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An Intronic 10-Base-Pair Deletion in a Class II Aβ Gene Affects RNA Processing

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Several biologically important examples of posttranscriptionally regulated genes have recently been described (T. Gerster, D. Picard, and W. Schaffner, Cell 45:45–52, 1986; R. Reeves, T. S. Elton, M. S. Nissen, D. Lehn, and K. R. Johnson, Proc. Natl. Acad. Sci. USA 84:6531–6535, 1987; H. A. Young, L. Varesio, and P. Hwu, Mol. Cell. Biol. 6:2253–2256, 1986). Little is known, however, regarding sequences that mediate posttranscriptional RNA stability. Characterization in our laboratory of a mutant murine B lymphoma, M12.C3, revealed a posttranscriptional defect affecting the synthesis of a major histocompatibility complex class II gene ($A\beta^d$) whose product normally controls both the specificity and magnitude of the immune response. Molecular studies revealed that the mutation responsible for diminished $A\beta^d$ gene expression was an intronic deletion of 10 base pairs (bp) located 99 bp 5′ of the third exon. This deletion lies in a region not known to be critical for accurate and efficient splicing. Furthermore, sequence analysis of amplified Aβ-specific cDNA demonstrated that the small number of $A\beta^d$ transcripts produced in the mutant cells was correctly spliced. It appears that the mechanism by which this intronic 10-bp deletion acts to decrease RNA stability is unlikely to be at the level of RNA splicing.

Significant progress has been achieved in identifying transcriptionally active DNA sequences; however, relatively little is known regarding sequences that act posttranscriptionally to regulate gene expression. Recently, it has become apparent that many genes are regulated at the posttranscriptional level (8, 24, 36). One notable instance involves the immunoglobulin heavy-chain gene. Gerster et al. (8) have found that the activity of the immunoglobulin heavy-chain gene enhancer and hence the transcription rate of the immunoglobulin heavy-chain gene are both high in pre-B-cell lines, although mRNA levels in these cells are relatively low. These studies indicate that increased levels of immunoglobulin gene expression in plasma cells are dependent upon posttranscriptional mechanisms. Furthermore, the induction of human beta and gamma interferon expression (22, 36), the developmental regulation of the *psp* gene in the mouse parotid gland (33), and the tissue-specific expression of glyceraldehyde 3-phosphate dehydrogenase in rat tissue (21) all appear to be partially controlled by posttranscriptional events.

The *cis*-acting sequences that act posttranscriptionally to regulate gene expression are not known for many of these cases. However, an AT-rich sequence has recently been identified that causes selective mRNA degradation when introduced into the 3′ untranslated region of the β-globin gene, which normally produces a stable mRNA (32). This AT-rich sequence has been found in the 3′ untranslated region of a number of posttranscriptionally regulated genes, including the human beta and gamma interferons as well as the bovine interleukin-2 gene (2, 25, 32). A labile nuclease in bovine lymphoid and CV-1 fibroblast cells that selectively destroys interleukin-2 mRNA and other mRNAs containing this 3′ AU-rich sequence may be involved in these events (24).

The sequence requirements, if any, for intranuclear stability or transport of heterogeneous nuclear RNA (hnRNA) to the splicing machinery are unknown, although consensus splice site sequences have been identified. Recently, an intronic point mutation within the c-Ha-*ras* oncogene has been shown to cause a 10-fold increase in expression that may be mediated by increased hnRNA stability (7). To our knowledge however, no intronic sequence has been identified that decreases hnRNA stability, although mutations in donor-acceptor splice sites do have profound posttranscriptional consequences (20, 31).

We report here the molecular characterization of a mutant B lymphoma cell line that produces only minimal levels of cytoplasmic mRNA encoded by the class II Aβ gene. The class II major histocompatibility complex (MHC) antigens are integral membrane-bound proteins encoded in the I region of the MHC. They are normally expressed on B lymphocytes, dendritic cells, and most cells of the mononuclear phagocyte system, and they control both the specificity and the intensity of the immune response. In this mutant B-cell line, the transcription rate for the class II Aβ gene is normal and the transcripts produced from the Aβ gene are correctly spliced. Sequence analysis and transfection experiments with recombinant Aβ genes demonstrate that a small intronic 10-base-pair (bp) deletion between the β1 and β2 exons is responsible for the failure of this B-cell line to produce Aβ protein. This intronic 10-bp sequence appears to be necessary for posttranscriptional stability of Aβ hnRNA in murine B cells.

