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Two α heavy chain disease proteins with different genomic deletions demonstrate that nonexpressed α heavy chain genes contain methylated bases

(myeloma mutants/differential DNA methylation/allelic exclusion)

WILLIAM DACKOWSKI AND SHERIE L. MORRISON

Department of Microbiology, and the Cancer Center, Institute of Cancer Research, College of Physicians & Surgeons of Columbia University, New York, New York 10032

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ABSTRACT **Two independently arising α heavy chain mutants have been found to synthesize heavy chains with CH_1 deletions of approximately equal extent. Both were isolated from heavy chain-producing variants of the mouse myeloma W3129 and demonstrate that it is possible to arrive at the heavy chain disease phenotype by the pathway H + L → H → ΔH. Analysis of genomic DNA by digestion with restriction endonucleases followed by molecular hybridization showed that one mutant (Δ37) had a deletion of approximately 0.2 kilobase and the second mutant (Δ15) had a deletion of approximately 0.5 kilobase. Mouse myeloma cells contain several α chain alleles but only one is expressed; the presence of the deletion in Δ37 and Δ15 made it possible to identify the restriction fragments from the expressed allele. Analysis of the fragments produced after cleavage with an isoschizomeric pair of restriction enzymes, *Msp* I and *Hpa* II, indicated that, in the W3129 cell line and its variants, the unexpressed α alleles contain methylated bases. The influence of methylation on gene expression remains to be elucidated.**

Immunoglobulin gene expression exhibits allelic exclusion—that is, although the lymphocyte is diploid with two copies of each gene, only one allele is functional at any one time. Similarly, the synthesis of functional κ and λ light (L) chains appears to be mutually exclusive. During the differentiation of the lymphocyte, heavy (H) and L chain rearrangements occur when the cell becomes committed to the synthesis of a particular H or L chain (1–4). Rearrangement *per se* is not the signal for expression because multiple rearrangements occur within many lymphocytes (5, 6), and both κ alleles are frequently rearranged in a cell synthesizing λ chains (7). It has been suggested that allelic exclusion results principally from recombinational errors during rearrangement and that abortively rearranged genes are not expressed (7). However, transcription has been shown to occur off unrearranged L chain genes (8), and κ mRNA and κ related proteins have been found in λ-producing cell lines (9).

H chain disease proteins in which deleted H chain is produced in the absence of L chain have been described in man and in mouse (10, 11). In man, the steps that lead to the production of deleted H chains are not precisely defined; however, in the murine example, the H chain disease arose in two steps: first, a deleted H (ΔH) and a normal L chain were synthesized, and then a secondary variant synthesizing only ΔH was identified (12). The mechanism for the generation of the deletion in the H chain remains obscure. However, because most H chain disease proteins resume normal sequence at an exon boundary (10, 11), it has been postulated that these provide signal or recognition points during gene rearrangement or RNA processing (13).

The present paper describes two independently arising mutants of the murine myeloma W3129, which produce α chain proteins with similar internal deletions and no L chain. Analysis of genomic DNA from these mutants by digestion with restriction endonucleases followed by molecular hybridization showed that both mutants result from deletions of genomic DNA of differing extents. The W3129 myeloma contains multiple copies of the α constant region genes. However, in the mutants, only the expressed allele contains a deletion and thus restriction fragments from the expressed gene can be distinguished from those of the unexpressed genes. Analysis of the restriction fragments produced by an isoschizomeric pair of restriction enzymes, *Hpa* II and *Msp* I, shows that the nonexpressed α chain alleles are covalently modified and contain methylated bases. It is hypothesized that methylation may be one mechanism whereby the cell ensures the nonexpression of the excluded alleles.

MATERIALS AND METHODS

Cells. The W3129 tumor (IgA, κ, anti-α1 → 6 dextran) was acquired from the Salk Institute and was adapted to continuous growth in tissue culture in this laboratory. Cells are maintained in suspension culture in Iscove's modified Dulbecco's medium supplemented with glutamine and nonessential amino acids (GIBCO), penicillin, streptomycin, and 10–20% heat-inactivated (56°C, 30 min) horse serum. Cells were cloned in 0.24% agarose (Sigma) with rat embryo fibroblast feeder layers (14). Variants that had lost or gained the ability to secrete H chain were identified by overlaying the growing clones with antiserum directed against H chain.

