Analysis of Somatic Mutations in κ Transgenes

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Summary

We have examined the nature and localization of somatic mutations in three κ transgenes cloned from IgG-secreting hybridomas. All of the mutations identified were single base substitutions. Mutations were localized to the variable (V) region and its flanking sequences. In every case, the nuclear matrix association region, κ enhancer, and C gene were spared. These data indicate that the rearranged κ gene contains the necessary sequences for targeting of the mutation process, and suggest that the observed localization of mutations to the V region reflects the inherent specificity of this mutation process.

Somatic mutation of Ig V genes after antigenic stimulation is an important mechanism responsible for diversification of V genes (reviewed in references 1 and 2). The mutations are generally single base substitutions and are localized to the V region and its flanking sequences (3, 4). The mechanism responsible for introduction of these mutations is unclear. In the chicken, it appears that upstream V or VD pseudogenes can function as potential donor sequences for H and L chain V gene conversions (5-7). However, in mammalian species, there is little evidence in support of a significant role for gene conversion (8, 9).

The overall frequency of mutations has been found to be similar for both productively and nonproductively rearranged V genes (10). There appears to be at least a 10-fold reduction in frequency of mutations in partially assembled V regions, i.e., genes arrested at the DJ stage (11, 12). Few (13), if any, somatic mutations are seen in germ line V genes (14). Thus, rearrangement of V genes seems to be important for targeting the mutator machinery and may be integral to its complete activation as well.

In an effort to examine the role of a rearrangement event in the acquisition of somatic mutations, we introduced a rearranged κ gene cloned from the myeloma MOPC-167 into the germline of mice (15). Analysis of hybridomas generated from these mice revealed the following: (a) ongoing rearrangement is not required for the introduction of somatic mutations; (b) targeting of the Ig transgene by the "mutator" is independent of its chromosomal location; and (c) based on preliminary sequence analysis, mutations were present in the transgene V regions, but not in the C regions (16). Preliminary evidence for somatic hypermutation of an H chain transgene has also been obtained (17).

In the present study, we examined the nature and localization of somatic mutations in three κ transgenes cloned from IgG-secreting hybridomas. Since the transgenes are present in multiple copies in these transgenic mice, this allows examination of the overall integrity of the somatic mutation process under conditions where selective pressures to produce functional Ig L chains have been greatly reduced.

Materials and Methods

Hybridomas. The hybridomas 3A1, CE9, and CF2 were generated from splenic B cells in mice carrying a rearranged κ transgene after hyperimmunization with phosphorylcholine (PC)-keyhole limpet hemocyanin (16). 3A1 was generated from mouse line 194-2, which contains three copies of the κ transgene (unpublished observation) along with the complete pUC18 vector, while CE9 and CF2 were generated from the 233-8 line that carries ~13 copies of the κ transgene with ~150 bp of pUC18. All of the hybridomas transcribe both the T15Vκ gene and the κ transgene, and secrete IgG that binds PC (16). They were selected for transgene mutation analysis because their endogenous Vκ gene was mutated, thus, they were known to have undergone a somatic hypermutation event (16).

Cloning and Sequencing. The construction of partial genomic libraries of 3A1, CE9, and CF2, and subcloning of 4.8-kb BamHI fragments containing the V and C regions of the κ transgenes has been described previously (16). The entire 4.8 kb of genomic clone pl-7 isolated from hybridoma CE9 was subcloned as fragments averaging 400 bp in length into Bluescript KS (Stratagene, San Diego, CA). Double-stranded dideoxynucleotide sequencing was performed with Sequenase, as outlined by the manufacturer (United States Biochemical Corp., Cleveland, OH), utilizing the KS and SK

