Myeloma Mutant with a Novel 3' Flanking Region: Loss of Normal Sequence and Insertion of Repetitive Elements Leads to Decreased Transcription but Normal Processing of the Alpha Heavy-Chain Gene Products

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We isolated and characterized LP1.2, a mouse myeloma mutant with a deletion of at least 4 kilobases (kb) immediately 3' of the alpha gene and introduction of at least 5 kb of novel (nonimmunoglobulin) sequence in its place. A 6.2-kb genomic *EcoRI* fragment from the mutated allele was cloned, and a subfragment was sequenced. The deletion begins 11 base pairs (bp) beyond the normal site of cleavage and polyadenylation for the secreted form of alpha mRNA. A short direct repeat, eight copies of the 17-mer GCCT ATAGAAGTAAGGA, is located at the junction of the alpha and novel sequences. The first 4 bp of the 17-mer are identical to the last 4 bp of the alpha sequence. Novel sequences downstream of the direct repeats in LP1.2 include a low-copy-number sequence flanked by two distinct, highly repetitive elements. The low-copy-number portion of the novel sequence appears on a single 30-kb *EcoRI* fragment in several myelomas and in liver DNA; one copy of this fragment has rearranged in cell line W3129, and this allele has rearranged a second time in LP1.2. LP1.2 contains low levels of apparently normal alpha protein and mRNA. The S1 nuclease protection of nuclear and cytoplasmic RNAs shows that cleavage and polyadenylation are efficient and accurate and that they occur without the accumulation of aberrant transcripts. Alpha transcription in isolated nuclei is decreased sevenfold in LP1.2 relative to its parent, which accounts for the low steady-state levels of cytoplasmic alpha mRNA and protein in LP1.2. Decreased alpha transcription could result either from the deletion of a positive regulator in the 3' flanking region or from the introduction of novel sequences which exert a negative effect.

Immunoglobulin genes are assembled from germ line variable-region and constant-region gene segments in developing B cells. Despite the fact that germline variable-region genes possess functional promoters (5), they are not expressed in myeloma cells (46, 79). Conversely, germ line constant-region genes that lack an immunoglobulin promoter are transcribed from secondary promoters in the 5' flanking region (54, 73). Recently, positive regulatory sequences, or enhancers, have been identified in the J-C intron of both mu and kappa genes (3, 29, 57). The activation of immunoglobulin genes may be accomplished by transposing the variable region with its immunoglobulin promoter to the vicinity of the constant-region enhancer.

Although immunoglobulin enhancers are required for the expression of linked genes introduced into lymphoid cells by transformation, the enhancer may not be required for the maintenance of high-level immunoglobulin expression. Recently, several myeloma mutants have been described in which the heavy-chain enhancer has been deleted (23, 42). Despite this deletion, normal levels of heavy-chain mRNA and protein are made by the cell. When the allele with the enhancer deletion is cloned from the genome and introduced into myeloma cells, it is transcriptionally inactive (80).

We chose to investigate immunoglobulin gene regulation by isolating mutants of immunoglobulin-producing cells which synthesize little or no heavy chain and then defining the genomic lesion which leads to the mutant phenotype. In this way, we hoped to identify and establish the function of sequences essential for immunoglobulin expression.

We report here the isolation and characterization of LP1.2, a mutant myeloma with very low levels of cytoplasmic alpha mRNA and protein. This mutant has been found to have deleted at least 4 kilobases (kb) of sequences normally present 3' of the α heavy-chain gene. In its place is a novel sequence which contains two different highly repeated elements and a unique sequence component. The unique sequence component does not characteristically rearrange in immunoglobulin-producing cells. However, it has been shown to rearrange twice in the myeloma W3129 and its derivatives. In LP1.2, the sequences 3' of those represented in the mature form of the alpha mRNA encoding secretory immunoglobulin A (IgA) are deleted. Despite this, LP1.2 transcripts are processed normally and have the same 3' end as do the wild-type transcripts. A transcriptional analysis of the mutant showed a decrease in the transcription rate of the same order of magnitude as the observed decrease in cytoplasmic mRNA. The decreased transcription rate of the alpha gene in LP1.2 could result from the deletion of a positive regulatory sequence from the 3' flanking region. Alternatively, the novel sequences introduced at the 3' end of the transcription unit may interfere with transcription.

MATERIALS AND METHODS

Cell lines and mutant isolation. Derivatives of the mouse myeloma cell line W3129, an IgA(κ) producer specific for $\alpha(1\rightarrow6)$ -linked dextrans, were used. All lines were grown in suspension in Iscove modified Dulbecco medium supplemented with glutamine and nonessential amino acids (GIBCO Laboratories), penicillin, streptomycin, and 10 to 20%

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heat-inactivated (56°C for 30 min) horse serum (Flow Laboratories, Inc.).

Selection of mutants. $\Delta 15.8$, a mutant of W3129 which synthesizes a heavy chain with a deletion in CH_1 and no light chain, was cloned in 0.24% agarose (Sigma Chemical Co.) over a feeder layer of rat embryo fibroblasts (17). After 2 to 4 days, the clones were overlaid with 0.1 ml of rabbit anti-mouse IgA in agarose. Several days thereafter antibody-antigen precipitates were visible in the agarose surrounding those clones that secreted IgA. Approximately 16 such clones were transferred to liquid media, and 9 of these were grown to mass culture. From four of these independent IgA-producing subclones of $\Delta 15.8$, nonsecreting variants were isolated, subcloned to ensure clonality, and analyzed. Of these variants, three were shown to have resulted from the loss of the rearranged alpha allele and were not studied further. The fourth, LP1.2, is described in detail below.

RNA. Cytoplasmic RNA was isolated as described before (47). To prepare nuclear RNA, nuclei were isolated as described for the nuclear-runoff assay. Nuclear RNA was prepared from 5×10^7 to 5×10^8 nuclei that had been lysed in 4 M guanidine thiocyanate (15). DNA was sheared first by vortexing and then by passage through 22- and 25-gauge needles. Nuclear guanidine thiocyanate lysates were layered over a 5.7 M CsCl (Suprapur; EM Reagents) cushion containing 0.1 M EDTA at pH 7.0. The CsCl solution was treated with diethylpyrocarbonate and autoclaved before use. RNA was pelleted by centrifugation for 19 h at 24,000 rpm in a Beckman Ultracentrifuge in an SW41 or SW28 swinging-bucket rotor at 15°C.

Of the total cytoplasmic RNA, 5 μ g was separated by electrophoresis in formaldehyde-agarose slab gels, transferred to nitrocellulose, and hybridized as described before (70).

S1 nuclease protection. DNA restriction fragments were end labeled with T4 DNA polymerase in a replacement reaction. Of the digested plasmid, 1 μ g was incubated at 37°C for 5 min with T4 DNA polymerase (P-L Biochemicals, Inc.) in 33 mM Tris acetate (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, 5 mM dithiothreitol, and 100 μ g of bovine serum albumin per ml. dATP, dTTP, and dGTP (P-L Biochemicals) were added to a final concentration of 100 μ M with 15 pmol of [32 P]dCTP (3,000 Ci/mmol; Amersham Corp.). After 10 min at 37°C, cold dCTP was added to 100 μ M and incubated for an additional 10 min.

