MOLECULAR AND CELLULAR BIOLOGY, July 1984, p. 1270–1277
0270-7306/84/071270-08$02.00/0

Vol. 4, No. 7

Loss of a Consensus Splice Signal in a Mutant Immunoglobulin Gene Eliminates the CH_1 Domain Exon from the mRNA

CURTIS R. BRANDT,[1]† SHERIE L. MORRISON,[1] BARBARA K. BIRSHTEIN,[2] AND CHRISTINE MILCAREK[1,3]*

Department of Microbiology, Columbia University, New York, New York 10032[1]; Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461[2]; and Department of Microbiology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261[3]

Received 15 December 1983/Accepted 12 March 1984

A series of mouse myeloma mutants, derived from a cell line of the murine MPC-11 tumor (γ2b, kappa), resemble human heavy-chain disease in their loss of an internal domain (exon). In these mutants, most of the γ2b CH_1 exon was present in the nuclear RNA but was removed during splicing to form the mature cytoplasmic RNA. Amino acid sequence studies of one mutant (10.1) are consistent with the loss of the complete CH_1 domain. A second mutant cell line (I17) derived from 10.1 and containing the same CH_1 alteration was shown by S1 nuclease protection experiments to have an additional mRNA deletion spanning the CH_2–CH_3 domain boundary. This second deletion was shown to result from a genomic alteration that provided a marker for the isolation of the expressed H-chain allele. To determine the basis of the CH_1 splicing defect, the I17 genome-expressed γ2b constant region DNA was cloned. Sequence studies showed a deletion of 99 nucleotides around the 3′ end of the CH_1 domain, which removed the splice site and flanking DNA, apparently causing the aberrant splicing of the RNA transcript. The sequence deleted in the mutant is flanked by short repeats of the octameric sequence CCAGCCAG in the wild-type gene. In the mutant, one copy of the repeat, in addition to the sequences between the repeats, has been lost.

Many eucaryotic genes have been found to contain intervening sequences (IVSs) which interrupt coding regions. The IVSs are transcribed with the coding sequences and are removed by splicing to generate mature functional RNA. Splicing has been shown to be important for regulation of gene expression in many cases (12). The study of splicing mutants will be important in elucidating the mechanism of splicing and how it is controlled. Some mutants with DNA alterations consistent with errors in splicing have recently been described (16, 17, 22, 23, 31, 37, 38). In this communication, we describe experiments showing that a series of immunoglobulin heavy-chain deletion mutants exhibit altered processing of the nuclear precursor RNA. These mouse myeloma mutants share some features with human heavy-chain disease.

Several human heavy-chain disease proteins have been shown to have internal deletions beginning or ending near the hinge (20, 21). The molecular explanation for these altered proteins remains obscure since the DNA and parental cell from which they arose are not available for study; altered RNA splicing may explain the origin of some. The myeloma cell culture system is well suited for elucidating the mechanisms by which mutants in immunoglobulin production arise and for generating mutants in gene expression. The mutation frequency in the immunoglobulin genes is high (1, 10); mutants can be readily identified and isolated (9). Parents and progeny can be compared directly to localize significant DNA alterations.

We have previously described the mRNA and proteins produced in a series of myeloma mutants derived from the MPC-11 cell line 45.6. Mutant 10.1 arose spontaneously from 45.6 and synthesizes a γ2b heavy chain with a deletion of at least a portion of the CH_1 domain (35, 36). We have shown that the mRNA of 10.1 is shorter than wild type and lacks at least a portion of the CH_1 domain. A mutant, I17, derived from 10.1, also produces H-chain mRNA with a similar CH_1 deletion. This mutant synthesizes heavy chains even smaller than that produced by 10.1, indicating that an additional alteration has occurred.

To determine how the CH_1 deletions arose in the γ2b mutants, we studied the steady-state nuclear RNAs from the wild-type and mutant cell lines. In this communication, the primary transcripts of the mutant are shown to contain CH_1 sequences subsequently deleted from the mRNA and protein. To determine whether the deletions arose from a mutation in an RNA splicing enzyme or a γ2b DNA alteration, the expressed allele in the mutant I17 was cloned and the 3′ end of CH_1 was sequenced. A short DNA deletion consistent with aberrant RNA processing was found.

MATERIALS AND METHODS

Cells. The mouse myeloma cell line 45.6 (immunoglobulin γ2B) was adapted to tissue culture from the MPC-11 tumor (27). Isolation of mutants has been described previously (9, 10). Cells were maintained in spinner or suspension culture in Iscoves modified Dulbecco medium (GIBCO Laboratories) supplemented with 5% heat-inactivated (56°C, 30 min) horse serum, nonessential amino acids, glutamine, penicillin, and streptomycin.

Isolation of RNA. Cells were pelleted and washed in wash buffer (0.15 M KCl, 4 mM magnesium acetate, and 10 mM Tris [pH 7.4]). They were then suspended in lysis buffer (150 mM KCl, 4 mM magnesium acetate, 0.05% Triton X-100, 10 mM Tris [pH 7.4], 250 mM sucrose, and 10 mM vanadium ribonucleoside complex) (3, 42), mixed gently for 3 min, and centrifuged at 2,000 rpm in a CRU-5000 centrifuge (Damon/IEC) to pellet nuclei. Cytoplasmic mRNA was obtained from the supernatant at this step. The lysis procedure was repeated, and the nuclear pellet was suspended in lysis buffer with Sarkosyl, EDTA, and proteinase K added to final concentrations of 1%, 0.01 M, and 10 μg/ml, respectively. Samples were incubated for 15 min at 37°C. Urea and CsCl were

* Corresponding author.
† Present address: Fred Hutchinson Cancer Institute, Seattle, WA 98104.

added to concentrations of 7.0 M and 0.5 g/ml, respectively, and samples were layered on 3-ml cushions of CsCl (1.35 g/ml) in 10 mM Tris (pH 7.4)–1 mM EDTA. The cytoplasmic samples were adjusted to 0.5 g of CsCl per ml and layered on 3-ml CsCl cushions (1.35 g/ml) in 10 mM Tris (pH 7.4)–1 mM EDTA. Gradients were centrifuged in an SW41 rotor at 30,000 rpm for 16 h. RNA pellets were suspended in binding buffer, and polyadenylate-containing RNA was isolated on oligodeoxythymidylate cellulose columns as previously described (34).