Antisera. Rabbits were injected with 1 mg of IgA protein in complete Freund's adjuvant in the footpads; they were bled repeatedly and given booster injections. Anti-idiotypic antiserum was prepared by passing anti-IgA through Sepharose to which was attached QUPC-52 protein (IgA, κ, anti-l → 6 dextran, idiotype different from W3129) or normal mouse serum protein.

DNA Isolation and Gel Analysis. High molecular weight DNA was isolated from the myeloma cells essentially as described by Wigler *et al.* (15). DNA was digested with restriction enzymes as directed by the supplier (Bethesda Research Laboratories, Rockville, MD, or New England BioLabs). DNA was applied, at 10 μg per lane, to a Tris/borate (16) agarose gel. Molecular hybridizations were done by standard procedures (15) except that 10% dextran sulfate was added. ^{32}P-Labeled nick-translated DNA probes (17) were prepared by using re-

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Abbreviations: kb, kilobase(s); Δ, deletion; H, heavy; L, light.

Table 1. Derivation and phenotype of cell lines

Cell line*	Parental cell line	Immunoglobulin secretion	Size, daltons†	
			H chain	L chain
W3129	—	H, L	55,000	25,000
R15	W3129	None	55,000	None
Δ15	R15	ΔH	39,000	None
R37	W3129	None	55,000	None
Δ37	R37	ΔH	39,000	None
R6.1	W3129	L	None	25,000

* Variants that had lost or gained the ability to synthesize or secrete H chain.

† H and L chain size were estimated by using $NaDodSO_4$/polyacrylamide gels.

striction enzyme fragments of DNA isolated from the recombinant plasmids.

RESULTS

Isolation of Deletion Mutants from W3129. Mutants that synthesized only H chains were isolated from W3129 by cloning the cells in soft agarose, overlaying the cells with antiserum specific for IgA, and identifying those clones over which there was no precipitate. The H chain-producing mutants synthesized intact H chains that were not secreted. To determine if these H chain producers could revert to the secretion of H chain, they were cloned in soft agarose and overlaid with antiserum directed against IgA, and the clones secreting Ig were recovered. These clones were of two different phenotypes: those that had resumed the synthesis of L chain (to be described elsewhere) and those that were synthesizing and secreting H chains smaller than those of wild type. Deletion mutants were isolated from two independently derived H chain producers, R15 and R37 (Table 1). These independently arising deletion mutants had the same apparent size (Fig. 1), approximately 39,000 daltons. Analysis of the mRNAs of mutants and wild type on agarose gels (data not shown) showed that the mRNAs from both deletion mutants were shortened by approximately 0.35 kilobase (kb). When the two ΔH mutants were compared by peptide map analysis (Fig. 2) no differences were observed. Thus, within the resolution of peptide map and $NaDodSO_4$ gel analysis, the independently arising mutants were of the same structure.

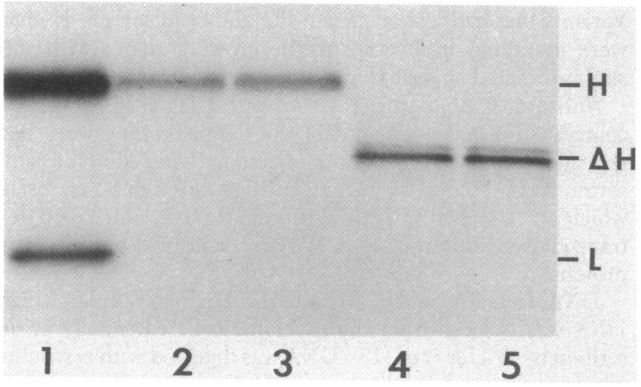

FIG. 1. $NaDodSO_4$ gel analysis of immunoglobulin immunoprecipitated from the cytoplasmic lysate of wild-type and mutant cell lines. Cells were labeled by growth in the presence of [^{14}C]valine, [^{14}C]threonine, and [^{14}C]leucine for 30 min, cytoplasmic lysates made, and the labeled immunoglobulin was immunoprecipitated as described (18, 19). Immunoprecipitates were treated with 0.15 M 2-mercaptoethanol at 37°C for 30 min and analyzed on 12% Tris/glycine gels (20). Lanes: 1, W3129; 2, R15; 3, R37; 4, Δ15; 5, Δ37.