Of the labeled DNA, 100 ng was ethanol precipitated with 5 μ g of the total cytoplasmic RNA or 30 μ g of the nuclear RNA, suspended in 6 μ l of 5 $\times$ buffer (1 $\times$ is 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) [pH 6.4], 1 mM EDTA, 0.4 M NaCl) (8) and 24 μ l of formamide, heated to 70°C for 10 min, transferred rapidly to a water bath at 50°C, and incubated overnight. Samples were digested at 37°C for 1 h as described before (8).

Transcription in isolated nuclei. Nuclei were isolated by using the procedure of Eberwine and Roberts (22) with minor modifications. To isolate nuclei, 5×10^7 cells were pelleted, washed in Iscove modified Dulbecco medium without serum, repelleted, suspended in 2 ml of AT buffer (0.3 M sucrose, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.15% Triton X-100), layered over a 0.8-ml cushion of AT buffer plus 0.4 M sucrose in a 4-ml polypropylene tube, and centrifuged for 10 min at 2,500 rpm in a DAMON IEC centrifuge at 4°C. The cytoplasmic fraction was removed, and RNA was extracted. The nuclear pellet was loosened, then suspended in 1 ml of AT buffer containing 0.3 M sucrose, transferred to a 1.5-ml Sarstedt tube, and centri-

fuged again for 10 min at 2,500 rpm. The nuclear pellet was loosened and suspended in 50 μ l [32 P]UTP (400 Ci/mM; Amersham), and the volume was measured. An equal volume of 2 $\times$ transcription buffer (usually 85 μ l) was added, mixed by pipette, and incubated at 29°C for 30 min. When the nuclei are being prepared, speed is essential, the nuclei must be kept cold at all times. All manipulations were done in the cold room by using prechilled pipettes, pipette tips, tubes, and solutions. It is also essential to loosen the cellular or nuclear pellet on each occasion to prevent clumping and cell lysis.

The 2 $\times$ transcription buffer contained 40 mM Tris (pH 8.3), 5 mM $MgCl_2$, 2 mM $MnCl_2$, 2.5 mM dithiothreitol, 32% glycerol, 80 mM $(NH_4)_2SO_4$, 0.6 mM ATP, 0.25 mM CTP, 0.25 mM GTP (nucleoside triphosphate purchased from P-L Biochemicals), 7 U of nucleoside 5' diphosphate kinase per ml, 2 mM phosphocreatine, 35 μ g of creatine phosphokinase per ml (enzymes from Boehringer Mannheim Biochemicals), 0.1 mM EDTA, and 2 μ l of RNasin (Promega Biotec) per batch.

Labeled RNA was isolated essentially as described before (22). Nuclei were lysed in 1% sodium dodecyl sulfate (SDS) and digested with 10 μ l of proteinase K (20 mg/ml) (Boehringer Mannheim) at 45°C for 30 min. Proteins were extracted twice with 1:1 phenol- $CHCl_3$; nucleic acids were precipitated in 5% trichloroacetic acid and collected on a BA85 24-mm filter (Schleicher & Schuell, Inc.). After being digested by RNase-free DNase (Worthington Diagnostics), RNA was eluted in SET buffer (1% SDS, 5 mM EDTA, 10 mM Tris [pH 8]), phenol extracted, and ethanol precipitated.

To prepare filters, electrophoresis through agarose gels was used to separate DNA fragments. The fragments were then blot transferred to Gene Screen Plus (New England Nuclear Corp.) membranes by using 10 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 14 to 24 h. Filters were air dried and prehybridized in 1% SDS-1 M NaCl-1 $\times$ Denhardt solution (0.02% each polyvinylpyrrolidone)-Ficoll (Pharmacia Fine Chemicals)-bovine serum albumin (Pentax fraction V)-50 mM Tris (pH 7.5) for 24 h at 60°C. [32 P]RNA was added to a final activity of from 1×10^6 to 4×10^6 cpm/ml of hybridization solution. After being hybridized for 3 to 4 days at 60°C, the filters were washed exactly as described in the Gene Screen Plus manual and were exposed to X-ray film with intensifying screens at -70°C for 2 to 12 h. X rays were scanned by using a densitometer to quantitate the hybridization of each band. As an internal positive control, a housekeeping gene, gamma actin, was used in these experiments.

DNA isolation and characterization. High-molecular-weight DNA was prepared essentially as described by Wigler et al. (78). Of the genomic DNA, 10 μ g was digested with 20 U of restriction enzyme for at least 1 h under conditions suggested by the supplier. Samples were electrophoresed through horizontal agarose slab gels in Tris borate (56) or Tris acetate buffer (40 mM Tris acetate, 2 mM EDTA [pH 8]). DNA was transferred to nitrocellulose filters (BA85 grade; Schleicher & Schuell) essentially as described before (64). Hybridization to nick-translated DNA probes (76) was performed as described before (77). All blots were washed to a stringency of 0.5 $\times$ SSC at 65°C.

DNA sequencing. The fragment to be sequenced was cloned into M13mp8 and M13mp9 (49). Single stranded templates were prepared (62) and sequenced by the dideoxy chain termination method (60, 62), the 17-base universal primer P-L Biochemicals) was used. The reaction products were separated on an 8 M urea-8% polyacrylamide gel in

Tris borate buffer and exposed to X-ray film at 70°C with an intensifying screen for 1 to 12 h.

Bacteriophage library preparation. High-molecular-weight genomic DNA from LP1.2 was digested to completion with *Eco*RI and separated on a 0.8% agarose gel. In the 4- to 7-kb region, 10 slices (3 mm) were cut from the gel, and DNA was extracted by adsorption onto glass powder (74). A portion of each fraction was electrophoresed in agarose, blot transferred to nitrocellulose, and hybridized to an alpha-specific probe (see Fig. 2c). In this way, the fraction containing the 6.2-kb *Eco*RI fragment of interest was clearly identified.

The fraction containing the 6.2-kb *Eco*RI fragment was ligated to *Eco*RI-digested λ gt10 (39) and packaged into phage coats in vitro (67). Plaque hybridization (6) was used to identify recombinant phage with alpha-specific inserts. Phage DNA was prepared from plate lysates as described before (14).

Plasmid construction and growth. Fragments were isolated from agarose gels by using glass powder (74) and were mixed with linearized vectors by using a vector/insert molar ratio of 1:6. Ligations were carried out at 12°C under conditions suggested by the supplier (Collaborative Research, Inc.). Ligated DNA was introduced into bacterial cells made competent by treatment with CaCl_2 (44). Plasmids were isolated from bacteria by lysis in sodium hydroxide-SDS (9), and plasmids carrying correct inserts were identified by filter hybridization (32).

