Somatic Mutations During an Immune Response in Xenopus Tadpoles

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The tadpole B-cell repertoire is less diverse than that of the adult frog; their antibodies are of lower affinity and are less heterogenous. In order to determine whether this difference is due to a lack of or a reduced rate of somatic hypermutation, we analyzed and compared cDNA sequences utilizing V_H1 elements with germline counterparts in isogenic LG7 tadpoles during an immune response. Indeed, tadpole V_H1 sequences contained somatic mutations. There were zero to 5 mutations per sequence, all single base-point mutations, with the high ratio of GC to AT base-pair alterations similar to that observed in adult frogs.

KEYWORDS: Antibody diversity, LG hybrid.

INTRODUCTION

The African clawed frog, Xenopus, provides an attractive model for studying the ontogeny of the immune system and much is known about the changes that occur at metamorphosis (reviewed by Flajnik et al., 1987). Xenopus larval antibody responses are not influenced maternally and are clearly distinct from adult antibody responses. The tadpole B-cell repertoire is less diverse than that of the adult frog, which is considered more limited than that of mammals (Du Pasquier et al., 1989). Tadpole antibodies are of lower affinity than adult antibodies specific for the same antigen and their isoelectric focusing patterns of antigen-specific antibodies are less heterogeneous (Du Pasquier et al., 1979; Hsu and Du Pasquier, 1984a, 1984b). These differences are not correlated with the stage of differentiation, not size or age. Very large, very old tadpoles produced by blocking metamorphosis with sodium perchlorate (Du Pasquier et al., 1985) make only tadpole-type antibodies (Hsu and Du Pasquier, 1992).

Xenopus immunoglobulin genes are organized and rearranged in a way similar to that of mammals (Schwager et al., 1988a) with multiple V_H, multiple D (≥ 16), multiple J (8–9), and a single constant region gene. Eleven V_H families (Haire et al., 1990) with ca. 2 to 32 members per family (Wilson et al., 1992b) offer a large diversity of viable region genes.

In Xenopus, as in mammals (Tonegawa, 1983), CDR3s are the most diverse. Nucleotides may be deleted from and/or added to V_H,D and D_lJ joints during rearrangement (Tonegawa, 1983; Lafaille et al., 1989). However, like in fetal and newborn mice (Feeney, 1990; Gu et al., 1990), Xenopus tadpoles lack N diversification, which might partially explain the low heterogeneity of tadpole antibodies (Schwager et al., 1991).

More recently, tadpole CDR3 were also found to be much shorter than adult CDR3 (Lee et al., 1993); although the differences in CDR3s might account for the observed lower heterogeneity of tadpole antibodies, they do not necessarily explain the lower affinity. Although we originally proposed that somatic mutation might play a reduced role in Xenopus antibody responses and thus limit the affinity maturation of Xenopus antibodies (Du Pasquier, 1982), our recent results suggest that this is not the case, at least in adult (Wilson et al., 1992a). The rate of somatic mutation in Xenopus adult is well within the range reported in mice (McKean et al., 1984; Wabl et al., 1985; Kocks and Rajewsky, 1989), but the mutants are apparently poorly selected. This situation could lead easily to the observed minimal affinity maturation of the antibody response of many cold-blooded vertebrates. Larvae have an even more restricted antibody repertoire and a
response characterized by an affinity lower than in the adult, at least in the case of the response to dinitrophenol coupled to keyhole limpet hemocyanin (DNP-KLH) (Hsu and Du Pasquier, 1984a). It is therefore possible that the difference between adults and larvae could be due to the absence of somatic mutation in tadpoles. To investigate this point, we analyzed cDNA sequences from immunized LG7 tadpoles. LG7 is an isogenic line derived from an X. laevis/X. gilli hybrid (Kobel and Du Pasquier, 1975), which is homozygous at the immunoglobulin (Ig) heavy chain locus (Wilson et al., 1992b).

