Spontaneous deletions in Ig heavy chain genes: flanking sequences influence splice site selection

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ABSTRACT

The cell line G403.4.7, isolated as a spontaneous variant of the MPC-11 derived myeloma G403.4, produces a truncated $\gamma 2b$ HC protein, but no light chain (LC), and a single $\gamma 2b$ specific transcript of 2.4kb. This $\gamma 2b$ transcript consists of the VDJ and C_H1 exons, the C_H1 to Hinge (Hi) intervening sequence (IVS) and Hi exon, part of the IVS between the two membrane exons M1 and M2, and most of the membrane 3' untranslated (UT) region. Even though the mature mRNA contains intronic sequences, it is abundant in the cytoplasm. Analysis of the $\gamma 2b$ genomic organization reveals that this unusual transcript results in part from two genomic deletions of 2.5kb and 588bp and in part from an altered splicing pattern. This altered splicing pattern is probably a consequence of the sequence alterations resulting from the genomic deletions. Analysis of these events provides some interesting insights into the mechanism of splice site selection and the evolution of introns and exons.

INTRODUCTION

MPC-11 is a $\gamma 2b$, κ producing myeloma. The Ig locus in this cell line has been shown to have a spontaneous mutation rate of 10^{-3} events/cell/generation (1). Because of this relatively high rate of mutation, it has been possible to follow the evolution of this specific locus. The study of spontaneously derived mutants of MPC-11 has led to insights into Ig protein assembly and transport, Ig specific and general recombination, and RNA processing (2, 3, 4, 5, 6, 7, 8, 9). In addition, certain derivatives have been used as murine models for human heavy chain disease (HCD) (10, 11, 12, 13).

The cell line G403 ($\gamma 2b^*$), derived in two steps from MPC-11 ($\gamma 2b$, κ), produces a HC lacking a C_H1 domain, (designated $\gamma 2b^*$) (10). The absence of C_H1 results from a 99 bp genomic deletion that removes the C_H1 donor splice site so that the C_H1 exon is skipped and the VDJ exon is spliced directly to the Hi exon (See Figure 1) (9). Exon skipping is frequently seen as a

consequence of the loss of a functional donor splice site (14). Lack of a C_H1 domain in the protein allows this aberrant HC to be secreted in the absence of a LC, presumably because it no longer binds heavy chain binding protein (3, 15). Here we describe another HC variant, G403.4.7, which contains additional genomic deletions 3' of the known splice signals and displays an alternate splicing pattern such that it now retains the C_H1 exon; this variant suggests that additional aspects of RNA sequence and structure may influence splice site selection.

MATERIALS AND METHODS

Cell Lines

The cell line 45.6 is the tissue culture derivative of the myeloma MPC-11 and produces a $\gamma 2b$, κ protein (16). 4TOO1 ($\gamma 2b$, κ), a drug marked derivative of 45.6, produces the same protein as the wild type tissue culture cell line and will be referred to as MPC-11 for convenience. 10.1 ($\gamma 2b^*$, κ) is a spontaneously arising variant of MPC-11 (*indicates that the $\gamma 2b$ HC protein does not contain the C_H1 domain) (10, 9). The myeloma G403.4 ($\gamma 2b^*$) was derived from 10.1 after mutagenesis with Melphalan and was identified by its lack of LC secretion (10). G403.4.7 arose spontaneously from G403.4 and was identified by overlaying with a polyclonal rabbit anti-mouse anti- $\gamma 2b$, κ antiserum in soft agarose. Clones which were not surrounded by an immunoprecipitate were picked and grown for further analysis in IMDM supplemented with 10% heat inactivated horse serum, non-essential amino acids, glutamine, penicillin, and streptomycin.

Northern analysis

Total cytoplasmic RNA was isolated from cells using the protocol described in Maniatis et. al.(17). Samples were fractionated on formaldehyde containing agarose gels and transferred to nitrocellulose filters as described previously (18). α - ^{32}P dCTP labeled probes were random primed according to the manufacturer's instructions (Pharmacia, Piscataway, NJ) and hybridized to filters at 65°C overnight (19).