RESULTS

Selection of mutants. The cell line $\Delta 15.8$, which synthesizes and secretes an alpha heavy chain with a deletion in CH_1 , was cloned in soft agarose to isolate myeloma cells deficient in their secretion of immunoglobulin. $\Delta 15.8$ was isolated in two steps from the myeloma W3129 (20). The interrelationship of the mutants is shown in Table 1. Growing clones were overlaid with rabbit anti-mouse IgA, and clones over which no antigen-antibody precipitate formed were identified. Of four independent nonsecreting variants that were isolated and analyzed, three had lost the productively rearranged alpha allele. The fourth, LP1.2, is described in detail below.

Biosynthetic labeling and immunoprecipitation of cytoplasmic proteins from LP1.2 and $\Delta 15.8$. To compare the synthesis of alpha chains in $\Delta 15.8$ and LP1.2, we incubated myeloma cells for 30 min in medium containing isotopically labeled amino acids. Cytoplasmic extracts were immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis. LP1.2 contained a small quantity of alpha heavy chain of the same apparent molecular weight as that made by $\Delta 15.8$ (Fig. 1). A pulse-chase experiment (data not shown) showed no evidence of protein degradation in LP1.2. The newly synthesized alpha protein was stable over a 3-h chase and assembled normally into H_2 dimers. The low level of protein present is therefore a consequence of decreased

FIG. 1. Immunoprecipitated cytoplasmic proteins from W3129 (W), LP1.2 (LP), and $\Delta 15.8$ ($\Delta 15$). For each cell line, approximately 3×10^6 cells were labeled for 30 min with [^{14}C]VTL as previously described (51), and a cytoplasmic fraction was immunoprecipitated with rabbit anti-mouse IgA and insoluble protein A. Labeled proteins were separated by SDS-polyacrylamide gel electrophoresis in phosphate buffer (43) under nonreducing conditions. Proteins were detected by the autoradiography of dried gels. Arrows at left indicated kappa light chains (L), alpha heavy chains (H), and heavy-chain dimers (H_2) made by W3129. Arrows at right indicate the shortened alpha heavy chain (ΔH) and heavy-chain dimers (ΔH_2) made by $\Delta 15.8$ and LP1.2.

synthesis. Analysis of the cytoplasmic mRNA of $\Delta 15.8$ and LP1.2 showed that both contain alpha mRNA of the same size, but $\Delta 15.8$ contains about 10-fold more alpha mRNA than does LP1.2. In both cases, the alpha mRNA was polyadenylated.

Characterization of the mutated allele. High-molecular-weight DNA was digested with *Eco*RI and hybridized to either the *Xho*I to *Eco*RI (XHO) fragment of the *Eco*RI to *Sma*I (SMA) fragment to locate the genomic lesion in LP1.2 (Fig. 2). In each cell line, two *Eco*RI fragments hybridize to the XHO fragment (Fig. 2a). These are the 10-kb fragment from the unexpressed allele and the approximately 5-kb fragment from the rearranged and expressed allele. The 5-kb fragments in LP1.2 and $\Delta 15.8$ are of the same size and, because of the deletion in CH_1 (20), slightly smaller than the corresponding fragment from W3129. However, when the 3' *Eco*RI fragment was investigated by using the SMA probe, a novel 6.2-kb *Eco*RI fragment appeared in LP1.2 (Fig. 2b), indicating a change downstream of the *Eco*RI site in CH_2 . To define the change more precisely, this novel 6.2-kb *Eco*RI fragment was cloned into λ gt10 and then subcloned into pBR325.

Restriction map of the 6.2-*Eco*RI fragment. A restriction map of the novel fragment is compared with the known restriction map of the alpha gene in Fig. 3. The *Hind*III site 900 base pairs (bp) downstream of the *Eco*RI site in CH_2 is missing in the mutated allele, and a new *Msp*I site has appeared within 1 kb of the *Eco*RI site. It is interesting to note that restriction sites found in the latter two-thirds of the fragment show no relationship to sites in the wild-type gene.

TABLE 1. Derivation of mutant cell line

Cell line	Parent	Characteristics
W3129		IgA(κ) myeloma specific for $\alpha(1 \rightarrow 6)$ dextran
R15	W3129	Synthesis of alpha heavy chain but not light chain; however, contains kappa mRNA
$\Delta 15.8$	R15	Synthesis of alpha chain lacking CH_1
LP1.2	$\Delta 15.8$	Decreased synthesis of alpha heavy chain lacking CH_1

FIG. 2. Genomic Southern blot. High-molecular-weight DNA was digested with *EcoRI* and hybridized to the XHO fragment (a) or to the SMA fragment (b). Lanes: 1, W3129; 2, LP1.2; 3, $\Delta 15.8$. For both panels, arrows at left indicate Charon 4A *Bam*HI molecular weight markers. Those to the right are Charon 4A *Sac*I markers. (c) Restriction maps of the wild-type alpha constant region (IgA) and the $\Delta 15.8$ allele. Solid boxes denote exons. Thin lines in $\Delta 15.8$ indicate genomic deletions of CH₁ ($\Delta 15$) and a part of CH₃ (ΔW) (see text). The site at which the secreted form of alpha mRNA is cleaved and polyadenylated is labeled cD. DNA fragments used as hybridization probes are indicated above the wild-type (IgA) map at the top of the figure. Restriction enzymes are *Xho*I (Xh), *Msp*I (M), *Hinc*II (Hc), *Eco*RI (E), *Pvu*II (Pv), *Hind*III (H), *Bgl*II (Bg), *Sma*I (S), *Pst*I (P), and *Xba*I (X).

A *Pvu*II-*Msp*I fragment containing both alpha and novel sequences was cloned into M13, and both strands were sequenced by using the dideoxy termination method described by Sanger et al. (60). The sequence is 389 bp long and departs from published alpha sequences at two points (Fig. 3). First, there is a deletion of 55 bp and an addition of one base in CH₃ at nucleotide 1455 (numbering system from 73), predicting an in-frame deletion of amino acids 446 to 463 and the substitution of a serine for a glycine at position 464. This deletion is present in W3129, is unrelated to the LP1.2 phenotype, and will be described in detail elsewhere (manuscript in preparation). Beyond the deletion in CH₃, the sequence continues in agreement with published cDNA and genomic sequences with an intact polyadenylation (pA) addition signal and a 3' untranslated region (72). The sequence diverges from the published alpha genomic sequence 11 bp beyond the site of polyadenylation. The new sequence begins with eight direct repeats of 17 bp each (Fig. 3). The

eight repeats are identical to the bracketed sequence except for the fifth, which reads GCCTATA--AGTAAGGA, and the eighth, which is missing the final A. The starred bases GCCT within the first repeat are common to both the repeat and to the wild-type alpha sequence at that location. The next base following these starred bases is the first base that diverges from published alpha sequences. The additional 60 bp of novel sequence following the direct repeats are unrelated to published alpha sequences. The presence of the 11 bp of genomic sequence following the cDNA cleavage site, retention of the CH₂-CH₃ intron, and the absence of a poly(A) tail, shows that the mutation is not the result of the insertion and expression of an alpha cDNA.