MATERIALS AND METHODS

Animals and cDNA Library Construction

Twelve isogenic LG7 tadpoles (X. laevis/X. gilli; Kobel and Du Pasquier, 1975) were blocked at stage 52 using sodium perchlorate, as previously described (Du Pasquier et al., 1985). After 6 months, the animals were hyperimmunized with DNP-KLH according to a protocol known to produce the best antibody responses in tadpoles (Hsu and Du Pasquier, 1984a). Four weeks after the last injection, 5 animals were sacrificed and about 1 µg mRNA was prepared from their pooled livers and spleens using a Fast Track mRNA kit (Invitrogen). LG7 tadpole cDNA was synthesized with a kit from Pharmacia and cloned into the lambda Zap II vector system from Stratagene. The unamplified library, which contained 1 x 10^7 recombinant phage clones, was screened at high stringency with Xenopus Cµ, Cδ and V_H, V_H5 probes (kindly provided by our colleague J. Schwager). The probes were labeled by random priming (Feinberg and Vogelstein, 1983) and hybridization conditions were as described by Wilson et al. (1986). Positive clones were plaque purified and subcloned into Bluescript according to the manufacturer's (Strategene) recommended protocol.

Sequencing and Computer Analysis

The double-stranded recombinant plasmid cDNAs were sequenced on both strands by primer extension with the dideoxynucleotide triphosphate chain-termination-reaction method (Sanger et al., 1977). Synthetic oligonucleotides and universal primers were prepared by H.R. Kiefer's laboratory (Basel Institute for Immunology). We consider the error frequency of the modified T7 polymerase in our sequencing reactions to be negligible, because 2 independent Cµ cDNAs were sequenced (4830 bp each) with no errors.

All computer programs were written by C. Steinberg (Basel Institute for Immunology). DNA sequences were aligned in pairs with a program based on the algorithm of Needleman and Wuntsch (1970). Each sequence was aligned to a master, and the pairwise alignments were used as input for a multiple alignment based on a heuristic algorithm.

RESULTS

Analysis of Expressed V_H1 cDNAs

From the unamplified library, we isolated and sequenced 5 µ and 6 u cDNAs containing V_H1 family elements. Only 1 of the µ cDNAs could be matched with a V_H1 germline sequence (Wilson et al., 1992b). The other 4 µ cDNAs were too short to be assigned; their sequences began in either CDR2 or FR3. In fact, many of the positive µ and u cDNAs represented short transcripts (for example, only 21 of 73 u positive cDNAs screened positive with a J consensus oligo).

All of the µ cDNAs had identifiable V_H1 germline gene counterparts. One of them (LG73) was unmaturated and corresponded to the germline gene LG7G341.

Five (4 u and 1 µ) out of the 7 analyzable V_H1 cDNAs form a related group, and they are shown in Fig. 1. cDNAs u8 and u4 are clearly derived from the GL7g21 genomic sequence. These 2 cDNAs are identical and presumably form a clone (clone 1), because both have identical V_H1 and DJ_H joints. As in our previous studies, we assume that if 2 similar genes (similar CDRs 1 and 2, similar FRs) have identical leader sequences, they are likely to be derived from the same germline V_H (Wilson et al., 1992a). Both u8 and u4 have a point mutation in their leader sequence; CTA becomes TTA. However, because of their close identity throughout the coding region to LG7g21 (only 2 point mutations), we match them with LG7g21. cDNAs µt14, µt12, and µt6 appear to be derived from a germline V_H1 related to LG7g21. These 3 cDNAs all rearrange to different DJ_H; Fig. 1 shows the differences that they share as compared to LG7g21. In this group, 8 nucleotide differences are scattered throughout the
FIGURE 1. Alignment of tadpole cDNAs related to the Xenopus germ line V_{μ}1 gene LG7g21. The master genomic sequence begins at the initiation codon ATG and continues down to the heptamer of its RSS. The leader intron is spliced out for the alignment. The cDNA sequences also begin at the initiation codon or at the first sequenced nucleotide of their leader and continue down to their CDR3 boundaries. Sequence identities are indicated by primes (') and gaps (-) are introduced to maximize homology. The CDR boundaries are marked and are according to Kabat et al. (1991). The GenBank accession number for LG7g21 is M94841.
coding region and 2 of the cDNAs, ut12 and ut6, have a CA at their DJH joints (see what follows). We have assigned these 2 nucleotides to the VH, assuming that deletion is occurring at VH in μt14, bringing the total of nucleotide differences to 10. In adult LG7 frogs (Wilson et al., 1992a), the average number of mutations per gene was 3, and it is unlikely that 3 different cDNAs would contain 10 identical point mutations, we have assumed that this group was derived from a new germline gene, which we have designated VHg21B. Thus, ut6 contains 2 mutations, as it differs from μt14 and μt12 by 2 nucleotides.