Abbreviations used in this paper: CDR, complementarity-determining region; FR, framework; PC, phosphorylcholine.
primers (Stratagene), as well as M13 and M13 reverse primers (New England Biolabs, Beverly, MA). Approximately 3.7 kb of nucleotide sequence was determined for each of the clones pIII-2 and pII-13 from hybridomas 3A1 and CF2, respectively, with the aid of the following primers: 5’d(TCTCTCTCTAGGATTCAAGTGG3’) , 5’d(CAGCAGCTACCTGAGCGT3’) , 5’d(GAGTGAACTCAGGATGC3’) , 5’d(TCTCTCTCTCTCTCTCAG3’) , 5’d(TTCTACAGGTCTCCTC3’) , 5’d(TTCCTACAGAGTCTCTC3’) , 5’d(TTCTATACAGTGTGTCCTCAGG3’) , 5’d(GACTGCCATCATCAG3’) , 5’d(GAGCTTCTGTCACATCTC3’) , 5’d(GCCAGCAAAAGTCATGG3’) , 5’d(T000TGCCAAAGGCAAC3’) , 5’d(TACTACCTCTGTCCC3’) , 5’d(GACT000AAACATCAGG3’) , 5’d(GAG000TTCCTTGTTAC3’) , 5’d(GCCACTCACAAGACA3’) .

Results and Discussion

To assess the nature and distribution of somatic mutations introduced into κ transgenes, genomic transgene clones known to contain V region substitutions, which had been isolated from three different splenic B cell hybridomas (16), were further examined. The history of the hybridomas is described in Materials and Methods. Transgenes were cloned from the genomic DNA of the hybridomas as 4.8-kb BarnHI fragments spanning from within the leader-Vκ intron to 3’ of Cκ (Fig. 1). The entire 4.8 kb of clone pI-7 (isolated from hybridoma CE9) was subcloned and sequenced. The region sequenced included most of the leader-Vκ intron (315 bp), the entire V region, the Jκ-Cκ intron, the Cκ gene, and ~1.3 kb 3’ of the C gene. A total of 27 nucleotide substitutions were identified based on the published germline sequence (Fig. 2). Of these, 15 were somatic mutations already present in the MOPC-167 κ gene used to make the transgenic mice. Thus, the pI-7 clone contains 12 new nucleotide substitutions. Two mutations are present in the leader-Vκ intron, 6 in the V region (three of which had previously been identified; reference 16), and four within 500 bp adjacent to the V gene in the J5-Cκ intron. Thus, 50% of the mutations occurred outside coding regions. Five of the six V region mutations are present in framework (FR) regions. Furthermore, all of these nucleotide substitutions result in amino acid replacements. The mutations at positions 561, 582, and 585 in FR1 result in changes from threonine to serine, proline to arginine, and valine to glycine, respectively. The base change at position 693 in FR2 replaces proline with leucine. The mutation at position 767 in FR3 leads to substitution of threonine with alanine. Many of these amino acid residue changes represent significant replacements within the framework regions of the V gene and could be potentially deleterious to the production of a functional antibody molecule. In fact, the sole nucleotide substitution present within a complementarity-determining region (CDR) is at position 658, and it results in the introduction of a translational termination codon. Thus, this κ transgene no longer retains the capacity to produce a functional κ protein.

Sequencing of genomic clone pII-13 (isolated from hybridoma CF2) was initiated 275 bases upstream of the V region and extended through the 3’ untranslated region of Cκ (~3.7 kb). A total of 21 nucleotide substitutions were observed in addition to the marker mutations present in the transgene (Fig. 2). Five mutations are present in the leader-Vκ intron, six within the V region (three of which were previously identified; reference 16), and 10 in the Jκ-Cκ intron. Based on this analysis, 71% of the newly introduced mutations are outside of the coding regions. Three of the six mutations found in the V region are in FR regions. Of these, the nucleotide substitution at position 591 in FR1 results in
in the replacement of serine with phenylalanine. The substitution at 556 in FR1 is silent. The mutation at 789 in FR3 causes the introduction of threonine in the place of serine. Both of the nucleotide substitutions within CDR3 are silent mutations.

Genomic clone PIII-2 (isolated from hybridoma 3A1) was sequenced from 270 bp 5' of the V gene through the 3' untranslated region of the Ck gene. In the ~3.7 kb sequenced, four new nucleotide substitutions were observed (Fig. 2). Of the four mutations, only one is within the V region, so 75% of the substitutions detected are outside of coding areas. The mutation in the V gene is present within CDR3, but is silent. The base substitution at position 838 was previously shown to have arisen in the B cell from which hybridoma 3A1 was derived. The mutation was detected in hybridoma 3A1 DNA, but was absent from kidney (germline) DNA and thus was a somatic event (16). The mutations at positions 942 and 1031 have since been shown to be somatic by sequence comparison of polymerase chain reaction-amplified kidney and 3A1 hybridoma DNA (data not shown). Whether the base substitution at position 390 represents a somatic mutation is currently being determined.