To determine whether the substitution of the 3' flanking sequence occurred as a result of deletion, insertion, or inversion, the *Sma*I to *Eco*RI (MB) fragment (Fig. 2c), which contains the membrane exon, was hybridized to genomic DNA from $\Delta 15.8$ and LP1.2. If an inversion or insertion had

FIG. 3. Restriction maps of the $\Delta 15.8$ alpha constant region and the LP1.2 allele including the 6.2-kb *EcoRI* (p6.2.11) fragment cloned in λ gt10. Solid boxes denote exons. Deletions found in $\Delta 15.8$ and in W3129 are indicated by short bars above the map. Novel sequences in LP1.2 are indicated by a thick line. The portion of p6.2.11 subcloned in M13 and sequenced (indicated by the arrow M13) is expanded at the bottom. The region of CH₃ absent from W3129 and its progeny (ΔW) is indicated by a thin line. The direct repeats are shown as open boxes. The polyadenylation signal AAATAA is labeled pA and the site of cleavage and polyadenylation is labeled cD. Hatched bars indicate the position of repetitive elements. Beneath the LP1.2 map is the sequence from the poly(A) signal to the *MspI* site. The bracketed sequence is repeated eight times; all repeats are identical except for the fifth, which reads GCCTATA--AGGTAAGGA, and the eighth, which lacks the final A. Restriction sites are *XhoI* (Xh), *EcoRI* (E), *PvuII* (Pv) and $\blacksquare$, *HindIII* (H), *BglII* (Bg), *SmaI* (S), *MspI* (M) and $\triangle$, *PstI* (P), *HincII* (Hc), *XbaI* (X), *BamHI* (B), *KpnI* (K), and *BstEII* ($\bullet$).

occurred, these downstream sequences should appear in a new context in LP1.2. Figure 4 shows that digestion with any one of five restriction enzymes and hybridization with the MB probe reveal no novel bands in LP1.2. The *BamHI* and *BglII* MB⁺ fragments from the rearranged alpha allele seen in $\Delta 15.8$ are missing in LP1.2. The 6.2-kb *EcoRI* fragment in LP1.2 does not hybridize to the MB probe. Therefore, a minimum of 3.8 kb of 3' flanking sequence have been deleted in LP1.2, including the entire MB fragment and most of the SMA fragment.

Characterization of novel sequences and their localization in the mouse genome: identification of repetitive elements. When the 6.2-kb *EcoRI* fragment was hybridized to filters containing genomic DNA, a diffuse background of hybridization was observed, with somewhat stronger hybridization to the 6.2-kb *EcoRI* fragment. To determine whether this resulted from hybridization to mouse repetitive elements and to localize any repetitive sequences within the *EcoRI* fragment, a repetitive mouse DNA probe was prepared by nick translation of genomic DNA from $\Delta 15.8$.

The 6.2-kb *EcoRI* fragment was digested with a panel of restriction enzymes singly and in pairs, and the fragments were hybridized to the repetitive sequence probe. Fragments

at the 5' and 3' ends of the insert contained mouse repetitive sequences; these are indicated by hatched bars beneath the map of LP1.2. A nonrepetitive central region extends from the first *XbaI* site 2 kb from the 5' end to the *BamHI* site 2 kb from the 3' end. The two repetitive elements are distinct and do not cross-hybridize (data not shown). Highly repetitive mouse DNA does not hybridize to the 300-bp *PvuII* fragment in p6.2.11, which includes all eight 17-bp direct repeats (Fig. 3). The direct repeats occur only once in p6.2.11, as shown by the lack of hybridization of the *PvuII* fragment to other regions of p6.2.11 (data not shown).

Localization of novel sequences in $\Delta 15.8$ and LP1.2 by using a unique sequence probe. The *PstI* fragment located between the highly repetitive sequences (Fig. 3) hybridizes to two *EcoRI* fragments of 30 and 20 kb in W3129 and $\Delta 15.8$ (Fig. 5A). DNA from the liver of BALB/c and C57BL/6 mice, from a myeloma or from three hybridomas, contains only one large *EcoRI* fragment of about 30 kb (Fig. 5B). On a separate filter (not shown), the myeloma J558 and three sequentially derived mutants show the same 30-kb fragment. In LP1.2, the 20-kb band unique to W3129 and $\Delta 15.8$ is replaced by the 6.2-kb band. Apparently, the unique sequence has undergone one rearrangement in W3129 to

FIG. 4. Southern blot analysis of genomic DNA from $\Delta 15.8$ and LP1.2. High-molecular-weight DNA was digested with one of five restriction enzymes and hybridized to the MB probe (Fig. 2c). Lanes 1, 3, 5, 7, and 9 contain DNA from $\Delta 15.8$; lanes 2, 4, 6, 8, and 10 contain DNA from LP1.2. Enzymes used were *Bam*HI (lanes 1 and 2), *Bgl*III (lanes 3 and 4), *Hind*III (lanes 5 and 6), *Eco*RI (lanes 7 and 8), and *Sac*I (lanes 9 and 10). In lanes 1 and 3, arrows point to fragments in $\Delta 15.8$ that have been lost in LP1.2 (see the text). The arrow in lane 8 indicates the approximate position of the novel 6.2-kb *Eco*RI fragment in LP1.2. Arrows at left indicate λ *Hind*III molecular weight markers.

produce the 20-kb fragment, and this fragment has in turn been rearranged in LP1.2 to form the 6.2-kb alpha-junction fragment. Rearrangement of this sequence does not appear to be characteristic of immunoglobulin-producing cells.

Cytoplasmic alpha mRNA has an unaltered 3' end. To determine whether the altered 3' flanking region in LP1.2 influences the site of cleavage and polyadenylation, the 3' ends of alpha mRNA were examined by S1 nuclease protection. When the mutant gene was end labeled with T4 DNA polymerase at its *Pvu*II site (Fig. 3), a 155-bp fragment was protected by RNA from W3129 or LP1.2 (Fig. 6, lanes 3 and 1, respectively). The mutant allele carries a small deletion in CH_3 which preexists in W3129 (Gregor and Morrison, manuscript in preparation); the discontinuity there between J558 mRNA and the cloned DNA was only partially digested (Fig. 4, lane 2). Increasing the S1 digestion temperature from 37 to 45°C increased the efficiency of digestion (data not shown). Other gels (not shown) include $\Delta 15.8$ RNA, and the same 155-bp fragment is protected. Thus, the alpha messages of LP1.2 terminate at or very close to nucleotide 1589 (numbering system from 72), the same site used in an alpha cDNA (2).