MATERIALS AND METHODS

RNA analysis. Total cellular RNA was isolated with the use of guanidine isothiocyanate (5). Samples (10 to 15 μg) of RNA were electrophoresed on 1% agarose gels containing

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formaldehyde and were transferred to nitrocellulose as described by Thomas (35). A 2.1-kilobase (kb) *Bam*HI DNA fragment derived from an $A\beta^k$ genomic clone (6) and a 2.0-kb *Bam*HI fragment derived from an $E\beta^d$ genomic clone (9) were used as probes to detect Aβ and Eβ sequences, respectively.

Nuclear runoff transcription. Nuclei were isolated by using a modification of the method of Groudine et al. (10). In vitro transcription and RNA purification were performed as described previously (10). Purified nuclear RNA was hybridized to denatured DNA probes (10 to 20 μg) immobilized to nitrocellulose filters using a Schleicher & Schuell slot blot apparatus. A 1.7-kb genomic *Hin*dIII fragment (ligated into the M13 vector) was used as a single-stranded probe for Aβ-specific RNA; this probe does not detect Eβ-derived RNAs. A rat housekeeping cDNA clone, A50 (18), was used as a positive control, and the single-stranded M13 vector alone served as a negative control. Each filter was hybridized for 36 to 48 h at 65°C with approximately 2.5×10^6 cpm of ^{32}P-labeled RNA. For washing, the filters were cut such that the rat-mouse hybrids (A50 probe) could be washed at a lower stringency (65°C, 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]), while the mouse-mouse hybrids were washed for 2 h at 65°C in 2× SSC.

Cloning and sequencing of the M12.C3 $A\beta^d$ gene. A plasmid genomic library was prepared (14) from size-selected, *Bam*HI-digested genomic DNA from the M12.C3 cell line. An Aβ-specific probe (2.1-kb *Bam*HI fragment of a genomic $A\beta^k$ clone [6]) was used to probe nitrocellulose filters from the genomic library, and a 10-kb Aβ clone was identified. A 2.9-kb *Sac*II-*Bam*HI fragment of the $A\beta^d$ clone including the β2, transmembrane, and first cytoplasmic domains was sequenced by the dideoxy-chain termination method with M13 vectors (29).

DNA-mediated transfection of the M12.C3 cell line. Plasmids were linearized with *Sal*I before transfection. Briefly, the M12.C3 cells were cotransfected as previously described (19) with 50 μg of $pA\beta^b$ (mutant, wild type, or exon-shuffled) and with 40 μg of pMSV-neo (17), which contains the neomycin resistance gene as a selectable marker. For shuffle B (see Fig. 2B), 50 μg of *Sal*I-linearized $pA\alpha^b$ was included in the cotransfection. The transfected cells were then seeded into 24-well tissue culture plates. At 48 to 72 h after transfection, a selection medium containing 0.20 mg of G418 neomycin (GIBCO) per ml was added to cultures, and 2 to 3 weeks later, the wells containing neomycin-resistant cells were expanded for fluorescence-activated cell sorting analysis using the 25-9-17 and 34-5-3 monoclonal antibodies (anti-I-$A^{b,d}$) to detect surface I-A^b or the MKD6 (anti-I-A^d) monoclonal antibody to detect surface I-A^d (1).

Sequencing of the M12.C3 $A\beta^d$ cDNA. The cDNA for the M12.$A\beta^d$ gene was produced by using the polymerase chain reaction (PCR) to amplify from M12.C3 $A\beta^d$ mRNA. In brief, 600 ng of poly(A)+ RNA was used for cDNA synthesis as previously described (11). Two oligonucleotides were used for amplifying the cDNA: 5′ CATTACCTGTGCCTT AGA 3′ (coding) and 3′ GGACGTACAGAGACGAA 5′ (noncoding), which bind upstream of the leader sequence and downstream of the second cytoplasmic sequence, respectively. PCR was carried out essentially as previously described (28). Briefly, a 50-μl reaction mixture was cycled 70 times, each cycle consisting of 45 s at 94°C for denaturation, 1 min at 53°C for annealing, and 10 min at 72°C for polymerization. The cDNA produced was then ligated into the M13 vector for sequence analysis. Internal oligonucleotide sequences as well as the universal primer were used for sequencing the entire cDNA, using the dideoxy-chain termination method (29).