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cDNA PCR

The PCR was performed essentially as described by Roth et. al. (20). Briefly, 10 μ g of total hot phenol RNA (21) was reverse transcribed in PCR buffer (10 $\times$ PCR buffer: 100mM Tris-HCl (pH 7.5), 500mM KCl, 15mM MgCl₂) for one hour at 42°C with 20 units of AMV-RT (Life Sciences, St. Petersburg, FL), using the 3' oligo primer (5' TTCCAGTTCATCTTAGGCAAA-CA 3') which is located 270 bp 3' of the second membrane exon and 6 bp 3' of a Bgl II site within the γ 2b membrane 3' UT. Half of the reverse transcriptase reaction was used for PCR. The PCR profile consisted of 1 minute at 94°C, 1 minute at 50°C, and 8 minutes at 72°C for a total of 25 cycles. The 5' oligo primer (5' CCAAGGCACCACTCTCACAG 3') was located 8 bp 5' of the end of J_H2. The 3' PCR oligo was the same as that used in the cDNA reaction. PCR products were digested with EcoR V and BamH I (Promega, Madison, WI) and cloned into Bluescript SK(+) (Stratagene, La Jolla, CA). Translation data were obtained from DNA Inspector.

Genomic PCR

PCR was performed as described by Saiki (22). Briefly, 100ng of undigested genomic DNA was used for PCR. The PCR profile consisted of 1 minute at 94°C, 1 minute at 50°C, and 2' at 72°C for a total of 25 cycles. The 5' oligo primer (5' CCAAGTAA-

CATCCAGCCTTCTC 3') was located in the C_H1 to Hi IVS, 5 bp 5' of the Hi exon and 1 bp 5' of an endogenous Pst I site. The 3' oligo primer was the same as the 3' oligo used in the cDNA PCR. Genomic PCR products were digested with Pst I and Bgl II (Promega, Madison, WI) and cloned into Bluescript SK+ (Stratagene, La Jolla, CA).

RESULTS

Isolation of G403.4.7 and HC protein characterization

In a search for mutants altered in immunoglobulin heavy chain expression, the cell line G403.4.7 was isolated as a non-secreting variant of G403.4 (a random subclone of G403). Analysis of immunoprecipitates prepared from supernatants of metabolically labeled cells confirmed that the G403.4.7 protein is not secreted and indicated that G403.4.7 produces a truncated γ 2b HC of approximately 23 kDa. (Data not shown.)

The G403.4.7 mRNA is composed of a mosaic of exon, intron, and 3' untranslated sequences

Northern analysis of the G403.4.7 HC transcript with pMK-3, an MPC-11 V region probe (23), identified an mRNA species that was larger than the parental secreted γ 2b mRNA (Figure 2A). This contrasts with the size of the protein product, which is reduced in size compared to the wild type protein. To further investigate the origin of the extra sequences, total cytoplasmic RNA was probed with a 725bp Bgl II γ 2b membrane 3' untranslated region probe (24). As shown in Figure 2B, this probe hybridizes to the same transcript in G403.4.7 identified

MPC-11

G403.4

G403.4.7

Figure 1. Splicing patterns of heavy chain transcripts in wild type and mutant genes. Exons are represented by boxes and introns by lines, except for a portion of the M1M2 IVS which is represented by a smaller box and designated MIVS. MPC-11 represents the wild type heavy chain splicing pattern. The MPC-11 pre-mRNA splices correctly to the C_H1 exon and is processed to produce secreted or membrane specific transcripts. The C_H1 exon is removed from the mature G403.4 mRNA, but is retained in the G403.4.7 mRNA. The * indicates the loss of the C_H1 5' donor splice site due to a pre-existing 99 base pair deletion (9).