Nuclear RNA species in LP1.2. To determine the steady-

state levels of alpha hnRNA precursors and mature, processed mRNA in the nuclei of LP1.2 and $\Delta 15.8$, nuclear RNA was prepared by using guanidine thiocyanate (15) and was analyzed by S1 nuclease protection (8). Our objective was to identify unspliced transcripts in LP1.2 and $\Delta 15.8$, compare the ratios of processed to unprocessed species, and search for transcripts extending beyond the normal site of cleavage and polyadenylation in $\Delta 15.8$ and LP1.2.

The three probes used for these experiments are illustrated at the bottom of Fig. 7. Plasmids p165 and p6.2.11 were modified by deletions. Consequently, protected fragments are easily distinguished from undigested probe, and all transcripts that span a part of the deletion protect a single-sized fragment, reducing the diffuse signal expected for heterogeneous transcripts to a single band.

When labeled at the *Eco*RI site in CH_2 , p165 Δ B distinguishes processed transcripts from those which retain the CH_2 - CH_3 intron. Processed transcripts protect 135 bp of the labeled fragment, and unspliced transcripts protect 600 bp. A 135-bp fragment representing processed transcripts is approximately three times stronger in $\Delta 15.8$ than it is in LP1.2 (Fig. 7A, lanes 2 and 3). Unprocessed transcripts are rare, but after long exposure of a 5% gel (Fig. 7B, lanes 2 and 3), the 600-bp species representing unspliced RNA is seen in both $\Delta 15.8$ and LP1.2. The relative amounts of spliced and unspliced RNA are approximately equal; therefore, removal of the CH_2 - CH_3 intron appears equally efficient in the two cell lines.

p6.2.11 Δ X was labeled at its *Bst*EII sites to identify extended transcripts in LP1.2. Correctly cleaved transcripts protect 135 bp of the 2-kb *Bst*EII fragment, and unprocessed transcripts protect up to 1.2 kb of the same fragment. When this probe was hybridized to LP1.2 nuclear RNA, the 135-bp fragment arising from cleavage at nucleotide 1589 was evident (Fig. 7A, lane 4), and no strong bands of a higher molecular weight were seen (Fig. 7C, lane 2). Therefore, in LP1.2, cleavage and polyadenylation occur efficiently and at the correct location.

p165 Δ H, end labeled at the unique *Bst*EII site in CH_3 , was used to identify extended transcripts in $\Delta 15.8$. Because p165 Δ H contains a wild-type alpha gene and both $\Delta 15.8$ and LP1.2 carry the deletion in CH_3 characteristic of W3129, these RNA-DNA hybrids were digested with exonuclease VII instead of S1 nuclease to prevent digestion of the resultant DNA loop. Correctly cleaved transcripts will protect 200 bp of the probe, and transcripts cleaved beyond this site but within the region deleted in p165 Δ H will protect 285 bp. Transcripts cleaved more than 1.5 kb from the alpha constant region will protect fragments up to 900 bp in size. Nuclear RNA from $\Delta 15.8$ and LP1.2 has similar protection patterns (Fig. 6D), and there was no accumulated unprocessed RNA in $\Delta 15.8$.

In summary, in both LP1.2 and $\Delta 15.8$, the primary transcripts were cleaved at nucleotide 1589 (± 3) in agreement with the published data (2). A small quantity of transcripts in which the intron between CH_2 and CH_3 was not removed was seen in both lines. There is no obvious difference in the processing patterns or the efficiency of processing of hnRNA in $\Delta 15.8$ and LP1.2, and therefore the loss of 3' sequences in LP1.2 has not significantly altered its hnRNA processing.

LP1.2 is deficient in alpha transcription. The transcription rates of the alpha gene in $\Delta 15.8$ and LP1.2 were compared by using runoff transcription in isolated nuclei. The nascent [32 P]RNA was assayed by hybridization to cloned DNA fragments immobilized on filters (described in the legend to Fig. 8). Each filter hybridized to pulse-labeled nuclear RNA

FIG. 5. Southern blot analysis of genomic DNA. Of the high-molecular-weight DNA, 10 μ g was digested with *Eco*RI, fractionated on agarose gels, transferred to nitrocellulose, and hybridized to the 2.4-kb unique sequence *Pst*I fragment (Fig. 3). (A) Lanes 1, W3129; 2, LP1.2; 3, Δ 15.8. Arrows at left indicate Charon 4A *Bam*HI molecular weight markers; those at right are Charon 4A *Sac*I markers. (B) Lanes 1, BALB/c liver; 2, C57BL/6 liver; 3, clone 5, a light-chain variant of MPC-11; 4 to 6, three IgA-producing hybridomas between clone 5 and BALB/c splenocytes. Arrows at right indicate λ *Hind*III molecular weight markers.

included pSV2-gpt *Bg*II cut with *Hind*III in lane a, gamma actin cut with *Bst*I and p6.2.11 cut with *Pvu*II in lane b, and p6.2.11 cut with *Hinc*II in lane c. To ensure that hybridization occurred in DNA excess so that the degree of hybridization is a true measure of the RNA available in solution, three duplicate DNA filters were hybridized to different amounts of [32 P]RNA. Filter 1 contained 1×10^6 cpm/ml, filter 2 contained 2×10^6 cpm/ml, and filter 3 contained 4×10^6 cpm/ml. These are labeled 1 $\times$, 2 $\times$, and 4 $\times$, respectively, in the autoradiogram in Fig. 8.

The alpha constant region is transcribed more efficiently in Δ 15.8 than it is in LP1.2. Visual examination of the *Hind*III B fragment which contains the alpha constant region (Fig. 8, lanes a) led to an estimate of a fourfold-higher transcription rate in Δ 15.8 versus LP1.2. However, the *Hind*III C fragment containing the membrane exon, which is deleted from the expressed LP1.2 allele, hybridizes to RNA prepared from LP1.2, indicating either that the nonproducing alpha alleles are transcribed in LP1.2 or that the membrane region contains repetitive elements. The latter hypothesis is unlikely because this region has been used many times as a probe in Southern blots for single-copy genes, and no evidence for nonspecific hybridization has been seen. Therefore, hybridization to the *Hind*III B fragment represents transcription from both the expressed and unexpressed alleles. If we assume that the mutation in LP1.2 does not affect the transcription rate of alpha genes located on different chromosomes, the transcription of unexpressed alleles is the same in Δ 15.8 and LP1.2. Therefore, the difference in the transcription rate of the expressed allele is greater than is indicated by direct comparison with the *Hind*III B hybridization signal in Δ 15.8 and LP1.2.

To calculate the true difference in the transcription rate from the expressed allele, the value obtained from a densitometer scan of the *Hind*III C fragment in Fig. 8 was

first normalized to actin and then subtracted from the value obtained from the *Hind*III B fragment. When this was done, the transcription rate of the expressed alpha allele in Δ 15.8 was found to be seven times greater than that of the alpha allele in LP1.2. Dot-blot analysis of cytoplasmic RNA also indicated a four- to eightfold decrease in the steady-state levels of alpha RNA in LP1.2 relative to Δ 15.8 (data not shown).