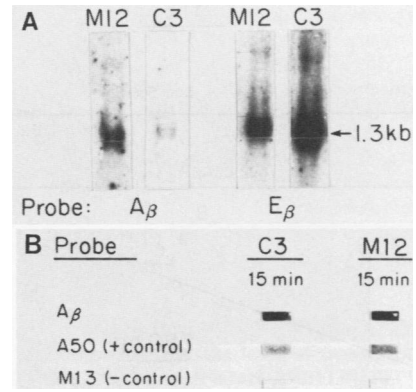

FIG. 1. (A) RNA analysis. Total cellular RNA was isolated with the use of guanidine isothiocyanate (5). Samples (10 to 15 μg) of RNA were electrophoresed on 1% agarose gels containing formaldehyde and were transferred to nitrocellulose as described by Thomas (35). Northern blots were hybridized to Aβ probe (lanes 1 and 2) or to Eβ probe (lanes 3 and 4). The Aβ probe used does not cross-react with I-E β-chain mRNAs. (B) Nuclear runoff transcription. Purified nuclear RNA was hybridized to denatured DNA probes (10 to 20 μg) immobilized to nitrocellulose filters, using a Schleicher & Schuell slot blot apparatus. A 1.7-kb genomic *Hin*dIII fragment (ligated into the M13 vector) was used as a single-stranded probe for Aβ-specific RNA; this probe does not detect Eβ-derived RNAs. A rat housekeeping cDNA clone, A50 (18), was used as a positive control, and the single-stranded M13 vector alone served as a negative control. Each filter was hybridized for 36 to 48 h at 65°C with approximately 2.0×10^6 cpm of ^{32}P-labeled RNA. Densitometric scanning was performed on Aβ bands from the 24-h exposure film and on A50 (positive control) bands from the 5-day exposure film to create electrical deflections that would allow for precise ratio calculations. M12, M12.4.1 (wild type); C3, M12.C3 (mutant).

RESULTS

The M12.C3 surface Ia^- phenotypic variant produces a posttranscriptionally unstable Aβ RNA. The M12.C3 B-lymphoma cell line is an I-A-negative variant of the M12.4.1 wild-type B lymphoma cell line. The M12.C3 mutant was produced by irradiation and negative immunoselection as described previously (9).

The results of Northern (RNA) blot analysis of total cellular RNA from both the wild-type M12.4.1 and variant M12.C3 B lymphoma cells are shown in Fig. 1A. Hybridization with Aβ-specific probes revealed that the level of Aβ mRNA in M12.C3 cells is greatly reduced compared with that in the wild-type M12.4.1 cells; however, both cells contain normal levels of Aα (9) and Eβ mRNAs. Approximately equal amounts of RNA were added to each lane, as demonstrated by the comparable signals generated from each lane after hybridization to a β2-microglobulin probe (data not shown). The rate of Aβ-specific transcription was analyzed by nuclear runoff assays, using a modification of the method described by Groudine et al. (10). Radioactive nuclear RNAs from mutant and wild-type cell lines were hybridized either to Aβ single-stranded DNA, to cDNA encoding a constitutively expressed housekeeping gene (A50 [18]; positive control), or to M13 single-stranded DNA (negative control). The intensities of the radioactive bands corresponding to Aβ RNA were comparable for both the wild-type and mutant cell lines after 15 min of in vitro transcription (Fig. 1B). Densitometric scanning revealed that