S1 nuclease analysis. Total cytoplasmic or nuclear RNA (20 to 40 μg) was hybridized to 1×10^4 to 5×10^4 cpm of specific end-labeled DNA fragment at 52°C in 80% formamide as described previously (4). After hybridization, samples were digested for 60 min at 37°C with 3×10^3 U of S1 nuclease (Boehringer Mannheim Biochemicals). The protected hybrids were analyzed on 8 M urea–5 or 8% acrylamide gels, 0.4 mm thick. End-labeled *Hae*III-, *Alu*I-, or *Hpa*II-digested pBR322 DNA or a sequencing reaction (M13 mp8 DNA) was used as size marker. DNA fragments were 5′ end labeled by using T4 polynucleotide kinase and [γ-^{32}P]ATP (32) with prior calf intestinal alkaline phosphatase treatment to remove 5′ phosphates (30). The 3′ end labeling was done with T4 polymerase and [α-^{32}P]dXTPs in either a fill-in or replacement reaction (30). Specific activities of the probes ranged from 1×10^5 to 5×10^5 cpm/pmol of end.

B13 probe. The probe B13 was obtained from a partial *Bam*HI digestion of I17-9 DNA subcloned into M13 mp9 *Bam*HI site. The insert consisted of two linked *Bam*HI fragments (see Fig. 1 and 3) containing not only most of the CH_1 coding region but also approximately 900 nucleotides 5′ of CH_1 in the long intervening sequence. The sequence was essentially identical to wild-type genomic CH_1 DNA, except for a 99-nucleotide deletion at the 3′ end of CH_1 (see below for I17-9 DNA sequencing). The replicative form of B13 was nick translated with [α-^{32}P]dXTPs and used as probe on RNA blots. The single-stranded phage DNA of B13 was copied by using the M13 "universal" primer and [α-^{32}P]dXTPs and was digested with *Bgl*II. The extended primer fragment was isolated from a gel and used for the S1 protection experiments.

Protein characterization. Myeloma protein 10.1 was purified as previously described (19). Mutant 10.1 synthesizes an immunoglobulin protein molecule with disulfide bonds present between H chains but absent between H and L chains. Heavy-chain dimers were separated from light chains by gel filtration either on Sephadex G-150 equilibrated in 8 M urea–0.1 M formic acid or on Sephacryl S-300 equilibrated in 8 M urea–0.1 M acetic acid.

Cyanogen bromide cleavage, separation of amino-terminal and carboxyl-terminal portions, and complete reduction and alkylation of disulfide bonds were carried out as described previously (5, 6). Fragments were separated by Sephadex G-75 chromatography in 8 M urea and 0.1 M formic acid. The 27,500-molecular-weight pool contained the C-terminal fragments II.1, II.2, and II.3 (see Fig. 2).The 40,000-molecular-weight pool contained the amino-terminal fragments I.5 and I.3 plus I.4 from MPC-11 and I.3′–I.4′ from 10.1. Cleavage of I.3′–I.4′ with arginine-specific protease from mouse submaxillary glands was carried out for 6 h in 0.05 M NH_4OH (pH 8.0) with acetic acid. Enzyme samples of 62 and 31 μg were added at 0 and 3 h, respectively. Tryptic digestion was carried out for 6 h at 37°C in 0.05 M NH_4OH (pH 8.0) with acetic acid, using two aliquots of 250 μg each of TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin. Tryptic digests of I.3′–I.4′ were fractionated on Sephadex G-50 in 0.05 M NH_4OH. Some pools were further purified by ion-exchange chromatography, using either DEAE-Sephadex or type P chromobeads (Technicon Instruments Corp., Inc.) with a pyridine-acetate gradient as described previously (19). Amino acid analysis, automated Edman degradation, and identification of the resulting phenylthiohydantoin derivatives were carried out as described previously (5, 6).

Genomic DNA isolation, gel analysis, and cloning. High-molecular-weight DNA from the myeloma mutants was isolated from whole cells (39) and digested with restriction enzymes as directed by the supplier (Bethesda Research Laboratories or New England BioLabs, Inc.). High-molecular-weight I17 genomic DNA was digested with *Eco*RI to completion. DNA fragments ranging from 6 to 7 kilobases were selected on sucrose gradients and cloned into the isolated arms of the vector λgt WES (28). The recombinant DNAs were packaged with λ extracts (30). The resulting library of 5×10^5 phage was screened by using the wild-type γ2b constant region DNA (47) generously supplied by P. Tucker. An *Eco*RI fragment of wild-type γ2b DNA was subcloned before use.

Sequencing. DNA fragments for sequencing were subcloned into M13 mp8 or M13 mp9 (33) in the *Bam*HI or *Pst*I site, using JM103 as the host bacteria. Appropriate γ^+ plaques were picked, and the single-stranded phage DNAs were sequenced by the dideoxy chain terminator method (41), using the 15-nucleotide single-strand "universal" primer (P-L Biochemicals, Inc.). Nucleotide sequences were analyzed with computer programs written by Staden (45, 46).