Thus, a total of 37 new mutations have been identified within the three k transgenes analyzed. No mutations were found to be in common between the transgenes. Furthermore, no insertions or deletions were observed in any of the cloned transgenes.

Examination of the overall distribution of mutations within the rearranged k transgenes reveals that the are localized to the V region and its flanking sequences (Fig. 1). Although we do not know the 5' boundary of the mutations, the majority of the Jk5-Ck intron mutations are within 500 bp 3' of the V region, beyond which the frequency of mutations is significantly reduced. The 3' most mutation observed (position 1708, pII-13) was <1 kb from the V region. The nuclear matrix association region (18) and the k transcriptional enhancer located upstream of the C region are spared. No mutations were detected in the C gene or the 3' untranslated region. In fact, analysis of clone pl-7 revealed that no mutations were present as far as 1.3 kb 3' of the C region. To our knowledge this represents one of the most complete analyses of somatic mutations across an Ig gene. Consequently, we have been able to precisely define the 3' boundary of the mutations introduced into these k transgenes. The range of newly introduced mutations in the "recycled" k transgenes is very similar to that reported for endogenous Ig genes (3, 4, 19–21). Furthermore, the transgene itself had been mutated in the B cell (myeloma) from which it was originally cloned (22, 23); the newly introduced mutations are very similar in distribution (Fig. 1) and nature (Table 1). While it is not possible to formally show that the same mechanism is responsible for the hypermutation of the k transgenes and endogenous Ig genes, we have no evidence against a common mechanism. Even though the k transgenes differ with respect to chromosomal location from endogenous Ig genes, proper targeting of the "mutator" is maintained. This suggests that features responsible for targeting the mutations to a rearranged Ig gene are present within the k transgene.

| Table 1. Newly Introduced Mutations in the k Transgenes |
|-----------------------------------------------|
| Substitutions | VC167* | pl-7 | pII-13 | pIII-2 |
| Transitions |
| A→G              | 3     | 1    | 5     | -     |
| G→A              | 3     | 1    | 6     | -     |
| C→T              | 3     | 2    | 1     | -     |
| T→C              | -     | -    | -     | -     |
| Total            | 9     | 4    | 12    | 0     |
| Transversions |
| C→A              | -     | -    | -     | -     |
| G→T              | -     | -    | 1     | -     |
| A→C              | 3     | -    | 3     | 1     |
| A→T              | 1     | -    | -     | -     |
| G→C              | -     | 2    | 4     | 2     |
| C→G              | -     | 3    | -     | -     |
| T→A              | 1     | -    | 1     | -     |
| T→G              | 1     | 3    | -     | -     |
| Total            | 6     | 8    | 9     | 4     |

* Mutations that were already present in the myeloma MOPC-167.

The precise mechanism responsible for introducing somatic mutations is unknown. Of interest, a large number of mutations were found in the Jk5-Ck intron of these k transgenes. This distribution is consistent with the available data concerning the localization of somatic mutations outside V regions in endogenous Ig genes (3, 4, 21). These findings are not easily reconciled with a gene conversion mechanism. The frequency of mutations does not seem to be related to the transcriptional activity of the B cell hybridoma; the level of steady-state transgenic k mRNA was approximately the same in all three hybridomas (24). Furthermore, in the case of hybridoma 3A1, mutated and unmutated transgenes appeared to be transcribed equally well (16). Thus, at this time, no link can be made between the mutation process and transcription of its target gene.

Data concerning the nature of the substitutions identified in the transgenes are presented in Table 1. A composite of the k transgenes reveals that transitions and transversions occur at approximately equal frequency. This holds true whether assessed for the entire genes or, in order to bypass possible selective pressures, for only silent changes and substitutions within the introns. The T → C transition is the only interchange that was not observed, but this transition has been seen in other mutated Ig genes (25).