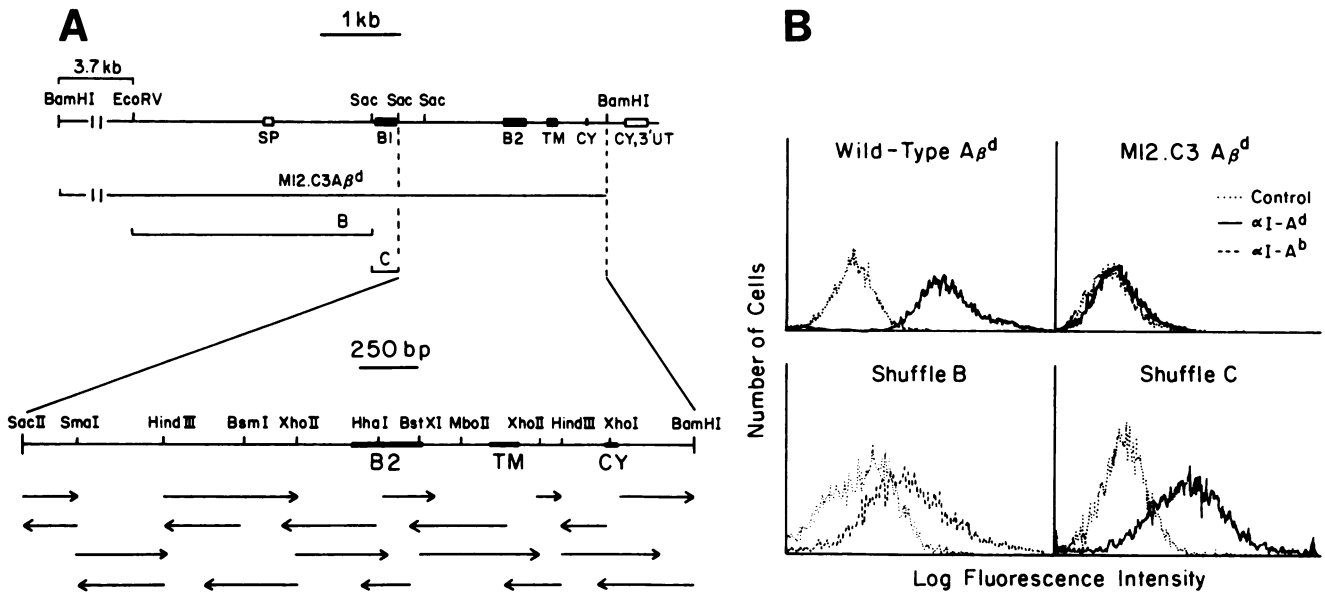

FIG. 2. (A) Molecular scheme of the M12.C3 Aβ^d gene. The genomic organization of the MHC class II Aβ gene is shown. Sequences from the M12.C3 Aβ^d gene used in transfection experiments are indicated: M12.C3Aβ^d, B, and C. The 2.9-kb *Sac*II-*Bam*HI fragment (not used in transfection experiments) of the Aβ^d clone including the β2, transmembrane, and first cytoplasmic domains was sequenced by the dideoxy-chain termination method with M13 vectors (29). (B) DNA-mediated transfection of the M12.C3 cell line. Aβ^b, Aβ^d, and mutant Aβ^d gene fragments were cloned in pBR327. The Aβ^d constructs consisted of a 10-kb *Bam*HI fragment containing the first five exons from the wild-type or M12.C3 Aβ^d genes fused with a 2.5-kb *Bam*HI fragment containing the final exon of the Aβ^k gene. The last exon did not affect the Aβ^d amino acid sequence, since the two haplotypes are identical in this region. In shuffle B, a hybrid gene containing the promoter and the first exon of the M12.C3 Aβ^d gene (a 3.2-kb *Eco*RV-*Sac*II fragment), and the last five exons of an Aβ^b gene construct was generated. Shuffle B was cotransfected into M12.C3 cells with an Aα^b gene construct to test whether the M12.C3 Aβ^d promoter region could result in expression of an Aβ^b molecule, as measured by the presence of cell surface I-A^b antigen. In shuffle C, a 0.5-kb *Sac*II fragment encoding the M12.C3 Aβ1 exon was placed in an Aβ^b gene construct in which the analogous segment of DNA encoding the Aβ1 exon had been removed. The M12.C3 DNA fragments used in shuffles B and C are indicated as B and C, respectively (Fig. 2A). The Aβ^b gene used in shuffles B and C is described elsewhere (1).

the ratio of Aβ/A50 was 1.10 for M12.4.1 and 1.28 for M12.C3, demonstrating that the M12.4.1 and M12.C3 Aβ^d genes are transcribed at similar rates. These nuclear runoff data, taken together with the Northern blot analysis, show that the M12.C3 cells transcribe a species of Aβ RNA that is unstable after transcription.