RESULTS

Mutants 10.1 and I17 lack CH_1 mRNA sequences. By hybridizing cytoplasmic mRNA with various fragments isolated from the immunoglobulin heavy-chain constant region cDNA plasmid, pγ2b[11], we previously demonstrated that all of the mutant heavy-chain mRNAs lack a portion of the sequences coding for the CH_1 domain (35). However, a small fragment of pγ2b[11], corresponding to 24 nucleotides of the 5′ half of CH_1, weakly hybridized to the mRNAs on Northern blots, leading us to conclude that a portion of CH_1 was still present in the mutants. We were able to reexamine this question in two ways with the cloned DNA from the CH_1 exon. A probe, B13, which encompasses almost all of CH_1 and 900 nucleotides of the IVS to the 5′ side of CH_1, was prepared (see above). Mutant cytoplasmic polyadenylate-selected RNA was electrophoresed under denaturing conditions, blotted to nitrocellulose, and hybridized with ^{32}P-labeled B13 or pγ2b[11] DNA (48). The B13 probe did not hybridize with the mutant mRNAs. Even with exposures 5 to 10 times as long as necessary to see bands with wild-type RNA, no hybridization of mutant mRNAs with B13 probe was detected (C. R. Brandt, Ph.D. thesis, Columbia University, New York, N.Y., 1983).

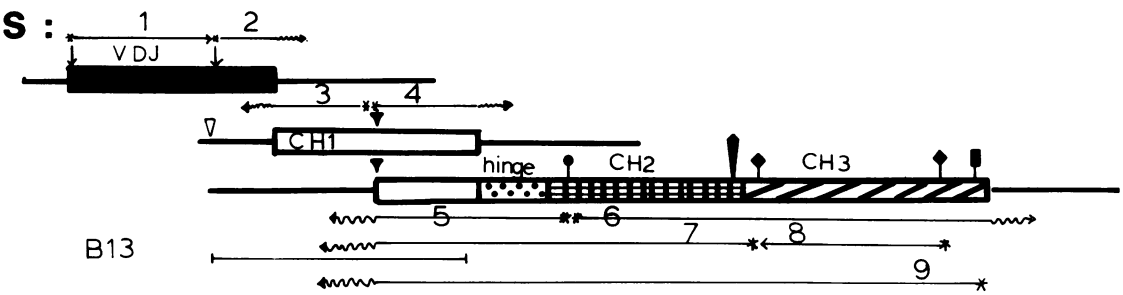
To increase the sensitivity of the technique, a ^{32}P-labeled, single-stranded, message-complementary probe was prepared from clone B13, corresponding to most of CH_1 and a small portion of the IVS before it. The probe was hybridized to cytoplasmic mRNA, and the hybrids were treated with S1 nuclease. The size of the DNA probe protected by wild-type or mutant mRNAs was determined by gel electrophoresis. The results (Fig. 1) showed that the wild-type mRNA protected a fragment of 260 nucleotides, whereas 10.1 and I17 mRNAs effected no protection of the probe. In control experiments, the mutant mRNAs were capable of protecting

probes complementary to other regions of the gene (Fig. 1). We therefore concluded that no detectable CH_1 sequences remained in the mutant mRNAs.

S1 nuclease analyses define the boundaries of the deletions. To map the boundaries of the CH_1 deletion more precisely, cytoplasmic RNA was hybridized with various cloned DNA probes, the sources of which are shown in Fig. 1. The sizes of the end-labeled DNAs protected by the RNA after S1 nuclease digestion were determined by gel electrophoresis, and the data are summarized in Fig. 1. For example, pγ2b[11], the C region cDNA clone, was digested with *Xho*I, and the DNA was 5′ end labeled with polynucleotide kinase and [γ-^{32}P]ATP. The resultant probe, no. 5 in Fig. 1, was hybridized with RNA from wild-type or mutant cells. With wild-type cytoplasmic RNA, a fragment of 259 nucleotides is protected from S1 digestion. This fragment corresponds to protection of all of the immunoglobulin DNA which is present in the cDNA plasmid 5′ of the *Xho*I site (48). With cytoplasmic RNA from 10.1 or I17, a fragment of 77 to 79 nucleotides is protected, showing the presence of the hinge but the absence of sequences 5′ of hinge. When the 3′ end of the V_H region was mapped by using probes 1 and 2, mutants and wild type gave the same sized protected fragments. This result indicates that the 3′ boundary of the rearranged VDJ exon is maintained in the three mutants, in the wild-type configuration. When probes within CH_1 were used in S1 protection experiments, wild-type cytoplasmic RNA gave the expected fragments, but cytoplasmic RNA from the mutants gave no fragments. We therefore concluded that CH_1 sequences were missing from mutant RNAs and that the 3′ end of the rearranged VDJ exon and the 5′ end of hinge were correctly spliced to each other, maintaining a correct reading frame in the protein. We cannot rule out the addition or deletion of a few nucleotides between the VDJ and hinge exons that might result from the abnormal splicing event occurring in these mutants, but this seems unlikely in view of the S1 results with probe B13 from CH_1.