Position of the Deletion in the Protein. The Δ15 and Δ37 deletion mutants did not exhibit any altered reactivity with anti-IgA Fc, and when Fc was prepared from them (21) it had the same apparent molecular weight as Fc prepared from wild-type W3129. Thus, the Fc of the deletion mutants appears to be intact. *In vitro* complementation between the ΔH and L chain from W3129 restored reactivity with anti-idiotypic antiserum specific for W3129. In addition, *in vitro* complemented molecules regained ability to bind antigen. Thus, at least enough of the variable region was intact in the deletion mutants to maintain idiotypic determinants and permit antigen–antibody interaction. The most probable position of the deletion therefore is CH_1.

Position of the Deletion in the Genomic DNA. Southern blot analysis of the genomic DNA was undertaken to determine the position and extent of the putative deletions. A restriction endonuclease map of the rearranged α gene of W3129 is shown in Fig. 3. Two nick-translated probes were used. One probe was the 0.8-kb *Xho–Eco*RI fragment (named *Xho* probe) containing CH_1 and part of CH_2 isolated from a cloned embryonic α chain gene; the second probe was the 1.6-kb *Eco*RI–*Sma* fragment (named *Sma* probe) isolated from a cloned 4.4-kb *Eco*RI piece; this contains part of CH_2, CH_3, and 3′ nontranslated sequences. Both α clones were the generous gifts of S.-P. Kwan and M. Scharff.

Cleavage of W3129 with *Eco*RI and hybridization with the *Xho* probe revealed two fragments, one ≈10 kb and one 5.1 kb (Fig. 4, lane 2). The embryonic *Eco*RI fragment, is ≈10 kb. Therefore, W3129 appears to contain at least two α chain genes. Cleavage of W3129 with *Eco*RI and hybridization with the *Sma* probe revealed only one fragment, 4.4 kb (data not shown). Hybridization of the *Sma* probe to *Eco*RI-cleaved DNA from Δ37 and Δ15 revealed no differences from wild type. However, when the *Eco*RI-cleaved DNAs were hybridized to the *Xho* probe, a smaller fragment was detected in Δ37 and Δ15 (Fig. 4, lanes 1 and 3). These fragments were approximately 0.3 kb shorter for Δ37 and 0.5 kb shorter for Δ15. These data suggest genomic deletions in Δ37 and Δ15.

Analysis with a series of restriction enzymes was undertaken to map the deletion. After cleavage of the genomic DNA with *Hha* I and hybridization with the *Xho* probe, the 1.3-kb fragment from W3129 was found to be replaced by fragments of 0.8 and 1.1 kb in Δ15 and Δ37, respectively (Fig. 4, lanes 4 and 6). Therefore, Δ15 and Δ37 contain deletions within the 1.3-kb *Hha* fragment of approximately 0.5 and 0.2 kb, respectively. Cleavage with *Hinc*II yielded two fragments from W3129, a 1.05-kb fragment from the 5′ side and a 3.3-kb fragment from the 3′ side of the *Hinc*II site (Fig. 4, lane 7). In Δ37, a new 0.85-kb piece was present, suggesting that Δ37 has a deletion of 0.2 kb 5′ to the CH_1 *Hinc*II site. In Δ15, a new 4-kb fragment was found that hybridized to both the *Xho* and *Sma* probes, suggesting that in Δ15 the CH_1 *Hinc*II site has been deleted. Analysis of double digests with *Xho*/*Eco*RI and *Xho*/*Hha* I verified that Δ15 has a deletion of 0.4–0.5 kb between the *Xho* and *Eco*RI sites, suggesting that most of the CH_1 encoding region and portions of the intervening sequences are missing. For Δ37, approximately 0.2 kb is deleted. The results of the restriction mapping are summarized in Fig. 3.