Figure 2. G403.4.7 mRNA is larger than the secreted form of wild type γ 2b mRNA and contains IVS as well as γ 2b membrane specific 3' untranslated sequences. 10 μ g of total cytoplasmic RNA was electrophoresed on formaldehyde containing gels, blotted to nitrocellulose, and probed with α -³²P labeled probes derived from either the MPC11 V region (A), the γ 2b membrane 3' untranslated region (B), or the C_H1 to Hi IVS (C). Specific probes are diagrammed next to each autoradiogram. (Not drawn to scale). B=BamH I, B2=Bgl II, E=EcoR I, P=Pst I.

by the V region probe. As expected, it also hybridizes to the less abundant membrane specific transcripts present in MPC-11 and G403.4. This Northern blot was re-probed with IVS specific sequences and as seen in Figure 2C, a 230 bp BamH I to Pst I C_H1 -Hi IVS probe also hybridizes to the G403.4.7 HC transcript. The increase in size seen in the G403.4.7 mRNA species can be partially accounted for by the presence of membrane 3' UT and C_H1 to Hi IVS sequences. The cell line G403.4, from which G403.4.7 was cloned, produces two $\gamma 2b$ messages, one of which co-migrates with the G403.4.7 mRNA, indicating that it consists of a mixed population containing both the original cell line and the mutant (Figure 2B and 2C).

Since the presence of membrane 3' UT and IVS hybridizing sequences was unexpected, PCR using J_H2 and 3' UT specific primers was performed on cDNA made from total RNA. Figure 3 shows the sequence of the 882bp cDNA PCR product; the structure is shown diagrammatically in Figure 1. The most striking aspect of the sequence is its patchwork composition: the cDNA contains the complete C_H1 exon and C_H1 to Hi IVS (with the pre-existing 99bp deletion), a complete Hi exon, 20 bp of the Hi to C_H2 IVS, 172 bp of the M1 to M2 IVS, and 87 bp of the membrane 3' UT. The sequence of each of these individual segments is identical to the analogous wild type segment, with the exception of an A residue near the M1M2 IVS to membrane 3' UT border. In contrast to the G403.4.7 mRNA, the parental G403.4 HC mRNA does not contain the C_H1 exon or the C_H1 to Hi IVS, nor does it contain the M1M2 IVS, while it does

possess the C_H2 and C_H3 exons. Only the membrane specific parental mRNA contains the M1 and M2 exons.

Translation of the G403.4.7 cDNA in the wild type reading frame predicts a 26kD protein product which terminates 78 bp 3' of the last C_H1 codon. The molecular weight of the HC protein immunoprecipitated from this cell line is consistent with this prediction.

G403.4.7 contains two additional genomic deletions that remove most of the constant region and the membrane exons

Southern analysis of EcoR I digested genomic DNA with a probe representing the entire $\gamma 2b$ constant region probe identified a 3kb deletion in the constant region of G403.4.7 (data not shown). In order to determine if the altered transcript resulted from the genomic deletion, the genomic DNA surrounding this region was cloned using PCR. Specific primers were positioned in the C_H1 to Hi IVS and in the membrane 3' UT (Figure 4A, primers are indicated by brackets). The DNA sequence from the Hi to the membrane 3' UT is identical to the analogous region in the cDNA (Figure 3) and indicates that this allele contains two genomic deletions of 2.5kb and 588bp, respectively. The 5' breakpoint of the 2.5kb deletion begins 20 bp 3' of the Hi exon, while the 3' breakpoint is located 14 bp 3' of the first membrane exon (M1). The 588bp deletion begins 172 bp downstream of the 2.5kb deletion and removes the entire M2 exon and 167 bp of the membrane 3' UT. A comparison of the flanking sequences surrounding the two sets of breakpoints is shown in Figure 4B.

Figure 3. Partial sequence of the G403.4.7 expressed heavy chain. Nucleotide sequence of cDNA and genomic PCR products. Exons are represented in capital letters, while introns are represented by lower case letters. The stop codon for the wild type reading frame is underlined. An arrow marks the first nucleotide in the C_H1 exon. Nucleotides which may represent sites of recombination are shown in bold face type (mb = membrane). The C_H1 5' donor splice site is missing due to a pre-existing 99bp deletion (9).

