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 heterogeneous. 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_HD and DJ_H 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

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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 = (H) 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 1×10^7 recombinant phage clones, was screened at high stringency with *Xenopus* C μ , C ν and V_H1, 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 (Stratagene) 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 C μ 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 ν 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 ν cDNAs represented short transcripts (for example, only 21 of 73 ν positive cDNAs screened positive with a J consensus oligo).

All of the ν cDNAs had identifiable V_H1 germline gene counterparts. One of them (LG7t3) was unmutated and corresponded to the germline gene LG7G341.

Five (4 ν and 1 μ) out of the 7 analyzable V_H1 cDNAs form a related group, and they are shown in Fig. 1. cDNAs vt8 and vt4 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_HD 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 vt8 and vt4 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 μ t14, vt12, and vt6 appear to be derived from a germline V_H1 related to LG7g21. These 3 cDNAs all rearrange to different DJ_Hs; 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* germline V_H1 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, $\text{vt}12$ and $\text{vt}6$, have a CA at their DJ_H joints (see what follows). We have assigned these 2 nucleotides to the V_H , assuming that deletion is occurring at V_HD in $\mu\text{t}14$, 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 because 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 $V_{H\text{g}21\text{B}}$. Thus, $\text{vt}6$ contains 2 mutations, as it differs from $\mu\text{t}14$ and $\text{vt}12$ by 2 nucleotides.

Finally, cDNA LG7t13 is a somatic mutant of the germline gene $V_{H\text{g}346\text{B}}$.

Table 1 is a summary of the mutations found in all of the V_H1 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 CDRs, 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 $V_{H\text{g}21}$ and 1 at codons 52 and 53 in $V_{H\text{g}346\text{B}}$. All of the mutations involve an alteration at a GC base pair.

Analysis of the Tadpole V_H1 CDR3s

The entire CDR3 sequences for all the tadpole V_H1 cDNAs are shown in Fig. 3. The sequences are arranged according to V_H1 usage and are separated into segments derived from the germline V_H , J_H , and putative D. The J_H elements could be positively identified because the LG15 J_H area has been mapped and sequenced (Schwager et al., 1991). J_H5 is used most frequently, followed by J_H3 . All of the cDNAs, except for $\text{vt}3$, have shortened J_H s and lack

5 ($\text{vt}8$, $\text{vt}4$) to 7 ($\mu\text{t}14$) 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 J_H : TTC becomes TTT. This mutation is silent. No other mutations were observed in the J_H segments. Two of the V_H1 sequences, including one switched to v ($\text{vt}6$), were out of frame.

The *Xenopus* D cluster has yet to be mapped and only 1 D (D15) in the germline 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 $\text{vt}8$ and $\text{vt}4$, 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 V_H1 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 (Lafaille et al., 1989). Because the complete sequences through the recombination signal sequences (RSS) are known for the majority of the LG7 V_H1 s and for the J_H s, P nucleotides can be recognized. For example, no nucleotides are deleted from the 3' end of the cDNAs corresponding to $V_{H\text{g}21}$, $V_{H\text{g}346\text{B}}$, and $V_{H\text{g}341}$ nor are there any nucleotides deleted from the 5' end of J_H4 in t3. Thus, the C at the V_HD junctions of clone 1, complementing the G of $V_{H\text{g}21}$, can be a P nucleotide. Likewise, the C at the V_HD joint and the A at the DJ_H 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 V_HD junctions of $\mu\text{t}14$.

TABLE 1
Summary of Mutations

V_H	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 J_H Usage

Taking into account all the V_H sequences (21) available from this tadpole library screening, we found that the J_H usage preference was the same as in adult, namely, a strong preference for J_H3 usage could be detected (Fig. 4).

	L	CDR2	FR3	
	-15	53	97	
	L	S	A	
V _H g21	C T A	A G C	G C T	
	F	N	T	
vt8	T ' '	' A '	A ' '	Clone 1
	F	N	T	
vt4	T ' '	' A '	A ' '	
	CDR1	CDR2		
	31	53		
	S	S		
V _H g21B	A G C	A G C		
	T	T		
vt6	' C '	' C '		
	FR1	CDR2		
	30	52	53	
	T	S	S	
V _H g346B	A C T	A G C	A G C	
	S	N	R	
vt13	' G '	' A '	' ' A	

FIGURE 2. Tadpole cDNA codons in which mutations are found. Sequences are aligned under the corresponding germline V_H1 sequence; only codons in which mutations occur are shown. The name and isotype of each cDNA; v, or μ is listed in the left margin. The one-letter code for the encoded amino acid is shown in bold type above the codon. Numbers correspond to codon positions according to Kabat et al. (1991). The primes (') indicate sequence identity. The vertical lines flank the two identical V_H cDNAs that have identical D_{JH} rearrangements. These two cDNAs are clone 1 because they probably are derived from a single rearrangement event at IgH. The GenBank accession number for V_Hg346B is M94845.

Comparison with Adults

V_Hg21 was one of the most widely used V_H1 members in the adult anti-DNP response studied earlier (Wilson et al., 1992a). One of the mutations, reported in larval vt8 and 4, was identical (position 53 in CDR2 G→A), and the D usage (D_H1, D_H16) could also be considered similar, although the reading frames were different. D_H5 used in one gene (μ t14) was in the same reading frame as D_H5 used in another adult V_H1 cDNA using the V_Hg 44 germline gene segment. V_Hg346b, also found in tadpole cDNA vt13, was used in adult (6 clones), but all the mutations were different, and there were no similarities in the D region. V_Hg341 was expressed without mutation in tadpole (vt3), as it was in adult. Altogether, the V_H1 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_H1 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

	V _H	D plus N and P nucleotides ↓	J _H	
V _{Hg21}				
ut8	ACT AGA GAT G	<u>CG TAC CCT AGC GGG</u>	<u>TTT</u> GAC TAC	D1 J _H 3 Clone
ut4	ACT AGA GAT G	<u>CG TAC CCT AGC GGG</u>	<u>TTT</u> GAC TAC	D1 J _H 3 1
V _{Hg21B}				
ut14	GCT AG	G GCT <u>TAC TGG GGT GGG AGC</u>	G TAT TTC GAG CAC	D5 J _H 8 out of frame
ut12	GCT AGA CA	<u>A TGG GGT AGC AGG</u>	GCT TTC GAT TAC	D16 J _H 5
ut6	GCT AGA CA	C <u>CTA GCG GGT ACA</u> GGG GG	GCT TTC GAT TAC	D1 J _H 5 out of frame
V _{Hg346B}				
ut13	GCT AGA GA	G GGG GGG	GCT TTC GAT TAC	-- J _H 5
V _{Hg341}				
ut3	GCT ACA GAA G	CC CA	T GTC TAT CAC	-- J _H 4

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 VDJ 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).

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_Hs analyzed

from this library, picked at random by cross-hybridization with a V_H5 probe, were all μ , 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_H usage. Indeed, two V_H1 members prominently used in the adult response, $V_{HG} 44a$ and $V_{HG} 27$, were not used in tadpoles of the same genetic background.

Several other interesting points arise from this analysis of the immunized tadpole cDNA library. There is the same type of bias in J_H3 usage in adults and tadpoles. Some V_H seem not to be used at this stage of development. For instance, 23 cDNAs were isolated by cross-hybridization with a V_H5 probe, but after sequencing, none turned out to be V_H5 , an observation consistent with previous work on tadpole Ig sequences, where no V_H5 usage was detected. 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 (Jürgens 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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