RNA from 10.1 and I17 gave identical patterns with probes near the CH_1 deletion, but when mutant I17 was examined for sequences in CH_2 and CH_3, it showed differences from

(a) PROBES :



(b) Length of Probe Protected (in nucleotides)

	FRAGMENTS USED									
RNAs:	**1**	**2**	**3**	**4**	**B13**	**5**	**6**	**7**	**8**	**9**
Cytoplasmic										
45.6	202	148	130	160	260	259	834	591	311	1014
10.1	202	148	0	0	0	78	834	425	311	825
I17	202	148	0	0	0	78	224	0	285	370
Nuclear										
45.6	—	—	—	160	—	—	—	—	—	—
10.1/I17	—	—	—	130	—	—	—	—	—	—

FIG. 1. Location and size determination of DNA protected from S1 nuclease by cytoplasmic and nuclear RNA. (a) The map shows the origins of the various probes used. The fused VDJ region was obtained from phage RBL-216, the cloned, expressed immunoglobulin γ2b gene in 45.6 cells (26); the CH_1 probes were obtained from the embryonic γ2b constant region (47); and probes from hinge, CH_2, and CH_3 domains were obtained from pγ2b[11], the cDNA clone (47, 48). The *→ represents a 3′-OH-labeled fragment; ←* represents a 5′-P-labeled fragment. The B13 probe was labeled in M13 by primer extension. Symbols for restriction enzymes: ↓, *Pst*I; ▽, *Bgl*II; ▼, *Bam*HI; ⚲, *Xho*I; ♦, *Hin*cII; ♦, *Sac*I; ▮, *Kpn*I. By the use of the various fragments, the size of DNA protected from S1 treatment (see the text) was determined on acrylamide–8 M urea gels. (b) The DNA probes numbered in (a) were hybridized with wild-type or mutant cytoplasmic or nuclear RNA, and the sizes of the S1-protected fragments were determined on acrylamide gels.

TABLE 1. Predicted[a] and observed[b] amino acid compositions of 10.1 specific fragments I.3′–I.4′, R.2, and T1 derived from I.3′–I.4′

Amino acid[c]	Composition in: I.3′–I.4′[d]		R.2[e]		T1[f]	
	Predicted	Observed	Predicted	Observed	Predicted	Observed
CMCys	5	4.2	4	4.5	2	1.2
Asp	7	7.8	6	9.8	3	3.2
Thr	5	5.5	4	4.1	4	3.4
Ser	12	9.8	8	7.1	7	5.6
Hser	1	1.1	1	1.1	0	0
Glu	6	6.5	3	4.6	2	2.5
Pro	11	10.4	11	8.2	6	5.5
Gly	6	6.9	6	5.4	3	3.4
Ala	3	3.7	1	1.4	0	0
Val	3	3.1	3	3.2	1	1.5
Ileu	6	5.3	5	4.5	2	2.2
Leu	5	5.0	3	2.6	1	0.97
Tyr	4	4.2	3	2.4	1	0.97
Phe	3	3.2	3	3.6	1	0.86
His	2	2.0	1	1.0	0	0
Lys	3	3.4	3	3.0	1	1.0
Arg	1	1.3	—	—	0	0
Trp	1	ND	1	ND	1	ND

[a] Assuming a precise deletion of the CH_1 domain.
[b] Average of several determinations.
[c] Abbreviations: CMCys, carboxymethylcysteine; Hser, homoserine; Ileu, isoleucine.
[d] Obtained by complete CNBr reduction and alkylation, followed by gel filtration.
[e] R.1 and R.2 were obtained by arginine-specific proteolysis of isolated I.3′–I.4′ and purified by column chromatography.
[f] T1 was obtained by tryptic digestion of I.3′–I.4′ and purified from the other fragments (T2 to T7) by ion-exchange chromatography.

10.1. The S1 protection experiments (Fig. 1; see also Fig. 4) demonstrated that mutant I17 had a second alteration, so that approximately 100 nucleotides from the 3′ end of CH_2 and 26 to 56 nucleotides from the 5′ end of CH_3 were missing from the mRNA. This result demonstrated that the second deletion in I17 was not precisely at the domain boundaries. The fusion of CH_2 and CH_3 at the mRNA level was probably out of the correct reading frame, causing the shortened immunoglobulin protein of aberrant composition seen in I17 cells.

Heavy-chain protein of 10.1 is missing CH_1 domain sequences. The 10.1 heavy-chain protein was characterized to determine whether the CH_1 domain had been deleted. The 10.1 heavy-chain molecules (H_2) were isolated, subjected to CNBr cleavage, and fractionated by column chromatography. A novel peptide, I.3′–I.4′, was found in the amino-terminal fragments from 10.1, and I.3 and I.4 were missing (see Fig. 2). In MPC-11, the CH_1 domain contains a single methionine that separates CNBr-derived peptides I.3 and I.4; the absence of these two fragments from the CNBr digest of 10.1 and the appearance of the new fragment, I.3′–I.4′, indicated that the deletion must involve at least that region of the CH_1 domain. Fragments I.5, II.2, II.1, and II.3 were isolated from 10.1; these four fragments have amino acid compositions and automated Edman degradation patterns identical to those from MPC-11 (data not shown).

Assuming a precise deletion of the CH_1 domain in 10.1, the sequence of fragment I.3′–I.4′ was predicted, and from it an amino acid composition was calculated (Table 1). The amino acid composition of I.3′–I.4′ was determined (Table 1), and together with the molecular weight of I.3′–I.4′, it was consistent with a deletion of the CH_1 domain.

Automated Edman degradation of I.3′–I.4′ from 10.1 showed that it contained the N-terminal sequence of I.3 as in MPC-11. Both the predicted sequence and the actual amino acid composition showed a single arginine in I.3′–I.4′. Treatment of the fragment with arginine-specific protease and subsequent gel filtration yielded two fragments, R.1 and R.2. Amino acid compositions of R.2 (Table 1) and R.1 (not shown) and sequencer analysis of R.2 (Fig. 2) demonstrated that R.1 contained the amino-terminal 17 residues of I.3′–I.4′ and R.2 contained the remainder of the fragment. The agreement between the predicted composition of I.3′–I.4′ and the observed summation of compositions R.1 and R.2 (which together totally account for I.3′–I.4′) is again consistent with precise deletion of the CH_1 domain.