Finally, cDNA LG7t13 is a somatic mutant of the germline gene VHg346B.

Table 1 is a summary of the mutations found in all of the VH1 cDNAs, and Fig. 2 gives the alignments of the cDNAs where mutations are found. Only the codons that contain mutations are shown. The FR and CDR boundaries, which are marked, were identified according to Kabat et al. (1991). Overall, there are 11 point mutations in 4 cDNAs; no deletion mutations were observed. Six of the mutations occur in the CDR3s, 3 in the FRs, and 2 in the leaders; all code for replacement changes. In addition, all of the mutated CDR codons are serines, and 4 of them create changes in charge—2 at codon 53 in VHg21 and 1 at codons 52 and 53 in VHg346B. All of the mutations involve an alteration at a GC base pair.

### Analysis of the Tadpole VH1 CDR3s

The entire CDR3 sequences for all the tadpole VH1 cDNAs are shown in Fig. 3. The sequences are arranged according to VH1 usage and are separated into segments derived from the germline VH1, JH1, and putative D. The JH elements could be positively identified because the LG15 JH area has been mapped and sequenced (Schwager et al., 1991). JH5 is used most frequently, followed by JH3. All of the cDNAs, except for ut3, have shortened JH5 and lack 5 (ut8, ut4) to 7 (μt14) bases at their 5' ends; these were deleted during rearrangement (Schwager et al., 1991). The 2 cDNAs of clone 1 contain a point mutation in JH; TTC becomes TTT. This mutation is silent. No other mutations were observed in the JH segments. Two of the VH1 sequences, including one switched to μ (ut6), were out of frame.

The Xenopus D cluster has yet to be mapped and only 1 D (D15) in the germ line has been sequenced (clone G2; Schwager et al., 1991). However, on the basis of core sequence identities, 16 putative D elements have been deduced from cDNA sequences (Schwager et al., 1988a, 1988b; Hsu et al., 1989; Wilson et al., 1992a). Five out of the 7 LG7 tadpole cDNAs have identifiable D core sequences (underlined in Fig. 3); and if assignment of D1 is correct for cDNAs ut8 and ut4, there is a point mutation in clone 1; GCT becomes CCT. This replacement change brings the total number of mutation to 15. Previously, we reported (Wilson et al., 1992a) that point mutations were rare in Xenopus CDR3s; only 1 putative point mutation in a D1 segment was found in a survey of 61 adult LG7 VH1 cDNAs.

In addition, the LG7 tadpole sequences confirm the findings of Schwager et al. (1991) that Xenopus tadpole CDR3s are diversified by P nucleotide additions (Laface et al., 1989). Because the complete sequences through the recombination signal sequences (RSS) are known for the majority of the LG7 VH1s and for the JHs, P nucleotides can be recognized. For example, no nucleotides are deleted from the 3' end of the cDNAs corresponding to VHg21, VHg346B, and VHg341 nor are there any nucleotides deleted from the 5' end of JH4 in t3. Thus, the C at the VHJH junctions of clone 1, complementing the G of VHg21, can be a P nucleotide. Likewise, the C at the VHJH joint and the A at the DJH joint of t3 can be attributed to P. Because of the lack of germline D sequences, the presence of N diversity is more difficult to detect; however, based on the core identity of D5, N nucleotides may be present at the VHJH junctions of μt14.