The transcription rate of the p6.2.11 *Hinc*II B fragment containing the unique portion of p6.2.11 is increased fourfold in LP1.2 compared with Δ 15.8 (Fig. 8), probably as a consequence of its proximity to an active transcription unit and resultant readthrough transcription. The repetitive elements are expressed in both cell lines to approximately the same extent.

Methylation of the variant allele. Methylation of cytidine residues correlates with gene expression in many eukaryotic genes, in which functional genes are relatively undermethylated compared with transcriptionally silent genes (53). The methylation state of a given gene can be determined by comparing DNA fragments generated by the enzymes *Hpa*II and *Msp*I, both of which recognize the sequence CCGG. However, *Hpa*II is inhibited by the methylation of the second cytidine residue: the sequence 5'-C^mCGG-3' is cleaved by *Msp*I but not by *Hpa*II.

Digestion of Δ 15.8 genomic DNA with *Msp*I and hybridization to the XHO fragment (Fig. 2c) reveals fragments of 3.8, 2.5, and 1.5 kb (20) (Fig. 9). The 3.8-kb fragment is a junction fragment from the deleted alpha allele and the two smaller fragments are from the wild-type germ line alleles (20). Digestion with *Hpa*II yields only the 3.8-kb fragment and some high-molecular-weight species. The yield of the 3.8-kb fragment is the same with either *Hpa*II or *Msp*I, showing that the expressed allele is unmethylated while the unexpressed alleles are methylated in Δ 15.8.

FIG. 6. S1 nuclease protection. Of the cloned mutant gene, 100 ng was endlabeled at its *PvuII* sites by using T4 DNA polymerase and was hybridized to 5 μ g of total cytoplasmic RNA. RNA-DNA hybrids were digested with 1,000 U of S1 nuclease, and protected DNA fragments were separated on an 8% polyacrylamide-urea gel and detected by autoradiography. RNA sources were LP1.2 (lane 1), J558 (lane 2), and W3129 (lane 3). Size markers were provided by end-labeled *HpaII* fragments from pBR322 and are indicated by arrows.

LP1.2 is a subclone of the original isolate, 7.1. Methylation of the alpha alleles in 7.1 and three subclones (7.1.2, 7.1.6, and 7.1.7) was examined by the digestion of DNA with *HpaII* or *MspI* and hybridization to the XHO fragment (Fig. 6). Digestion with *HpaII* yielded two hybridizing fragments of 2.8 and 3.9 kb. Examination of the cloned mutant allele (Fig. 3) showed that the 3.9-kb fragment is different from the 3.8-kb fragment seen in Δ 15.8. Digestion with *MspI* yielded only the 2.8-kb fragment and the 2.5- and 1.5-kb fragments from the unexpressed allele. In 7.1 and its subclones, therefore, the 3.9-kb *HpaII* fragment must contain a CCGG sequence located about 1.1 kb from one end of the fragment which is methylated in approximately 70% of the gene copies. In 7.1.7, the ratio of germ line to expressed alleles has increased relative to 7.1.2 and 7.1.6, and it appears to be a mixed clone which has lost the expressed allele. Subclone

7.1.2 (LP1.2) was used in all comparisons with Δ 15.8 and W3129.

DISCUSSION

To gain a greater understanding of the regulation of immunoglobulin expression, we isolated mutants of mouse myeloma cells which are deficient in their expression of immunoglobulin. One such mutant, LP1.2, has a decreased steady-state level of heavy-chain mRNA; this decreased steady-state level was shown to result from decreased alpha transcription. The cytoplasmic mRNA was polyadenylated and appeared identical to the mRNA present in the parental cell line from which the mutant was isolated.

Examination of the expressed alpha gene in LP1.2 showed that the sequences normally present 3' of the alpha chain gene had been deleted and that a novel sequence was present 11 bp beyond the normal site for cleavage and polyadenylation. A short direct repeat, eight copies of the 17-mer GCCTATAGAAGTAAGGA, is located at the junction of the alpha and novel sequences. The first 4 bp of the 17-mer are identical to the last 4 bp of the alpha sequence. This short stretch of homology between the immunoglobulin and novel sequences may have played a role in the mutation event since short sequence homologies are involved in the generation of deletions both in bacteria (1) and HeLa cells (40).

Novel sequences downstream of the direct repeat include a region of a unique, or low-copy-number, sequence flanked by two distinct highly repetitive elements. This unique sequence, which does not normally move in mouse myelomas, has been altered twice in a family of sequentially derived cell lines. An interpretation of this selective mobility is that the unique sequence has been altered in W3129, either by deletion, insertion, or transposition so that it is capable of rearrangement in LP1.2. For example, one or both of the repetitive elements may have been introduced near the unique sequence in W3129 and may promote recombination.

The two highly repetitive elements 3' of the mutant gene are distinct from one another and show no cross-hybridization. These have not been sequenced, so it is not known if they are related to any of the known mouse repetitive sequences. However, the restriction map of p6.2.11 shows no relationship to published maps of mouse repetitive DNA or to known mobile elements such as intracisternal A particles, which have transposed to the kappa locus in two documented cases (37). The 17-bp direct repeat is not part of any highly repetitive element. Search of the data base in Genbank failed to find this sequence among any of the known mouse sequences.

The modification of the region 3' of the alpha gene in LP1.2 does not appear to alter either the fidelity or efficiency of 3' cleavage and polyadenylation. Examination of nuclear RNA showed a complete absence of transcripts with uncleaved 3' ends in both Δ 15.8 and LP1.2. In both Δ 15.8 and LP1.2, the site of polyadenylation was identical to the site previously identified for IgA. An increasing body of evidence points to a requirement for sequences downstream of RNA cleavage sites which promote cleavage and polyadenylation (7, 28, 48, 59). Deletion of the 3' flanking sequence from the adenovirus E2A gene resulted in the accumulation of uncleaved transcripts (48). The placement of the lesion in LP1.2 just 11 bp beyond the end of the 3' UT region does not appear to remove any of these necessary processing signals. It is noteworthy that the pentamer CAGTG appears 4 bp 3' of the RNA cleavage site and is retained in LP1.2. The pentamer CAPyTG has been implicated in RNA cleavage (7).