Methylation of the Unexpressed Allele. Digestion with *Eco*RI suggested that W3129 and its mutants contain at least two α chain alleles (Fig. 4, lanes 1–3). However, after digestion with *Hha* I, no residual 1.3-kb fragment from the nonexpressed allele was seen (Fig. 4, lanes 4–6). The recognition sequence for *Hha* I is G-C-G-C, and it will not cleave the sequence G-^{m}C-G-C; it thus is possible that methylated bases are present in the unexpressed α chain genes and they are not cleaved by

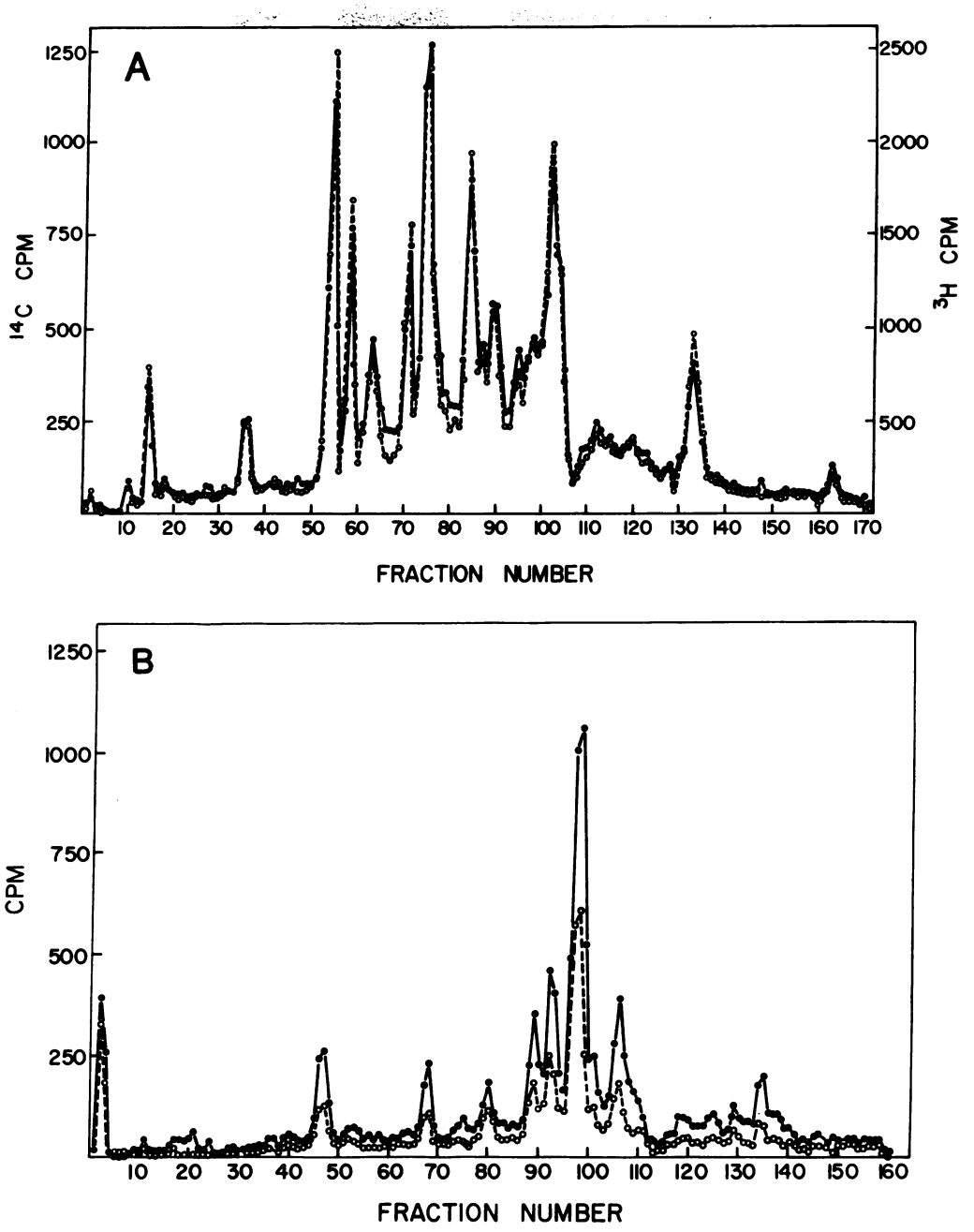

FIG. 2. Analysis of the tryptic peptides from the heavy chains of Δ15 and Δ37. The ^{14}C- and ^{3}H-labeled H chains were prepared and resuspended in 0.05 M NH_4CO_3 at pH 8.0, and trypsin (0.5 mg) was added. Digestion was carried out for 5–18 hr, with two additions of trypsin (0.5 mg each); samples were diluted with an equal volume of glacial acetic acid and applied to a 1 × 25 cm column containing a Technicon P2 resin warmed to 60°C. Fractions were eluted with a pyridine acetate gradient and the position of the peaks was determined by using a Beckman liquid scintillation counter; all results are corrected for background and for spill of ^{14}C into the ^{3}H channel. (*A*) With lysine as label. ●——●, ^{14}C-Labeled Δ15; ○---○, ^{3}H-labeled Δ37. (*B*) With arginine as label. ●——●, ^{3}H-Labeled Δ15; ○---○, ^{14}C-labeled Δ37.