Loss of I-A expression is caused by a genetic defect in the Aβ gene. Transfection of M12.C3 cells with a wild-type Aβ gene resulted in normal expression of Aβ protein (Fig. 2B). This suggests that the molecular defect lies within the M12.C3 Aβ gene and does not affect another gene whose product might be necessary for the posttranscriptional processing of Aβ RNA. To analyze the mutant Aβ^d gene, an Aβ-specific probe was used to identify a genomic clone from an M12.C3 genomic library. To determine the location of the genetic defect in the M12.C3 Aβ gene, DNA-mediated transfection

TABLE 1. Summary of the mutations identified in the M12.C3 Aβ^d gene

Location	Aβ^d sequence[a]	M12.C3 Aβ^d sequence[b]	M12.C3 versus Aβ^d sequence	M12.C3 versus Aβ^b sequence[c]
503 bp 5′ of β2 exon	GGACGA	GG$_{\blacktriangle}$GA	2-bp deletion	−
99 bp 5′ of β2 exon	GATCTTCATTCACT	GA$_{\blacktriangle}$CT	10-bp deletion	+
91 bp 5′ of β2 exon	CAGTT	CA$_{\blacktriangle}$TT	1-bp deletion	−
48 bp 3′ of β2 exon	GGGAT	GGAAT	1-bp change	+
64 bp 5′ of TM exon	GAGGTTTTT	GAAACTTGT	4-bp change	−
27 bp 5′ of CY exon	ACCTT	ACTTT	1-bp change	+
16 bp 3′ of CY exon	CTAT	CT^{T}AT	1-bp insertion	−
231 bp 3′ of CY exon	AGTTGTTAG	ATGGGTT^{T}AG	3-bp change and 1-bp insertion	−
261 bp 3′ of CY exon	TTTGC	TT$_{\blacktriangle}$GC	1-bp deletion	−
333 bp 3′ of CY exon	GATAT	GAGAT	1-bp change	−

[a] A wild-type Aβ^d sequence has been published previously (13). All sequence data presented read 5′ to 3′.

[b] Base changes identified in the M12.C3 Aβ^d sequence upon comparison with the published Aβ^d sequence are underlined twice. Deletions are indicated (▲). Insertions are shown in superscript.

[c] Symbols: −, identity between the M12.C3 Aβ^d sequence and the published Aβ^d sequence (12); +, difference(s) between the M12.C3 Aβ^d and Aβ^b sequences and the published Aβ^d sequence (13).