### Table 1

| VH1 | Total cDNAs | Point mutations | Base changes |
|-----|-------------|----------------|-------------|
| g21 | 2           | 6              | 2C→T, 4G→A |
| g21B| 3           | 2              | 2G→C       |
| g346| 1           | 3              | 1C→G, 1C→A, 1G→C |
| g341| 1           | 0              |             |

### Tadpole JH Usage

Taking into account all the VH1 sequences (21) available from this tadpole library screening, we found that the JH usage preference was the same as in adult, namely, a strong preference for JH3 usage could be detected (Fig. 4).
Comparison with Adults

V_{H}g21 was one of the most widely used V_{H}1 members in the adult anti-DNP response studied earlier (Wilson et al., 1992a). One of the mutations, reported in larval ut8 and 4, was identical (position 53 in CDR2 G→A), and the D usage (D_{H}I, D_{H}16) could also be considered similar, although the reading frames were different. D_{H}5 used in one gene (mut14) was in the same reading frame as D_{H}5 used in another adult V_{H}1 cDNA using the V_{H}g44 germline gene segment. V_{H}g346b, also found in tadpole cDNA ut13, was used in adult (6 clones), but all the mutations were different, and there were no similarities in the D region. V_{H}g341 was expressed without mutation in tadpole (ut3), as it was in adult. Altogether, the V_{H}1 member usage found in immune adults and immune tadpoles is similar; the same subset is used in adults and tadpoles. This predominance of certain V_{H}1 members among cDNA obtained in two different libraries made from immune animals together with the discovery of one identical mutation in CDR2 of tadpole and adult genes could indicate where selection takes place.

DISCUSSION

Somatic mutation in the immunoglobulin genes of a cold-blooded vertebrate was first described in the amphibian Xenopus (Wilson et al., 1992a). It was concluded that antibody diversity in such species as amphibians and probably fish was not limited by the availability of mutants, but the ability to select them properly. The cited work left open the possibility that during ontogeny of the immune systems, the ability to produce somatic mutants appeared after metamorphosis. The present work demonstrates that this is not the case. Tadpole B cells are
FIGURE 3. The CDR3 sequences of the tadpole V_{H1} cDNAs grouped according to the germline V_{H} used (Wilson et al., 1992b). The codons between the invariant cysteine that marks the end of FR3 and the invariant tryptophan that marks the beginning of FR4 are grouped according to their derivation: V_{H} (left), D (middle), and J_{H} segments (right). Identifiable D nucleotides are underlined and the names of the D and J_{H} segments (according to Schwager et al., 1991; Wilson et al., 1992a) are listed in the right margin. The name of the germline V_{H} gene counterpart is at the top of each group. Point mutations in clone 1 are double underlined. The GenBank accession number for V_{H341} is M94843.

FIGURE 4. J_{H} usage in tadpole VDJC rearrangements in LG7. The numbers on the abscissa refer to the J_{H} number. The relative position of the J_{H} on the chromosome segment is given underneath (from Schwager et al., 1991).

| V_{H21} | D plus N and P nucleotides | J_{H} |
|---------|---------------------------|-------|
| vt8     | ACT AGA GAT G             | TTT GAC TAC | D1 J_{H3} l Clone |
| vt4     | ACT AGA GAT G             | TTT GAC TAC | D1 J_{H3} 1 1 |

| V_{H21B} | D plus N and P nucleotides | J_{H} |
|----------|---------------------------|-------|
| μ14      | GCT AG                    | G GCT TAC TGG GCT GGG AGG | G TAT TIC GAG CAC D5 J_{H8} out of frame |
| vt12     | GCT AGA CA                | A TGG GCT ACC AGG          | GCT TTC GAT TAC D16 J_{H5} |
| vt6      | GCT AGA CA                | C TCA CCC GCT ACA GGG GG   | GCT TTC GAT TAC D1 J_{H5} out of frame |