Hha I. No isoschizomer for *Hha* I that will cleave the methylated recognition sequence is available.

To determine if differential base methylation occurs in the expressed and nonexpressed α alleles, an isoschizomeric pair of restriction enzymes, *Hpa* II and *Msp* I, was used. Each enzyme cleaves the sequence 5′ C-C-G-G 3′. *Hpa* II digests this sequence only if the penultimate cystosine is not methylated; *Msp* I digests if the penultimate cytosine is methylated. Cleavge with *Hpa* II generated several fragments from W3129 which hybridized to the *Xho* piece: one 1.6 kb, one 2.6 kb, and several larger fragments. The 2.6-kb piece also hybridized to the *Sma* probe, indicating that it is from the 3′ side of the *Hpa* II site. With Δ37, the 1.6-kb fragment was replaced by a 1.4-kb fragment (Fig. 5, lane 3, indicated by open arrowhead) indicating a ≈ 0.2-kb deletion in the 5′ *Hpa* II fragment. In Δ15, both the 2.6- and 1.6-kb fragments were missing, and there was a new ≈ 4-kb piece that hybridized with both probes (Fig. 5, lane 1, indicated by solid arrowhead). Therefore, Δ15 appears to have lost the *Hpa*II site in CH_1. In Δ15, Δ37, and W3129 there were additional large fragments that hybridized with both probes. After cleavage with *Msp* I the large bands disappeared. In Δ15 and Δ37, new small bands appeared that were identical in migration to those seen in W3129. However, the bands that contained the deletion were not altered in mobility. Therefore, the

