of nuclear-encoded mitochondrial proteins, precursors of these proteins must first be guided from the cytosol and imported into the mitochondrial matrix and proteolytically processed into a mature subunit (10, 13). In the case of IVD, mature subunits are then assembled into tetramers. The information necessary for correct targeting of the precursor protein to the mitochondria is contained solely within the leader peptide (9, 10). The specificity of the mitochondrial peptidases that cleave the leader peptide also appears to depend in large part on the sequence of the leader peptide and adjacent amino-terminal region (14–16). These sequences are normal in type II IVD. In this work we have shown that after the import of de novo-translated variant pIVD, only its mature counterpart was detected inside the mitochondria as in the case of normal IVD, indicating that cleavage of mutant pIVD was indeed normal. These data are further supported by the experiment using purified leader peptide proteases.

In spite of the normal processing after mitochondrial uptake, the in vitro expression studies presented here indicate that the efficiency of the overall mitochondrial import-processing of type II variant IVD precursor is only 30% of control. Such reduced efficiency may result from a defect in one of the remaining steps in the processing pathway, such as binding to the receptor or cytosolic import factors, or energy-dependent internalization. We have shown here that binding of variant pIVD to the mitochondrial surface at 0 °C was only 22–41% of normal pIVD. Furthermore, variant pIVD that is bound to the mitochondrial surface at 0 °C appears to be more readily released than its normal counterpart by centrifugation and re-suspension of the mitochondrial pellet and can be competed off of the receptor more easily than normal pIVD. These data suggest that binding of variant pIVD to the mitochondrial import receptor is weaker than for normal pIVD. Thus, the analysis of the various steps of the import-processing pathway in vitro indicates that the ineffective mitochondrial import of type II variant pIVD is most likely due to its defective binding to the mitochondrial import receptor per se (11, 12). However, our data do not exclude the possibility that the association of type II variant pIVD with cytoplasmic proteins involved in mitochondrial transport (chaperonins) is also inhibited, leading to the lack of maintenance of a proper physical state for subsequent association with the mitochondrial import receptor (17, 18). Direct binding experiments with normal and type II variant pIVD and purified transport factors will be necessary to resolve this definitively.

Several mechanisms for the defective binding of type II variant pIVD to the mitochondrial import receptor can be considered. The sequence of the deleted 30 amino acids may play a direct role in the recognition and/or binding of precursor to the import receptor on the mitochondrial surface. Alternatively, this may be the result of the altered tertiary structure of the variant IVD protein. Computer analysis of type II variant pIVD secondary structure predicts a disruption of an α-helical region immediately downstream of the amino terminus of the mature protein. Such a conformational change may interfere with interaction of the variant precursor with the mitochondrial receptor.

Only two naturally occurring mutations affecting the processing of nuclear-encoded mitochondrial proteins have previously been described in humans. In the first, a point muta-
tion in amino acid residue 319 of ornithine aminotransferase was shown to affect processing to mature form after uptake of precursor protein by mitochondria (34). This mutation was considerably further downstream from the mature amino terminus than seen in type II VD. A second mutation, detected in a variant human methionyl-CoA mutase, appeared to lead to the production of two smaller enzyme species which were not processed (35). The defect in this cell line has recently been shown to be due to a point mutation which changes the codon for glutamate 18 of the precursor sequence to a premature protein termination codon, with subsequent

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REFERENCES


Continued on next page.
Defective Mitochondrial Binding of Type II Variant IVD

Supplemental Material

The Variant Human Isotransyl-CoA Dehydrogenase Gene Responsible for Type II Isocitrate Lyase Dehydrogenase Deficiency Determines an RNA Splicing Error. Leading to the Deletion of the Essential Second Codon and Translation of the NcoI Coding Region (shown as hatched area), which was amplified for direct sequencing in two overlapping fragments with small amounts of untranscribed sequence at the 5' and 3' ends (Fig. 4A and C). Additionally, a smaller C fragment containing the identified type II IVD mutation was amplified to study YH834 cDNA (line c). A larger PCR fragment containing three fourths of the 3' region of the NcoI coding sequence was amplified to amplify the type II allele in YH834 (line e). A fragment of genomic DNA containing the NcoI polymorphism was amplified to identify the nucleotide in both IVD alleles of YH747 (line f).