Analysis of tryptic peptides gave further confirmation. The tryptic digest of I.3′–I.4′ was initially fractionated on Sephadex G-50; some pools were additionally purified by ion-exchange chromatography. Table 1 shows the amino acid composition of the T1 peptide. The compositions of the others (not shown) were used to determine their placement in the sequence of fragment I.3′–I.4′. All of the tryptic peptides isolated could be assigned to the variable region, the hinge region, or the CH_2, domain; none deriving from CH_1 domain sequences was isolated. Tryptic peptide T1, containing 35 amino acids, spans the deletion. Automated sequential degradation showed it to commence as shown in Fig. 2, and its amino acid composition is consistent with the

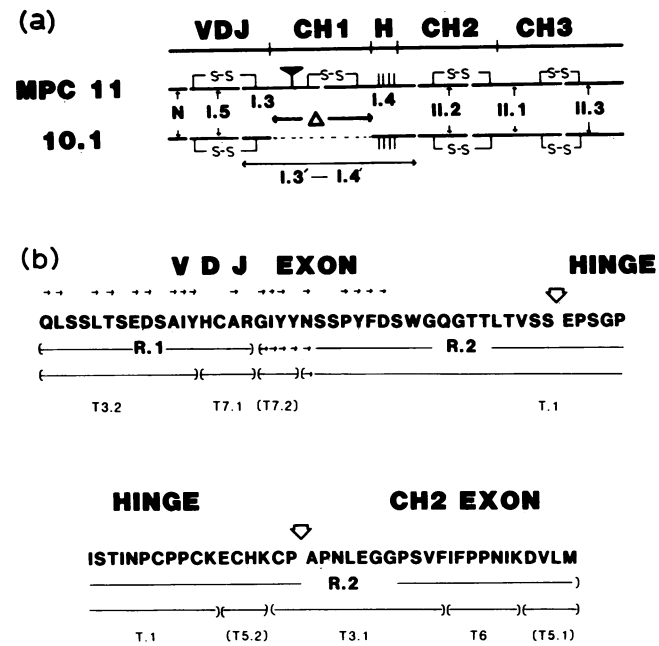
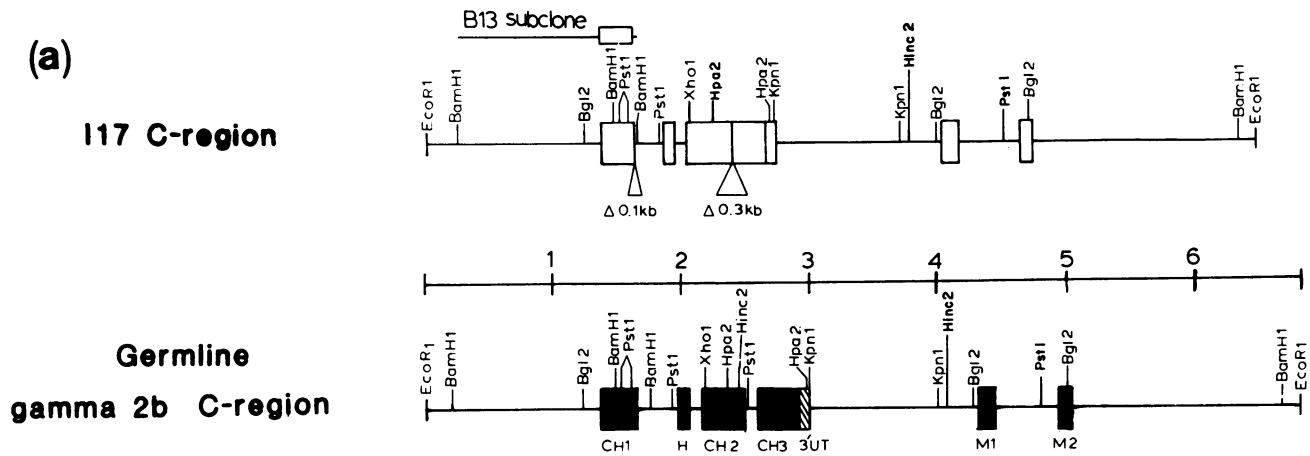


FIG. 2. Origins of the 10.1 heavy-chain fragments I.3′–I.4′, R.1, and R.2, and tryptic peptides. (a) Heavy-chain molecules (H_2) of 10.1 were isolated and cleaved with CNBr, and the material in the first peak from Sephadex G-150 was reduced, alkylated, and separated into I.5 and the novel I.3′–I.4′, which was found in 10.1, not in MPC-11. (b) The I.3′–I.4′ was subjected to arginine-specific proteolysis to obtain R.1 and R.2 and then to tryptic digestion to obtain fragments T-1 through T7-2. Arrows (→) mark residues identified by automated Edman degradation in the various peptides. Lines show residues identified by compositional homology (Table 1). Large arrows (⇔) demarcate the junction between the VDJ and hinge or hinge and CH_2 exons. Y indicates the site of carbohydrate attachment in MPC-11 heavy chains.

sequence shown. Of particular note is the absence of alanine from T1; alanine is the N-terminal residue in the CH_1 domain of MPC-11 H chain and all γ2b H chains. Together, these data permit us to conclude that 10.1 H-chain proteins contain a single deletion, comprising the precise CH_1 domain.