A

B

2.5kb deletion

HI IVS

CAGGTAAGTCACTACCAGAGG**CTC** *ggactcccaggagaatgta*
 ctcttcaaggttggcactgt**CTC** CAC**ACCC**TCGTGTGATAG

M1M2 IVS

588bp deletion

M1M2 IVS

CACATTTTGTCCCATACAGT**CT** *gcccttctgtctctacgtg*
 gagcaggctttacccagac **CT**AACACTTCCACAGCAGTTG

3'UT

Figure 4. Genomic Deletions in the G403.4.7 Expressed Heavy Chain. A: Schematic diagram of the extent of the genomic deletions in G403.4.7. The region cloned by PCR is flanked by brackets. B: Sequences flanking the deletions found in the G403.4.7 expressed $\gamma 2b$ allele. Nucleotides present in the final product are in capital letters. Nucleotides found in both donor sequences and the products are enlarged and bold. The inserted A residue in the 2.5kb deletion is enlarged and in italic. The sequence resembling the Ig switch region sequence is underlined.

Since both deletions share 2 to 3 bp in common, the exact site of recombination is difficult to determine. The 2.5kb deletion has an A residue near the region of recombination which is not present in the published M1M2 IVS sequence (25). This residue may mark the deletion breakpoint or it may be unrelated to the deletion and simply reflect an allelic sequence difference.

The G403.4.7 mRNA has an altered splicing pattern

The absence of the C_H2 , C_H3 , M1, and M2 exons in the mature G403.4.7 message can be explained by the two additional genomic deletions, but the presence of the C_H1 exon and the C_H1 to H1 intron indicates that, unlike the parental transcript, these sequences are not removed from the mature mRNA by splicing. Figure 1 outlines the splicing patterns in both the parent G403.4 and the mutant G403.4.7.

DISCUSSION

Somatic cell mutants provide useful tools for the investigation of the mechanism(s) of different cellular processes. When gene transfection is coupled with *in vitro* mutagenesis to study cellular processes, the prejudices of the investigator determine what mutations will be analyzed. In contrast, somatic mutants provide

a source of variation that is unbiased by preconceptions. In the past, mutant analysis has provided information about a variety of relevant events, including protein transport, DNA recombination, and RNA processing. In the present study, we describe a mutant which provides additional information about the mechanism of genomic rearrangements and splice site selection.

Analysis of the two spontaneous genomic deletions in G403.4.7 shows that neither is flanked by any extensive homology or obvious direct or inverted repeats. Both the 588bp and the 2.5kb deletions retain two or three nucleotides (CT or CTC) shared by both donor sequences. The presence of sequence homologies of between 1 and 5 nucleotides has been observed in approximately 60% of nonhomologous recombination junctions and suggests that the 2.5kb and the 588bp deletions are the result of illegitimate recombination (26, 27). It has been suggested that either topoisomerase I or II could mediate illegitimate recombination (28, 29). We did not find a topoisomerase II consensus site in the adjacent sequences, but the sequences CTC, GTC, and CCTT, which have been noted as preferential cleavage sites for rat topoisomerase I, are found within 6 bp of the overlap in three of the four donor sequences (28). In addition, the 2.5kb deletion is followed by the sequence CTCCCA, the complement of which, TGGGAG, is reminiscent of the pentameric TGGGG associated with switch recombination (30, 31). While it is unlikely that this single sequence could activate the switch recombination machinery, it is interesting to note that a subset of this sequence, TGG, has been identified at the site of Ig recombination in other myelomas (7). It has been suggested that the high rate of spontaneous mutation at the Ig locus in myelomas may be due to the abnormal activity of an Ig recombinase (6, 7, 32), and it is possible that this limited amount of homology is enough to facilitate these recombination events.

In the G403.4.7 mutant, the same splice signals are processed differently than they are in the parental message. While there is a growing understanding of the numerous factors involved in the assembly of splicing components, relatively little is known about the parameters which govern splice site selection (for reviews: 33, 34). The basic sequence requirements for pre-mRNA splicing consist of a 5' splice donor site, a branch point sequence (BPS), a polypyrimidine tract, and a 3' splice acceptor site. These regions by themselves are not sufficient for accurate splicing since numerous potential splice sites exist within a particular transcript, yet only the 'correct' sites are utilized. Splice site usage can be influenced by the content and context of the sequences mentioned above as well as by other less well defined elements within introns (35) and exons (36, 37, 38). In the G403.4.7 mutant, the sequence from C_H1 to 20 bp 3' of the H1 exon, a total of 595 bp, is unchanged and the H1 5' and 3' splice sites, the polypyrimidine tract and the BPS are intact. The only change we have detected is the juxtaposition of the M1M2 IVS and membrane 3' UT sequences to the H1- C_H2 IVS, a change that does not involve any of the known splice signals. Nevertheless, the H1 splice acceptor is not utilized in the mutant as it was in the parent G403.4, and instead the C_H1 acceptor site is now used for the VDJ splice. While the precise mechanism by which this downstream change influences splice site selection is not clear, there are several possible ways in which this alteration could either directly or indirectly affect the splicing of upstream sequences.