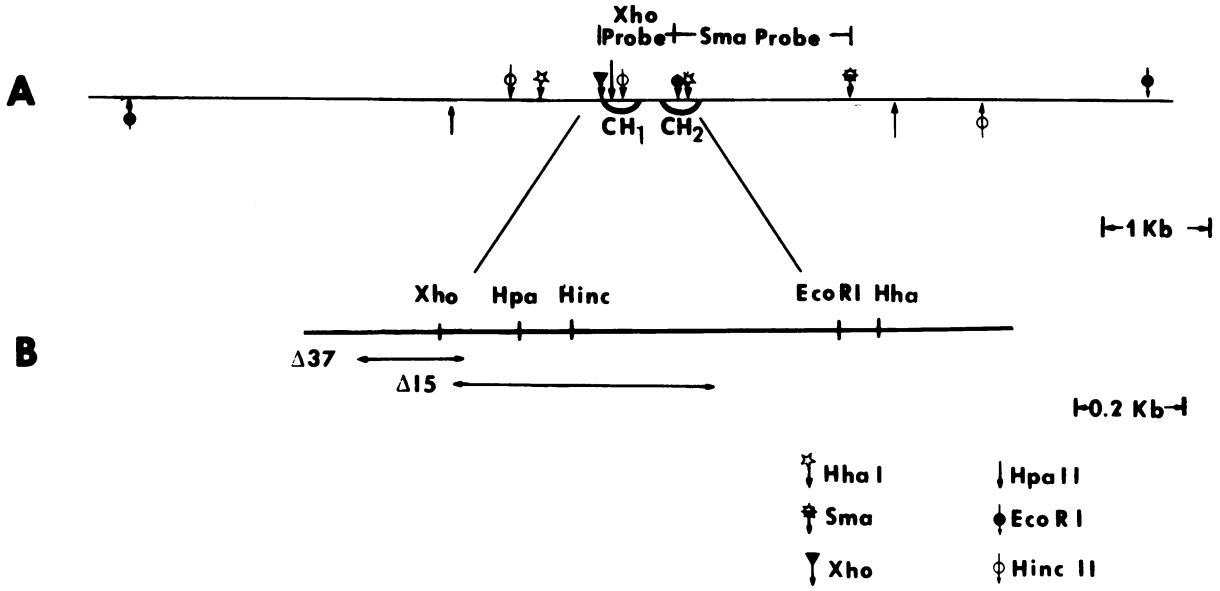

FIG. 3. Analysis of the genomic DNA of W3129, Δ37, and Δ15. (*A*) Restriction map of the α chain gene of W3129. Restriction sites from other reports (22–24) confirmed by the current investigation are shown above the line. Restriction sites determined from the current analyses are indicated below the line. The approximate positions of the exons are from Early *et al.* (23). The extent of the probes used is indicated. (*B*) Approximate extent of the genomic deletions of Δ37 and Δ15.

deleted transcribed genes show no evidence of methylation. However, methylated bases appear to be present in the nondeleted, nontranscribed genes. In cell line R6.1 (lane 7), which synthesizes no H chain, all α sequences contain methylated bases.

DISCUSSION

These studies of two ΔH mutants have enabled us to identify a potential new mechanism for the regulation of Ig gene expression and allelic exclusion. Although multiple α H chain alleles are present in the myeloma cells, only the expressed allele contains a deletion. Thus, restriction fragments from the expressed allele can be distinguished from those from the unexpressed allele, and it can be shown that the unexpressed α chain alleles contain methylated bases. In the expressed allele these bases are not methylated.

Many different deletion mutants of heavy chains have been described (10–12, 25–29). One γ H chain mutant, IF2, with an internal deletion of the entire CH_1 domain (27, 30) has been shown to result from a large genomic deletion. In addition to the CH_1 exon, part of CH_1–hinge intervening sequence and at least 3 kb of the 5′ intron are missing (31). The two deletions described in this report differ from each other and from IF2.

Δ37 and Δ15 both appear to synthesize identical shortened H chains, lacking about 16,000 daltons. Because α chains are glycoproteins it is not possible to determine from the molecular weight alone if exactly one exon is missing. However, within the resolution of $NaDodSO_4$ gels, the Fc portion of the molecule is normal in size and enough variable region of the H chain is present to maintain idiotypic determinants and antigen binding. The mRNA of the mutants is shortened by approximately 0.35 kb or enough to encode ≈120 amino acids, one domain.

Southern blot analysis indicates that, unlike IF2, Δ37 and

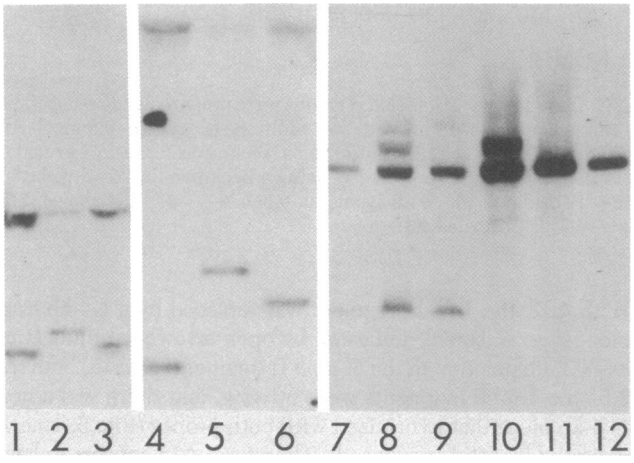

FIG. 4. Hybridization of ^{32}P-labeled *Xho* or *Sma* probe to restriction fragments of genomic DNA. DNA was digested with *Eco*RI (lanes 1–3), *Hha* I (lanes 4–6), or *Hinc*II (lane 7–12), electrophoresed through agarose, and transferred to nitrocellulose. Either the *Xho* (lanes 1–9) or *Sma* (lanes 10–12) probe was used for hybridization. W3129 (lanes 2, 5, 7, 11), Δ15 (lanes 1, 4, 8, 10), or Δ37 (lanes 3, 6, 9, 12) DNA was used.