CH_2–CH_3 deletion in mutant I17 provides a marker for cloning the expressed allele. To determine whether gross alterations of the DNA such as deletions or insertions around the CH_1 domain had occurred, two experiments were done. Polyadenylated nuclear RNA from the mutants was analyzed by Northern blots with CH_1-specific cloned probes. The 11-kilobase nuclear primary transcript was found to contain CH_1-hybridizable sequences (Brandt, Ph.D. thesis). These must be removed during the nuclear processing of the mRNA which is lacking CH_1. In addition, DNA from mutant and wild-type cells was analyzed by restriction digestion with numerous restriction enzymes and Southern blot hybridization. No differences were seen in the γ2b heavy-chain gene fragments originating from sites around CH_1 when we used *Bam*HI, *Hin*dIII, *Bgl*II, *Xho*I, *Eco*RI, and *Hin*fI and observed fragments from 500 to 20,000 nucleotides. However, γ2b-hybridizable fragments from I17 DNA cut with enzymes with sites near the ends of CH_2, and CH_3 or the IVS between them were different from those obtained with wild-type or 10.1 DNA and were consistent with a second alteration in I17 between CH_2 and CH_3 (Brandt, Ph.D. thesis). Although unrelated to the CH_1 alteration which appears to reside in the splicing of nuclear RNA, this CH_2–CH_3 alteration provided a convenient, *cis*-acting marker for the expressed allele in I17.

A 99-nucleotide deletion in CH_1 accounts for the splicing defect. The CH_2–CH_3 mutation in I17 was used to identify the expressed γ2b gene among the several nonproductively rearranged γ2b genes present in the 45.6–MPC-11 line (26). A library was constructed of phage λgt WES *Eco*RI arms and I17 *Eco*RI-digested, 6- to 7-kilobase size-selected genomic DNA. The phage library was screened with wild-type γ2b constant region DNA. The γ2b-positive clones were analyzed by restriction enzyme digestion and gel electrophoresis, and one clone, I17-9, showed significant differences from the wild-type γ2b constant region DNA. Restriction maps of the I17-9 and the wild-type γ2b constant regions are shown in Fig. 3. The sites for *Hin*cII and *Pst*I were absent from the CH_2–CH_3 regions, and several enzymes produced fragments smaller than wild type in the I17-9 DNA, as has been seen by Southern blot analysis of genomic I17 DNA (data not shown). Two features about the mutant DNA emerged from this map. The CH_2–CH_3 deletion seen in I17



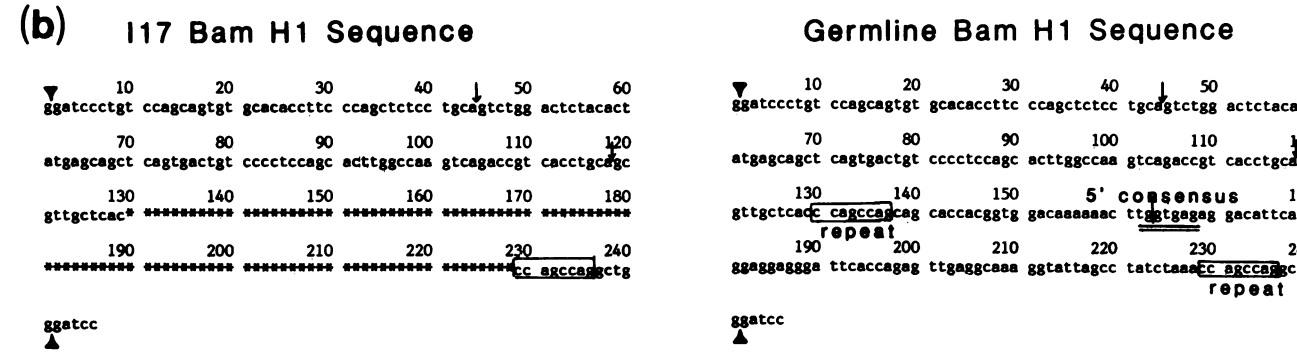


FIG. 3. Restriction map and sequence of the wild-type and I17 mutant γ2b constant region gene. (a) The wild-type γ2b constant region (47) subcloned from the phage into pBR325 is shown at bottom. The *Eco*RI fragment of the mutant I17 γ2b constant region, I17-9, is shown above, with deletions indicated by Δ. The location of the sequences in the M13mp9 subclone, B13, containing CH_1 coding and IVS sequences and isolated from I17-9, is also shown (see the text). (b) The sequence of the mutant I17 γ2b gene is compared with the wild-type sequences reported by two groups (47, 49). The deleted sequences in the mutant are denoted by the asterisks in the I17 sequence. The CCAGCCAG repeats are outlined with boxes. The normal consensus splice sequence is marked by a double underline, and the splice site is denoted by a vertical bar.