| V_{H346B} | D plus N and P nucleotides | J_{H} |
|-----------|---------------------------|-------|
| vt13     | GCT AGA GA                | G GGG GGG                        | GCT TTC GAT TAC -- J_{H5} |

| V_{H341} | D plus N and P nucleotides | J_{H} |
|----------|---------------------------|-------|
| ut3      | GCT ACA GAA G             | CC CA T GTC TAT CAC -- J_{H4} |

able to produce mutations that have the same characteristics as those of adults. Being limited in cell numbers when working with tadpoles, we cannot present any meaningful analysis of the R/S ratio or the position of the mutations within or outside the CDRs. However, all the bases altered by mutations were G or C, reflecting the same trend as the one observed in adults. The average number of mutations per V gene was of the same order of magnitude as in adults—1.5 in tadpole, 3 in adult. We therefore assume that the conditions of selection are also poor in tadpoles (like adults, tadpoles do not have bona fide germinal centers). The occurrence of somatic mutations in larvae raises an interesting question with respect to metamorphosis. In principle, there ought to be some mutants directed against future adult specificities. These would develop unnoticed and create autoimmune disorders when the corresponding epitope is generated. In this context, the poor ability to select mutants is perhaps advantageous. Being rarely selected, the potentially harmful mutant may not in fact be very dangerous.

Yet, as in adults, there must be some selection. Without clonal selection, no specific immune response would be noticeable. The finding of identical replacing mutations in adult and tadpole is also an indication that some gene products were selected. On the other hand, the similarity in D usage found in adult or tadpole without the conservation of the reading frame does not suggest a very strong selection by the antigen on the part of the antibody coded by this region. That some selection has taken place at the T-cell level is also shown by the fact that V_{H1} rearrangements have switched, a highly T-cell-dependent event, to the second isotype IgY, even in tadpoles where this switch is rather poor (Hsu and Du Pasquier, 1984a, 1984b). Other V_{H}s analyzed
from this library, picked at random by cross-
hybridization with a V\textsubscript{H}5 probe, were all \(\mu\), and,
when comparison with germline genes was possible,
unmutated. As a result of the present work, it is
more difficult to explain the difference between
larvae and adult antibody responses. However,
the difference in T-cell help (Hsu and Du Pasquier,
1984a, 1984b) might play a role in the development
of the response. The tadpole response would be
measured at a stage corresponding to an early adult
response. Another factor that could play a role is V\textsubscript{H}
usage. Indeed, two V\textsubscript{H}1 members prominently used
in the adult response, V\textsubscript{1Hg} 44a and V\textsubscript{1Hg} 27, were
not used in tadpoles of the same genetic back-
ground.

Several other interesting points arise from this
analysis of the immunized tadpole cDNA library.
There is the same type of bias in J\textsubscript{H3} usage in adults
and tadpoles. Some V\textsubscript{H} seem not to be used at this
stage of development. For instance, 23 cDNAs were
isolated by cross-hybridization with a V\textsubscript{H}5 probe,
but after sequencing, none turned out to be V\textsubscript{H}5, an
observation consistent with previous work on tad-
pole Ig sequences, where no V\textsubscript{H}5 usage was de-
tected. The tadpole sequences presented here show
as already observed (Schwager et al., 1991) that N
diversity is very limited at this stage of ontogeny.

Finally, the observations that tadpole B cells can
achieve somatic hypermutation and do not express
CD5 (J"urgens et al., in press, 1995) does not fit well
with the notion that larval B cells correspond to the
Ly-1 B cells (now called B-1 cells; Kantor, 1991) of
mammals, which is considered to be a primitive cell
type (Herzenberg and Herzenberg, 1989).

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