The genomic deletions in G403.4.7 not only juxtapose new sequences, but they also remove substrates normally used in the

splicing of wild type HC mRNA. The deletion of the C_H2 exon in particular removes a potential splice from Hi to C_H2 and may indirectly influence splice site selection by disrupting an important splicing event. The ordered splicing of downstream exons influences subsequent splicing to upstream exons in both the rat β -tropomyosin and preprotachykinin genes (39, 40), and it is possible that the deletion of the C_H2 exon and hence the loss of the splice from Hi to C_H2 in G403.4.7 alters the subsequent splicing efficiency between VDJ and C_H1 or between VDJ and Hi.

Alternatively, the new sequences 3' of the Hi exon could directly influence the choice of splice sites, possibly by altering RNA secondary structure. Analysis of the predicted RNA folding pattern for G403.4.7 suggests that the G403.4.7 mRNA does fold differently than the parental mRNA (data not shown). The influence of secondary structure on splice site usage has been documented *in vitro* (41, 42, 43, 44, 45, 46) and recently *in vivo* with the chicken β -tropomyosin gene (47). In the β -tropomyosin gene, RNA secondary structure has been shown to inhibit the formation of splicing complexes (46). It is possible that RNA secondary structure in the G403.4.7 mutant permits or denies communication between the different signals which participate in the splice site selection process.

An intriguing possibility is that the recognition of splice sites is altered by the new proximity of the membrane 3' UT sequences, (including the poly(A) signal). In the 'exon definition' model of splicing proposed by Robberson et al (48), exons are recognized and defined by splicing components which bind the 3' end of an intron and search for a downstream 5' donor splice site. Only exons flanked by donor and acceptor sites which are approximately 300 nucleotides apart allow stable pre-splicing complex formation. One exception to this size limit would be 3' terminal exons which begin with a 3' splice site and end with a poly(A) signal and are usually longer than internal exons. In the G403.4.7 mutant, the C_H1 exon, the C_H1 to Hi IVS, and the Hi exon could now be recognized as a new 3' terminal exon, possibly due to the presence of the membrane 3'UT. With this altered 'exon definition', the Hi acceptor site may no longer be recognized as a viable splice site.

A consequence of the altered splice is that the C_H1 to Hi IVS is present in the mature mRNA; even though the HC mRNA contains an IVS sequence, it is still very abundant in the cytoplasm. There are several examples of IVS containing cytoplasmic messages, including rat γ fibronectin, Drosophila P-element, human L-myc, and murine TNFb, but they are relatively rare for cellular genes (49, 50, 51, 52, 53, 54). In general, messages which would retain an IVS are retained and destroyed in the nucleus (55, 56, 57).

The G403.4.7 cell line provides a unique example of a spontaneously generated alternate splice. The fact that genomic rearrangement(s) can have such a profound effect on the splicing pattern of the resultant mRNA and ultimately on the structure of the protein product presents an interesting view of the creation of exons and of the possible origin and potential uses of open reading frames found within introns. The fates of the alternative protein products differ dramatically and serve to illustrate the power of the altered splice. The G403.4 protein does not have the C_H1 domain and is therefore secreted. The G403.4.7 protein contains the C_H1 domain and is not secreted. It is interesting to speculate that random genomic rearrangements could activate alternate splicing patterns and potentially access open reading frames to provide a source of diversity in the evolution of

proteins. Conversely, some open reading frames within introns may represent vestigial exons which were excluded due to a change in splicing patterns.

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