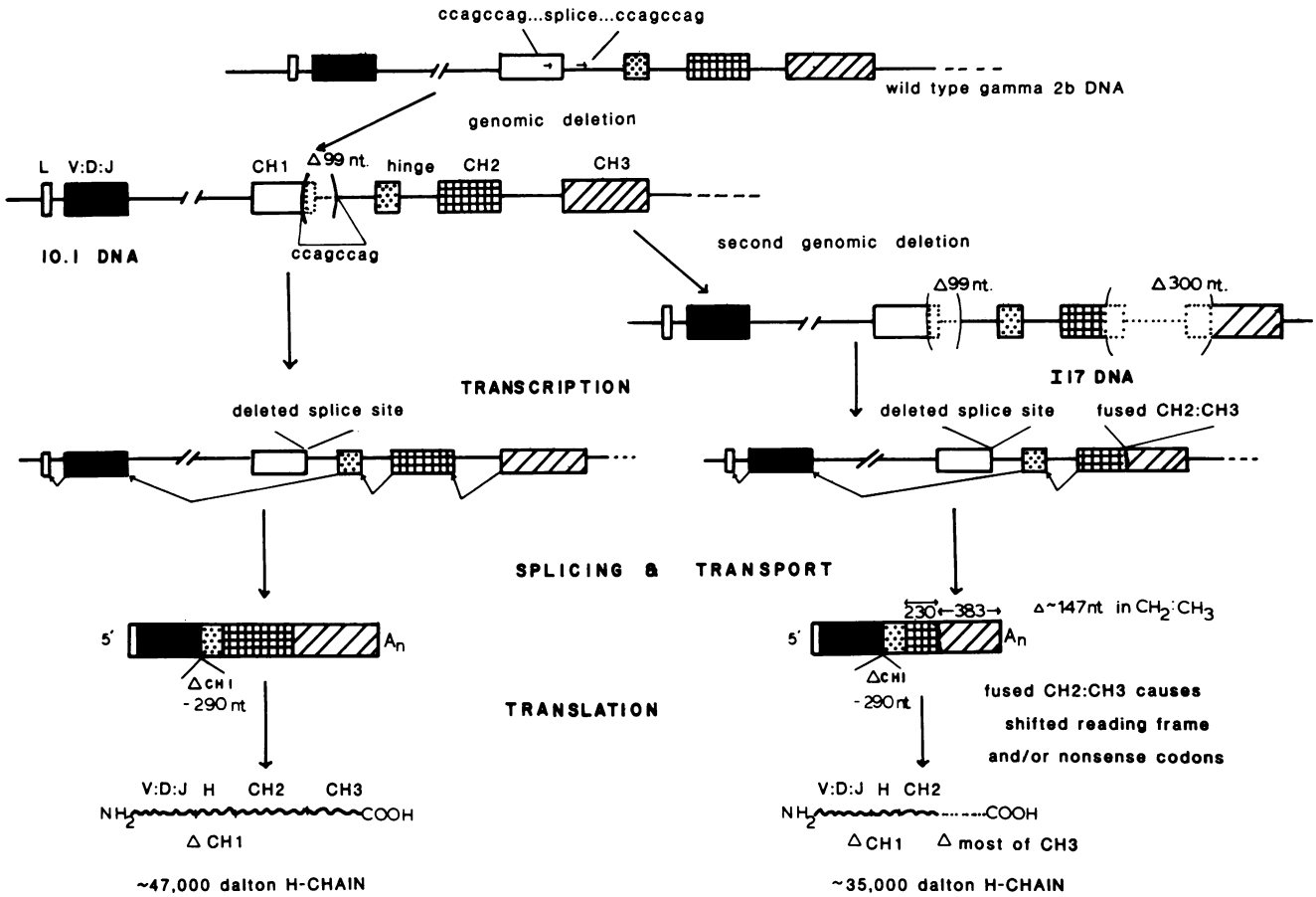


FIG. 4. Deletion of 99 nucleotides of genomic DNA produces mRNA and protein lacking CH_1 sequences.

mRNA correlated with a genomic deletion encompassing approximately the last 100 nucleotides of CH_2, the adjoining IVS, and the first 50 or so nucleotides of CH_3. Whether there is a one-to-one correspondence of mRNA and genomic deletion at this site has not yet been determined, but it seems very likely. Second, a small deletion of approximately 100 nucleotides was observed in the shortest *Bam*HI fragment arising from CH_1 and the adjoining IVS to the 3′ side. The Southern blot analyses of genomic DNA we had done were not designed to detect such small CH_1 alterations. The small, CH_1-derived *Bam*HI fragment was subcloned into M13 phage vectors and sequenced by the dideoxy method; the summarized sequence data are shown in Fig. 3. There is a 99-nucleotide deletion at the 3′ end of the CH_1 exon, starting 26 nucleotides before the splice site of the CH_1 and ending just before the *Bam*HI site in the CH_1–hinge IVS. The deletion is flanked in wild-type DNA by two directly repeated octamers, CCAGCCAG. One copy of the repeat and interposed DNA is removed in the mutant. We therefore concluded that the CH_1 defect in the mutants was due to the removal of the 5′ consensus splice sequence at the end of the CH_1 exon.

The 99-nucleotide deletion is also found in the genome. To determine whether the deletion of 99 nucleotides observed in the cloned DNA was also present in the myeloma mutants, nuclear RNA from the mutants was examined by S1 protection experiments. When a CH_1-derived *Bam*HI 3′-end-labeled fragment (probe no. 4 in Fig. 1) was hybridized with nuclear RNA from wild-type cells, a band of 160 nucleotides was observed (Fig. 1). This band was the same size as that seen with cytoplasmic RNA and must represent the large amount of processed but not transported mature mRNA in the nucleus seen by Northern blot analyses (Brandt, Ph.D. thesis). Nuclear but not cytoplasmic RNAs from the mutant cell lines 10.1 and I17 protected a fragment of 130 nucleotides (Fig. 1). Sequences from the *Bam*HI site in CH_1 to approximately 30 nucleotides 5′ of the normal CH_1–hinge splice site were present in the mutant nuclear RNA. However, the protection stopped within the first (5′-most) CCAGCCAG block, the site of the DNA deletion in the cloned DNA. We concluded that the 99 nucleotides missing in the cloned DNA were missing in the nuclear RNA synthesized by the mutant cells. Therefore, the deletion was already expressed in the myeloma mutants, before molecular cloning.