FIG. 7. S1 nuclease protection of nuclear RNA. The probes used for these experiments are shown at the bottom. The two starting vectors are in panel A, and their deletion variants are shown in panels B and C. Solid boxes denote exons. Deletions are indicated by dotted lines flanked by brackets. Arrows that are labeled cD indicate the site for cleavage and polyadenylation for secreted-form alpha mRNA. Restriction enzyme sites are *Eco*RI (E), *Bst*EII (B), *Sma*I (S), *Xba*I (X), and *Hind*III (H). Wavy lines above each probe are transcripts expected in nuclear RNA, and the solid bars below the map are the DNA fragments which may be protected by those transcripts. Question marks on some transcripts indicate they are of unknown length. RNA-DNA hybrids were digested with 1,000 U of S1 nuclease. Protected DNA fragments were separated on an 8% (A) or 5% (B through D) polyacrylamide-urea gel and detected by autoradiography. (A) Lanes: 1, pBR322 cleaved with *Hpa*II; 2 and 3, p165ΔB labeled at *Eco*RI hybridized with nuclear RNA from Δ15.8 (lane 2) or LP1.2 (lane 3); 4, p6.2.1ΔX end labeled at the *Bst*EII sites hybridized with nuclear RNA from LP1.2 (lane 2), Δ15.8 (lane 3), or tRNA (lane 4); lane 1 contains the undigested probe; lane 5 contains *Hpa*II-cut pBR322; lane 6 contains *Hae*III-cut pBR322. (D) p165ΔH labeled at its *Bst*EII site was hybridized with nuclear RNA from Δ15.8 (lane 1), LP1.2 (lane 2), and tRNA (lane 3); lane 4 contains *Hae*III-cut pBR322; lane 5 contains *Hpa*II-cut pBR322.

FIG. 8. Runoff transcripts in isolated nuclei. At the top are the restriction maps of plasmids pSV2-gpt *Bgl*II and p6.2.11 indicating fragments used as hybridization probes for nuclear transcription assay. The plasmid pSV2-gpt *Bgl*II contains the 15-kb *Bgl*II fragment from a genomic clone of an alpha allele from the myeloma S107 inserted into the *Bam*HI site of pSV2-gpt (52) (line a). The *Bgl*II fragment extends from the J_H exons through the constant region (α) and MB exon. Solid boxes indicate exons. The heavy-chain enhancer is indicated by the stippled box. Plasmid sequences are identified by a wavy line, and phage sequences are identified by dotted line. The starred arrow points to the cDNA cleavage site. Restriction enzyme sites are indicated by open stars (*Bam*HI), solid squares (*Eco*RI), and solid stars (*Xho*I); solid triangles indicate the hybrid *Bam*HI-*Bgl*II sites at the insert-vector junctions. Arrows pointing upward indicate *Hind*III sites. The eight *Hind*III fragments ranging in size from 600 to 6,000 bp are identified with uppercase letters beneath the map where A is the largest fragment and F and G are the smallest. The 4-kb B fragment containing CH_1 , CH_2 , CH_3 , and 2 kb of the J-C intron was used to compare polymerase loading of the alpha gene in LP1.2 and $\Delta 15.8$. The 2.85-kb *Hind*III C fragment includes the MB exon. Because this region has been deleted from the functional allele in LP1.2, it should hybridize only to RNA made from $\Delta 15.8$ nuclei. The plasmid p6.2.11, which contains the 6.2-kb *Eco*RI fragment characteristic of LP1.2 inserted into pSV2-gpt, was used to assay transcription of the novel sequences located 3' of the alpha gene in LP1.2 (line b-c). Solid boxes indicate exons. Plasmid sequences identified by a wavy line are on both sides of the insert for clarity. Repetitive elements are shown by cross-hatched bars below the map. The starred arrow indicates the cDNA cleavage site. *Eco*RI sites are

The decreased transcription seen after the substitution of novel sequences 3' of the alpha chain gene could result either from the deletion of sequences that stimulate transcription or from the insertion of sequences that depress transcription. Our experiments do not permit us to distinguish between these two possibilities.

Precedence exists for mutants in noncoding sequences which affect transcription. A gamma-beta thalassemia has been described (31) in which at one allele, the epsilon, gamma, and delta genes have been substituted by novel sequences. The lesion does not affect the beta-globin structural exons, and at least 2 kb of 5' flanking sequences are intact. However, the beta-globin gene remains transcriptionally silent. The cloned gene is expressed in transient assays in HeLa cells. There is an essential difference between this thalassemia and LP1.2 in that the thalassemia results from an inherited mutation which prevents the expression of the entire globin locus and so blocks the normal pathway of beta-globin expression. In LP1.2, the transcription of an active gene is decreased by the introduction of a new DNA sequence: this may explain why expression is only partially inhibited.

Three cases have been described where a decreased expression of a gene results from the insertion of a retrovirus into noncoding sequences (11, 37). In one instance, the Moloney leukemia virus was introduced into the mouse germ line (11), into the first intron of the $\alpha 1$ collagen gene, resulting in the complete inhibition of transcription of the gene, which is normally expressed in early-stage embryos. Hawley et al. (37) describe two transpositions of intracisternal A particles into the L-V and J-C introns in two kappa-producing myelomas. Here, kappa transcripts were detected at 10 to 20% of wild-type levels in Northern blots of total poly(A)⁺ mRNA. Transcripts from the J-C insertion mutant are translated into protein: transcripts from the L-V intron insertion mutant appear abnormal. The rate of transcription was not determined for these mutants, so it is possible that transcription is normal but processing is aberrant. The mutants described above suggest that the insertion or substitution of foreign DNA into intervening sequence or near a gene in the germ line prevents expression completely, but that a similar insertion event into an expressed gene results in only the partial suppression of expression.

Several classes of positive regulatory sequences have been described. Enhancer elements have been identified in several animal viruses (33, 75) and in the immunoglobulin

FIG. 9. Genomic Southern blots of DNA from $\Delta 15.8$ and three subclones of the variant 7.1. A 10- μ g amount of the high-molecular-weight genomic DNA was digested with *Hpa*II (H) or *Msp*I (M) and hybridized to the XHO probe (Fig. 2c). The 3.6-kb fragment migrates slightly differently after *Hpa*II or *Msp*I digestion because of the different salt content in the restriction enzyme buffers. Samples are DNA from $\Delta 15.8$ and three subclones of 7.1. Arrows at right indicate Charon 4A *Bam*HI molecular weight markers.

heavy- (3, 29) and kappa light-chain genes (57). They act in a bidirectional manner to increase the transcriptional activity of a nearby promoter over at least several kilobases (3, 4, 29, 50, 75), apparently by increasing the polymerase loading of DNA in their vicinity (71). The known heavy-chain and kappa light-chain enhancers are located in the major intron in each gene (3, 57). There are no data proving the existence of additional enhancer elements 3' of immunoglobulin genes.