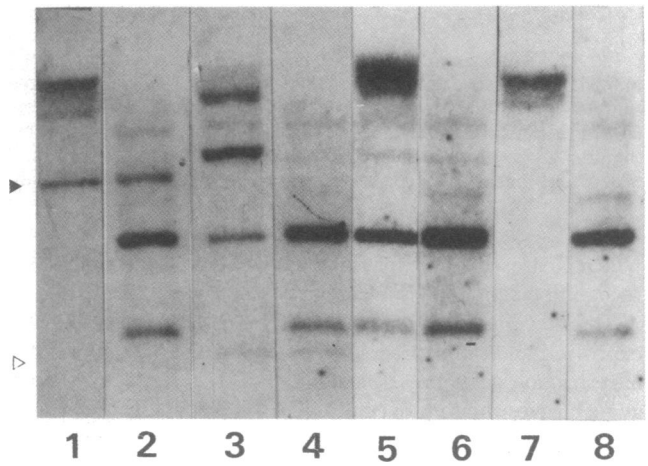

FIG. 5. Hybridization of ^{32}P-labeled *Xho* probe to restriction fragments of genomic DNA. DNA was digested with either *Msp* I (lanes 2, 4, 6, 8) or *Hpa* II (lanes 1, 3, 5, 7), electrophoresed through agarose, and transferred to nitrocellulose. W3129 (lanes 5, 6), Δ15 lanes (1, 2), Δ37 (lanes 3, 4), or R6.1 (lanes 7, 8) DNA was used.

Δ15 contain small genomic deletions of different extents. Both deletions lie 5′ to the *Eco*RI and *Hha* I sites in CH_2 (Fig. 3). The deletion in Δ15 appears to begin 3′ to the *Xho* site and is large enough to encompass all of the CH_1 exon plus portions of the intervening sequences. Δ37 appears to include the *Xho* site but not to remove the entire CH_1 exon, suggesting that information is discarded during RNA processing. The L chain fragment of MPC-11 provides a precedent for such events (32, 33); in that case, the absence of the J segment from the DNA results in the removal of the variable region of the H chain during RNA splicing. Although Δ15 and Δ37 differ in extent, their transcripts are processed to give rise to mRNAs and proteins of the same sizes.

IF2 has been proposed to arise by the illegitimate use of switch signals. The proposed switch signals lie about 2 kb 3′ to the *Eco*RI site in the V–CH_1 intervening sequence. Therefore, the nearest they could lie to the α chain gene of W3129 is about 2.3 Kb. But neither Δ37 nor Δ15 has deletions in this region. If Δ15 and Δ37 arise by the illegitimate use of switch signals, these signals must be different both from those used in IF2 and from each other. On the other hand, Δ15 and Δ37 may arise by a recombination/excision mechanism between regions of homology within the α chain gene.

Immunoglobulin-producing cells exhibit the phenomenon of allelic exclusion in that only one functional H and L chain is produced from the many available alleles. Allelic exclusion of L chains has been extensively investigated and it has been hypothesized that aberrant V–J joining leads to nonexpression. It has been shown in two cases that incorrect joining leads either to nonsense (34) or missense (35) mutations in the L chain gene and hence no functional protein is seen. In the present study, we examined the expression of α H chains in mouse myeloma cells containing more than one α allele and observed that the unexpressed α chain genes have methylated cytosine residues. This observation was possible because in the mutants the expressed genes contain deletions and the restriction fragments from the gene directing their transcription are clearly defined. The fragments are of the same size after cleavage with *Msp* I or *Hpa* II, isoschizomers that distinguish between methylated sequences; however, the sizes of the restriction fragments from the unexpressed allele are quite different after cleavage with these enzymes. Therefore, the unexpressed α chain allele appears to be methylated. This inverse relationship between expression and methylation has previously been observed for viral genes (36), some differentiated proteins (37, 38), rRNA (39), and genes turned off after malignant transformation (40). It is yet to be demonstrated whether methylation is the cause or consequence of the lack of expression. However, it does suggest that covalent modification of DNA by methylation may provide a second mechanism, in addition to abortive recombination, to ensure the expression of only one immunoglobulin allele.

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