DISCUSSION

The mutants 10.1 and I17, derived from 10.1, share the common defect that they synthesize mRNA lacking the CH_1 domain. Cloning and sequence studies of the I17 gene have shown that it contains a deletion that removes the donor splice junction at the end of the CH_1 exon. Analysis of nuclear RNA by S1 nuclease protection experiments shows this deletion to be common to both mutants. The deletion is 99 nucleotides in length and is bounded by an octanucleotide direct repeat which is itself a tandem duplication. The absence of the appropriate splice junction leads to altered processing of the nuclear transcript; the entire CH_1 domain

is missing from the mature cytoplasmic mRNA, and a splice from the 3′ end of the V:D:J fused exon to the 5′ end of hinge occurs (Fig. 4). This splice restores the correct reading frame of the protein. Amino acid sequence studies of 10.1 are consistent with a precise deletion of the CH_1 domain. Loss of CH_1 results in the absence of the cysteine residue involved in formation of the disulfide linkage between heavy and light chains in the parental MPC-11 protein. This accounts for the previously observed (36) loss from 10.1 of cysteine-containing peptides which are involved in interchain disulfide bridges.

In the wild-type γ2b CH_1 exon, many matches to the consensus (29, 40) splice sequence besides the normally used one can be found. When the normally used consensus sequence is lost, these internal sites are bypassed in favor of the 3′ end of the V_H exon over 6 kb upstream. Special conformational features of the nuclear RNA precursor may preclude splices occurring within the long IVS between V and CH_1. Alternatively, the selection scheme for isolating the mutants, involving the recognition of the proteins by immunoglobulin-specific antisera, may have revealed only those splicing mutations restoring correct reading frame without insertion of blocks of intron-encoded sequences. The proteins synthesized by these mutants resemble human and mouse heavy-chain disease proteins that have internal deletions affecting one or more domains. Similar selection may have been exerted in the heavy-chain disease proteins, for example, by secretion mechanisms that recognize one or more determinants or domains of the intact immunoglobulin protein.

Several mechanisms could lead to shortened proteins. These include nonsense mutations, genomic deletions, some of which precisely correspond to the protein deletion, and other genomic changes that result in altered RNA processing. Some examples of nonsense mutations leading to shortened immunoglobulin chains include the mouse IgA myeloma mutants T7.2 and SIU9 isolated from the cell lines W3082 and S107, respectively (50), and an MPC-11 variant in which deletion of two adenines from the DNA has resulted in the excision of the CH_3 domain (24). Cloning and sequencing of the gene coding for the shortened γ1H chain of the murine mutant IF2 have shown that it contains an extensive deletion which removes the entire CH_1 domain and a large portion of the IVS between J and CH_1. The deletion is thought to result from an aberrant switch recombination event (13, 14). Deletion of only a portion of the CH_1 domain produces mRNA completely lacking CH_1 in some α heavy-chain mutants (11).

Other genomic changes that result in altered RNA processing include point mutations within and small deletions at or near the consensus splice sequence (31). For example, cloning of an alpha 2 globin gene from a thalassemia patient has revealed that a 5-nucleotide deletion (TGAGG) renders a 5′ consensus splice sequence nonfunctional (38). A 25-nucleotide deletion of the 3′ end of the IVS in an alpha-globin gene causes one form of alpha-thalessemia (37). The mutation responsible for albuminemia in rats has been determined to be a 7-nucleotide deletion of intron H1 which alters the 5′ consensus splice sequence and results in a lack of albumin mRNA (16, 17). MPC-11 cells synthesize a kappa light-chain fragment that is missing the V region. Cloning and sequencing of the gene have revealed that an aberrant rearrangement removed the J segment and its 5′ consensus splice sequence. When the precursor transcript is spliced, the entire V region is deleted (8, 43, 44). The resulting mRNA codes for the leader peptide and kappa constant region sequences (25).

The splicing mutation characterized in this article differs from the other deletion splicing mutants in several respects. Few of these other splicing mutants have stable functional mRNAs. In 10.1 and I17 cells, not only is the mRNA stable enough to detect, but it is also translated into protein. In addition, the deletion we describe is longer than the others (which are all less than 10 nucleotides) and involves direct repeats. Deletions of sequences flanked by short direct repeats have been described previously in β-globin genes (15), bacteria, and phage. Ten of 18 *lacI* DNA deletions (2, 18) occurred at sequences having three or four copies of the sequence CTGG. This is the complement of the sequences flanking the CH_1 deletion in the I17 heavy-chain gene (CCAGCCAG). These *lacI* deletions occur 25 times more frequently in $RecA^+$ hosts (2).

Three mechanisms have been proposed to account for deletions between short repeated sequences: unequal chromatid crossing over (7, 15), hairpin loops bringing the two repeats close together, and slipped mispairing during replication (2, 15, 18). A computer scan of the 99-nucleotide deletion in CH_1 revealed no substantial hairpin structures. We cannot distinguish between unequal crossing over and slipped mispairing during replication unless the reciprocal unequal crossover events are found. Eucaryotic RecA-like molecules could act in any of these three hypothetical mechanisms and could be responsible for the particular deletion we have described as well as the high mutation frequencies (1, 9, 10) observed in the myeloma cells in general.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA-36606 and CA00758 (R.C.D.A.) to C.M., AI-12509 and -10702 to B.K.B., and CA-14051 to S.L.M., all from the National Institutes of Health.

We thank Richard Remnick for screening the λ phage libraries of genomic DNA, and Letitia Wimms for assistance with the somatic cell hybridization.

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