In the vast majority of plasmacytomas and in Burkitt lymphomas, the cellular counterpart of a viral transforming gene, *c-myc* (19), has been translocated to the immunoglobulin heavy- or light-chain locus and is transcriptionally active (13, 36, 63, 65, 68, 69). It is believed that this activation of transcription is a consequence of translocation to the new position, presumably because the *c-myc* gene is now near a positive regulatory element. In two cases, the translocation

indicated by solid squares. Arrows pointing downward indicate *Pvu*II sites. *Pvu*II fragments are identified with upper case letters above the map (line b). There are five *Pvu*II fragments. The 5-kb A fragment includes the 3' half of the 6.2-kb insert with the 3' repetitive element. The 3.3-kb B fragment includes 2.7 kb of pSV2-gpt and 600 bp of the alpha constant region. The 1.2-kb C fragment contains the 5' repetitive element from p6.2.11. Arrows pointing upwards indicate *Hinc*II sites. *Hinc*II fragments are identified with uppercase letters beneath the map (line c). *Hinc*II digestion of p6.2.11 generates seven fragments. The 2.7-kb B fragment contains the middle, nonrepetitive portion of the 6.2-kb insert; the 2.1-kb C fragment from the 5' end of the insert includes 600 bp of the alpha constant region and the 5' repetitive element; the 1-kb D fragment is from the 3' end of the insert; and the 800-bp E fragment contains the 3' repetitive element. The gamma actin clone (generous gift of L. Kedes) has a 1.2-kb cDNA insert in the 2.7-kb expression vector pCD (not shown). Digestion with *Bst*I liberates the insert. This digest was mixed with *Pvu*II-digested p6.2.11, and both samples were run together in lane b of the gel. Between lines a and b-c are photographs of the restriction fragments after electrophoresis through agarose gels and ethidium bromide staining. Each lane is presented separately, and the DNA fragments are identified with reference to the maps drawn in lines a and b-c. In lane b, pCD is the vector and λ A is the insert of the gamma actin cDNA. The rightmost picture shows the pattern of the entire strip, which was hybridized to pulse-labeled nascent nuclear RNA. Fragments which hybridize to ³²P-labeled nuclear RNA are indicated by a black dot. Fragments which hybridize primarily to RNA from one cell line are indicated by a black dot followed by a Δ ($\Delta 15.8$) or an LP (LP1.2) to indicate the cell line. Labeled RNA was hybridized to DNA filters prepared as described. After stringent washing, membranes were exposed to X-ray film. The three membranes on the left were hybridized to RNA from $\Delta 15.8$ nuclei; those on the right were hybridized to RNA from LP1.2 nuclei. For each cell, three hybridization solutions were prepared with different amounts of labeled RNA. Membranes labeled 1 $\times$ were hybridized to 1 $\times$ 10⁶ cpm/ml, those labeled 2 $\times$ were hybridized to 2 $\times$ 10⁶ cpm/ml, and those labeled 4 $\times$ were hybridized to 4 $\times$ 10⁶ cpm/ml. All hybridization volumes were 3 ml. Lanes a, b, and c on each membrane correspond to lanes identified with the same letters in the ethidium bromide-stained agarose gel.

preserves the heavy-chain enhancer (25, 38), but usually the enhancer is deleted. Frequently the *c-myc* gene is translocated to the unproductive alpha allele (13, 21, 36, 63). In one case, the translocation to alpha was followed by the deletion of the alpha constant-region exons (18). The new breakpoint lies between the *Bgl*I and *Bst*EII sites in the CH₃-MB intron (58). Therefore, if an alpha-associated activator exists for *c-myc*, it must lie 3' of this breakpoint and within sequences deleted in LP1.2.

A critical question that remains unanswered is how do regulatory sequences function? Recent data suggest that regulatory sequences bind protein (16, 24). The internal control region of the *Xenopus* 5S rRNA gene is activated by binding the transcription factor TFIIA (12). The binding of TFIIA allows the formation of a highly stable transcription complex (10) which serves two functions: active transcription after association with RNA polymerase III and maintenance of the expressed state (27, 61). RNA polymerase II transcription units may require two separate sequences to achieve the same effect: one to activate transcription by the formation of transcription complexes and the other to maintain expression.

Enhancers have been postulated to promote RNA polymerase II entry into the double helix, perhaps by the local melting of DNA. Another possibility is that enhancers are nucleation sites for the formation of active transcription complexes that are recognized by RNA polymerase II. They would therefore be necessary for the initiation, but not the maintenance of, transcription. A second sequence would serve to facilitate or maintain transcription.

This second hypothesis may better explain some recent observations. Several immunoglobulin mutants have been described in which the heavy-chain enhancer has been deleted (23, 42). Despite this, normal levels of heavy-chain mRNA and protein are made by the cell. When the deleted allele is cloned from the genome and introduced into recipient myeloma cells, it is transcriptionally inactive (80). Enhancers have previously been shown to be necessary to establish expression after immunoglobulin gene transfection (3, 55).

Recent transfection experiments have suggested the existence of at least two regulatory regions within an immunoglobulin gene: the enhancer and an intragenic sequence (30). In the absence of the intragenic sequence, expression following transfection takes place but is reduced. In the transfection experiments, it was not demonstrated whether the reduced expression was the result of decreased transcription or the decreased accumulation of transcripts.

The sequences missing from LP1.2 would be good candidates for sequences facilitating or serving a maintenance function during expression. As noted earlier, the *c-myc* gene is activated by its translocation to this region of the alpha locus. The *c-myc* gene has its own regulatory regions which initiate transcription in a cell-cycle-dependent manner (41): the translocated *c-myc* gene differs in that once transcription is initiated, it is continuously maintained (68). This continued expression could result from the presence of maintenance regulatory sequences.

An additional category of 3' sequence which must be considered is the transcription terminator. Loss of a terminator in the 3' flanking region of alpha could result in the loss of active transcription complexes by migration into sequences that disrupt the complex. Alternatively, transcription may be inefficient as a result of the retarded cycling of the transcription complex because of the extended transcription unit. Precedence for a transcription terminator in immu-

noglobulin is found in the μ - δ intron in which transcription through this region is seen in early-stage B lymphocytes, some of which express both μ and δ proteins, but not in μ -producing myelomas (45). Also, terminators have recently been identified in the amylase gene (34) and in the beta-major globin (26). However, there is no evidence for a transcription terminator in the 3' flanking sequence in Δ 15.8, and the transcription assay clearly shows transcription of the membrane exons in this line. Therefore, any terminator must lie 3' of the alpha membrane exons.

The methylation of cytidine residues has been correlated with gene expression in a number of systems. In general, methylation is inversely correlated with expression (31, 53, 78). The mechanism by which methylation is established is not known, but a maintenance methylase recognizes the hemi-methylated sequence ^mCpG/GpC found in newly replicated DNA and methylates the unmodified strand (35, 66). Without such a maintenance methylase, continued DNA replication would result in the rapid loss of methylation as the hemimethylated daughter cells give rise to unmethylated progeny.

In view of this, it is difficult to explain the partially methylated *Msp*I site located within the novel sequence in LP1.2, immediately 3' of the direct repeats bordering the recombination site. A second *Msp*I site 800 bp further into the novel sequence is unmethylated in all cases, as is the *Msp*I site 5' of CH₁. Partial methylation is not normally seen: maintenance methylation is expected to result in either complete or nonexistent methylation at a given site in a cloned population of cells. This partially methylated state persists since the original isolate and two randomly picked subclones show the same partial methylation. Since each subclone is the progeny of a single cell, which must be either methylated or unmethylated, the persistence of the partially methylated state may indicate an active process.

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