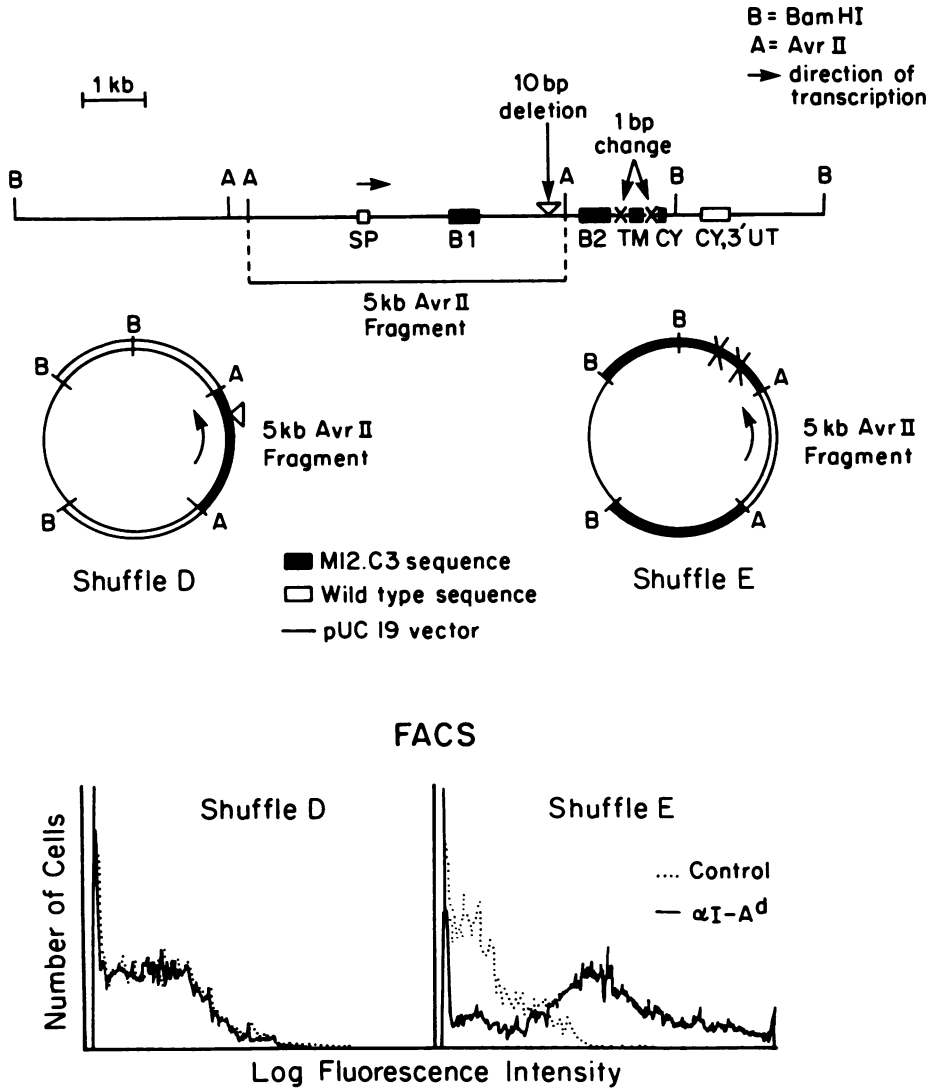

FIG. 3. DNA-mediated transfection of the M12.C3 cell line. Two additional plasmid constructs (shuffles D and E) were made by using the pUC19 vector. In shuffle D, the distal 2.5-kb *Bam*HI fragment containing the last exon is from the $A\beta^k$ gene whose sequence is identical to that of the wild-type $A\beta^d$ gene in this region. Transfection of the M12.C3 cells was performed as described in Materials and Methods, except that 5, instead of 40, μg of pMSV-neo (17) was used for cotransfection and the selection medium containing 0.20 mg of G418 neomycin (GIBCO) per ml was added 24, instead of 48 to 72, h after transfection.

of the M12.C3 cells with recombinant Aβ genes was performed. Transfection experiments were useful in evaluating the posttranscriptional stability of recombinant Aβ genes in M12.C3 cells, because the level of recombinant Aβ mRNA correlates with functional cell surface expression of I-A (data not shown). Three different plasmid constructs of the M12.C3 Aβ gene were tested (Fig. 2). To determine whether there was a functionally important mutation in the 3′ untranslated region, a 10-kb *Bam*HI fragment containing the M12.C3 Aβ gene up to the 3′ region of the cytoplasmic domain exon was ligated to the extreme 3′ end of a wild-type $A\beta^k$ gene. This construct, when transfected into M12.C3 cells, failed to result in the expression of I-A at the cell surface (0 of 12 neomycin-resistant cell lines were positive, in contrast to the wild-type Aβ construct of which 12 of 12 neomycin-resistant cell lines were positive). Two M12.C3 Aβ fragments labeled B and C (Fig. 2A) were used to make two additional constructs (shuffles B and C) to determine whether the 5′ region (up to and including the polymorphic β1 domain) of the M12.C3 Aβ gene was defective. Both shuffles B and C resulted in wild-type levels of cell surface I-A when transfected into M12.C3 cells (Fig. 2B). These experiments localize the defect in the M12.C3 $A\beta^d$ gene to a region of DNA that is downstream from the Aβ1 domain exon and upstream from the second cytoplasmic domain exon.