Amplicon of genomic DNA. The 212 bp fragment containing the NcoI polymorphism was amplified to amplify the type II allele in YH834 (lines d and e). A fragment of genomic DNA containing the NcoI polymorphism was amplified to identify the nucleotide in both IVD alleles of YH747 (line f). Amplification of genomic DNA. The 212 bp fragment containing the NcoI polymorphism was amplified to amplify the type II allele in YH834 (line f).

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Study of Genomic DNA and Origin of the 90 Base Deletion  

IVD genomic sequence data indicate that the deletion seen in type II variant IVD cDNA precisely corresponds to the sequence of the second coding exon of the gene (R. Perinovi and K. Tanaka, unpublished data). Such a change at the cDNA level can be explained either by a deletion encompassing all or part of introns 1 and 2, leaving exons 1 and 3 intact, or by a mutation leading to the aberrant splicing of exon 1 to exon 3, entirely deleting exon 2. Southern blotting experiments were first performed to evaluate the possibility of a deletion. Hind III does not cleave within the IVD coding sequences, and when control genomic DNA is digested with this enzyme and a Southern blot is probed with a 330 bp cDNA IVD fragment containing all of exons 1-3 and part of exon 4, two bands of 6200 and 900 bp are seen (Fig. 5, lanes 1 and 2). Using exon specific oligonucleotides as hybridization probes, we have determined that the 900 bp fragment contains all of exons 2 and 3, but no other coding sequences (data not shown). This fragment thus encompasses the region detected in type II cDNA. Digestion of YH834 with Hind III, followed by Southern blotting and hybridization with the same 330 bp 5'-probe yielded a pattern indistinguishable from control. Specifically, the 900 bp fragment was of the same intensity as control, and no new junction fragment indicative of a deletion was seen (Fig. 5, lane 1). Bgl I normally has only one restriction site in exon 2 and one in exon 4. Thus, if exon 2 were deleted in the type II IVD genomic DNA, the disappearance of a Bgl I band and the appearance of a new larger one would be expected in Southern blots from YH747. To study the intron 1 acceptor site, a 100 bp section, defined as in Fig. 1A, lane b, was amplified. For the study of donor and acceptor sites for intron 2 splicing, a 360 bp fragment of genomic DNA, encompassing the entire exon 2 (nt 145-234), intron 2 (213 bp) and entire exon 3 (nt 235-287) was amplified using a pair of 21mer primers as illustrated in Fig. 1B, lane a. Using these primers, single fragments of approximately 360 bp and 120 bp, respectively were amplified from both control and mutant genomic DNA. The sequences of the intron/exon junctions and consensus splice sites, as well as the entire intron 2 sequence and 55 bp of the 3'-portion of intron 1, were identical in amplified DNA from normal and mutant DNA.

Demonstration of Two Distinct Type 2 Variant IVD Alleles With Silent Polymorphisms at Position 723  

YH747 was previously considered to be homozygous for the type II IVD allele, since only type II variant IVD protein and cDNA were detectable in this cell line. To determine whether or not this cell line is truly homozygous at the IVD locus, we focused on the single base polymorphism at position 723 of the cDNA (Fig. 4A). Amplification of this region from YH747 genomic DNA using the primers shown in Fig. 1A, lane a, followed by direct sequencing, unexpectedly resulted in the identification of both a T and a G allele at position 723 (Fig. 4B), indicating that YH747 must contain two IVD alleles.

In the type II variant IVD allele in YH834, the nucleotide at position 723 in the type I allele was C (Fig. 4C), as in the normal sequence rather than the T found in the type II variant IVD cDNA from YH747 (Fig. 4A).

Figure 5. Southern Blot Analysis of YH834 and YH1074 Genomic DNA. Genomic DNA from YH834 or YH1074 was digested with either Hind III or Bgl I, run on a 0.8% agarose gel, and analyzed by Southern hybridization using a 360 bp probe corresponding to the 3'-most coding segment of IVD cDNA. Lanes 1 and 2 show YH1074 and YH834 respectively digested with Hind III. Lanes 3 and 4 show YH1074 and YH834 respectively digested with Bgl I.

Figure 7. Susceptibility of Normal and Type II Variant IVDs to Mitochondrial Leader Peptide Processing Peptidases. [35S]Methionine labeled normal and type II variant IVDs produced via in vitro transcription/translation were incubated with purified mitochondrial processing peptidase (MPP) or mitochondrial intermediate peptidase (MIP), or with both, and incubated at 37°C for 1 hr. The products were analyzed by SDS-PAGE and autoradiography. Addition of no addition of the peptidases is indicated at the top of each lane.