The frequency of mutations introduced into the V regions of the transgenes pl-7 and pII-13 is 1.7%, slightly higher than the 1.1% seen in the MOPC-167 k gene. The frequency for pIII-2 is ~0.3%. Of the cloned transgenes, the overall number of mutations observed in pIII-2 is reduced as well.
Figure 2. Nucleotide sequence of rearranged MOPC-167 k gene beginning at position 222 of the germline sequence (23). Comparison of the MOPC-167 k gene sequence with the Ja5-Ca intron germline sequence (19, 28, 29) confirmed differences previously observed by Gearhart and Bogenhagen (3), and revealed further alterations. The nature of the changes and their positions are listed below. Changes from the published sequence, including previously undetermined sequences, denoted as X in reference 28, are as follows: at position 1428, T-X deleted (del); 1435, X = GGCTG; 1443, A = del; 1444, A = del; 1463, A = del; 1637, C = del; 1706, X = del; 1762, X = del; 1832, X = A; 1847, XXXXX = C; 1860, G = del; 1865, T = del; 1878, T = del; 1913, X = TAAG; 1921, X = del; 1940, XX = CAT; 1974, A = del. We do not know whether the observed alterations represent errors in the published sequence or somatic mutations that occurred in MOPC-167. However, in all cases, the insertions and deletions were common to all of the cloned transgenes. The Va167 Ja5 coding region is boxed, and complementarity-determining regions (30) are underlined. FR1 begins at position 548. The Ja5 segment begins at position 848. Sequence analysis of pIII-2 and pII-13 begins at position 267 and 262, respectively. Nucleotide substitutions already present in the MOPC-167 k gene are indicated by asterisks. Base substitutions in the cloned transgenes pIII-2, pl-7, and pII-13 are identified. Mutations resulting in amino acid replacements are underlined. The nucleotide substitution that gave rise to a termination codon is boxed.

Clones pl-7 and pII-13 were isolated from hybridomas generated from a line carrying 13 copies of the k gene, while pIII-2 was cloned from a hybridoma generated from a line with three copies of the k gene. Whether there are consistently fewer mutations introduced into k transgenes in B cells from the three copy line is currently being investigated.

The analysis of somatic mutation through the use of Ig transgenes provides an opportunity not only to examine the minimal elements required for targeting of the "mutator", but also to examine its specificity for V and surrounding regions. Is the observed V region localization a result of selective pressures to produce intact C regions, or alternatively, a reflection of the inherent specificity of the mutator mechanism? The presence of multiple copies of the transgene might be expected to reduce the selective pressure on any given copy within the array, because introduction of mutations leading...
to a loss of function of one copy would be compensated for by the presence of other functional copies. Whereas a normal B cell may be unable to tolerate a mutation leading to a loss in surface Ig, in a transgenic mouse, expression of multiple copies of an Ig transgene would allow for continued survival of a transgenic B cell. In fact, evidence has been obtained that multiple copies of the κ transgenes are transcribed (16, 24). Even under these conditions, mutations are highly localized to the V regions.

Furthermore, analysis of clone pl-7 from CE9 revealed the presence of a stop codon in the V region. Clearly, we have no way of knowing when the stop codon was introduced. However, the continued production of transgenic κ by hybridoma CE9 (24) in conjunction with the high ratio of FR/CDR (5:1) mutations and the high (5:0) replacement/substitution ratio observed in the FR regions of pl-7 is consistent with the notion of reduced selective pressure on this transgene. Examination of the other cloned transgenes leads to a similar conclusion. All of the mutations within the CDRs of plII-2 and plII-13 are silent. Furthermore, plII-13 is characterized by a high ratio of FR/CDR mutations as well as a high ratio of replacements/substitutions within framework regions. This is in marked contrast to the pattern of mutations expected after extensive antigenic selection (26, 27). Nevertheless, proper targeting of the mutation process has been maintained. Therefore, the observed localization of mutations to the V region area, and apparent sparing of the C genes in normal B cells (3, 4, 19, 20), does not appear to be the result of selective pressure to produce a functional surface Ig molecule, but rather, a reflection of the specificity of the mutator mechanism. Experiments are currently under way to determine the requirements for targeting of the somatic hypermutation mechanism.

We thank Maria Glymour for her assistance in generating the figures and Dr. Peter Engler for his careful review of this manuscript.

This work was supported by National Institutes of Health Grant GM-38649. J. Hackett is a Leukemia Society of America Fellow. B. Rogerson is supported by a fellowship from the Cancer Research Institute.

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Received for publication 18 December 1989 and in revised form 20 March 1990.

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