A specific intronic 10-bp deletion in the Aβ gene causes loss of I-A expression. To identify the molecular defect in the region localized by the recombinant DNA experiments, the 3′ region of the M12.C3 Aβ gene including intronic regions was sequenced. The strategy for sequencing this 2.9-kb region is shown in Fig. 2A. The M12.C3 $A\beta^d$ gene sequence differs from the published $A\beta^d$ sequence (13) at 10 separate sites—all within introns (Table 1). In an effort to eliminate differences that were unlikely to have functional consequences, we compared the M12.C3 $A\beta^d$ sequence to the published sequence (12) of a closely related allele (the $A\beta^b$ haplotype) of this highly polymorphic gene. This comparison

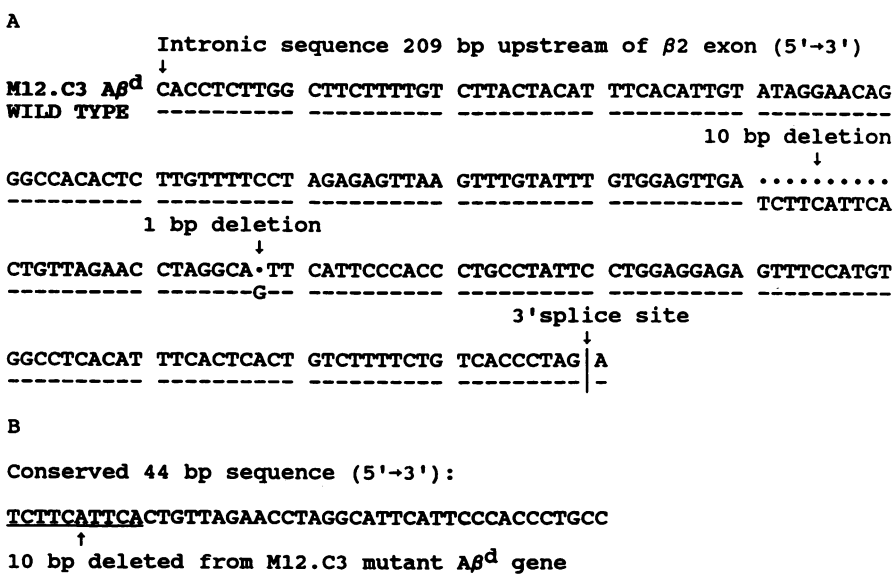

FIG. 4. (A) Intronic sequence flanking the 10-bp deletion. The sequence 100 bp upstream and 100 bp downstream from the 10-bp deletion site is shown. Identity between the wild-type $A\beta^d$ sequence (13) and the mutant M12.C3 $A\beta^d$ sequence is indicated (–) under the mutant sequence. The 3′ splice site corresponds to the β2 exon junction. The 1-bp deletion found 81 bp 5′ of the β2 exon is identical to that of the wild-type $A\beta^b$ sequence (12). (B) Conserved intronic 44-bp sequence. The 10-bp sequence that was deleted from the M12.C3 $A\beta^d$ gene is underlined.

revealed that for 7 of the 10 differences between the M12.C3 $A\beta^d$ and published $A\beta^d$ genes, the M12.C3 $A\beta^d$ residues were identical to the *b* haplotype (Table 1). These changes are not likely to be functionally significant because $A\beta^b$ RNA is processed normally. The three remaining differences included: a 10-bp deletion located 99 bp 5′ of the β2 exon, a G→A base change 48 bp downstream from the β2 exon, and a C→T base change 27 bp upstream from the first cytoplasmic domain exon.

To determine which of these three differences was responsible for the altered phenotype, two additional recombinant DNA molecules were constructed and transfected into M12.C3 cells. A 5-kb segment of M12.C3 Aβ DNA containing the 10-bp deletion was ligated into the wild-type $A\beta^d$ gene (shuffle D) (Fig. 3). When this construct was transfected into M12.C3 cells, there was no I-A cell surface expression (29 of 29 neomycin-resistant cell lines were negative) as determined by fluorescence-activated cell sorting analysis. This experiment demonstrates that the 10-bp deletion in the M12.C3 Aβ gene can prevent the expression of Aβ protein in this cell line. To determine the effect of the other two differences, a construct was made (Fig. 3, shuffle E) that effectively contained the M12.C3 $A\beta^d$ mutant sequence without the 10-bp deletion. M12.C3 cells transfected with construct E expressed wild-type levels of I-A on the cell surface as determined by fluorescence-activated cell sorting analysis (15 of 22 neomycin-resistant cell lines were positive). These experiments show that the intronic 10-bp deletion identified in the M12.C3 Aβ gene can, by itself, account for the loss of I-A expression seen in the mutant cells.

The M12.C3 Aβ mRNA is correctly spliced. The mechanism by which this intronic deletion results in posttranscriptional instability is not clear. One possibility is that splicing is disrupted. We postulated that incorrectly spliced forms of Aβ-derived RNAs might be present in extremely low levels and would be detectable by amplification of these rare species. Aβ-specific cDNA was synthesized in vitro (11) from M12.C3 cytoplasmic RNA and then amplified by PCR. The amplified DNA was ligated into the M13 vector for sequence analysis. No differences between the M12.C3 $A\beta^d$ cDNA (three clones analyzed) and the wild-type M12.4.1 $A\beta^d$ sequence were observed (data not shown). While the M12.C3 $A\beta^d$ primary transcript can be correctly spliced, it is possible that incorrectly spliced mRNAs could be formed and so rapidly degraded in the nucleus that they escape detection by PCR. However, the amplification experiments with M12.C3 cell RNA demonstrated only one species of Aβ-specific mRNA in any appreciable amount, as only one band of DNA was visible in 10 separate PCR trials, using either total or cytoplasmic RNA. Theoretically, only 0.05 ng of pure Aβ-specific mRNA would be required to produce a significant amount of DNA (30). Thus, the correctly spliced product predominates over any incorrectly spliced form.

DISCUSSION

The molecular analysis of an I-A-negative mutant B lymphoma cell line demonstrated that a 10-bp deletion in the intron of a mammalian gene can act posttranscriptionally to result in greatly diminished steady-state mRNA levels and hence undetectable levels of the polypeptide encoded by that gene. The deletion is located in the second intron of the polymorphic murine class II MHC gene, Aβ, 99 bp 5′ of the third exon (encoding the β2 domain); the deletion did not involve the donor or acceptor splice sites in that intron, nor did it create obvious cryptic splice sites (Fig. 4A). Sequence analysis of cDNA produced by PCR from the small amount of Aβ mRNA present in the mutant cell line demonstrated that the species of mRNA produced was correctly spliced and identical to the wild-type sequence.

While the accuracy of splicing may not be compromised by this intronic mutation, it is possible that the efficiency of the splicing reaction is affected. However, the 10-bp deletion (found 99 bp 5′ of the 3′ splice site) is not in any of the three locations commonly believed to be important for efficient splicing (3, 20, 31). This deletion might affect branch point

selection, but it is located 60 to 80 bp 5′ of the branch site found in many other introns (23). Furthermore, although the relative importance of a specific branch point sequence is unknown for class II genes, several studies of β-globin gene expression (27) have shown that small reductions in splicing efficiency associated with mutations in the authentic branch point sequence do not affect the level of mature cytoplasmic mRNA.

The most likely possibility is that the 10-bp sequence affects the stability of Aβ-specific hnRNA in the nucleus. The 10-bp deletion might destroy a necessary regulatory sequence or it may create a destabilizing sequence in the Aβ RNA. This area of posttranscriptional gene regulation is largely unexplored; however, interactions between conserved hnRNA sequences and nuclear factors (such as small nuclear ribonucleoprotein particles) may impart stability on the inherently unstable hnRNA (15, 26, 31). Very recent work has shown that hnRNP C, A1, and D specifically bind defined regions within the introns of mRNA precursors (16, 34). It is interesting that the 10-bp deletion sequence appears in the GenBank 45 times, although statistically (4), the sequence would appear only 12 times by chance alone ($P < 0.0001$). In addition, the 10-bp deletion is found in a 44-bp region (Fig. 4B) that is >98% conserved in both of the other haplotypes (*b* and *k*) of the murine MHC class II Aβ genes that have been sequenced to date. Furthermore, of the seven human class II (human leukocyte antigen) genes whose entire genomic sequence is known, six contain a 22-bp portion of the murine 44-bp sequence that is >70% conserved within an intronic region. These data suggest a functional significance for the intronic region in which the 10-bp deletion was identified. One possibility, which future experiments will address, is that this sequence is recognized by a nuclear factor whose role in RNA processing is to stabilize hnRNA.

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