complex interactions between *Xenopus* proteins and the bacterial mutagenesis proteins to abrogate replication arrest.

Although entering apoptosis might be appropriate for cells with heavily damaged genomes, there may be a local checkpoint mechanism in vertebrates that permits replication past small numbers of unrepairable lesions. Our results suggest that some eukaryotic cells contain a protein or proteins that prevent replication on damaged ss DNA and that other cells might possess a bypass system related to that found in prokaryotes.

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27. We thank J. K. Saxena, who participated in the early injection experiments with irradiated ss M13; J. Hauser for constructing the T7 mucA′ and mucB plasmids; V. Doseeva for testing replication of UV-damaged ss M13 with multiple oocyte nuclear extracts and transformation of DNA recovered from injected oocytes into competent cells; T. Horii for providing a *Xenopus RAD51* cDNA clone; Y. Shiloach and the NIDDK Biotechnology Unit for large-scale bacterial preparations; R. S. Lloyd for providing T4 UV endonuclease; and T. Kunkel, N. Nossal, and S. H. Wilson for comments on the manuscript.

8 March 1996; accepted 25 March 1996

Thymine-Thymine Dimer Bypass by Yeast DNA Polymerase ζ

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The *REV3* and *REV7* genes of the yeast *Saccharomyces cerevisiae* are required for DNA damage–induced mutagenesis. The Rev3 and Rev7 proteins were shown to form a complex with DNA polymerase activity. This polymerase replicated past a thymine-thymine *cis-syn* cyclobutane dimer, a lesion that normally severely inhibits replication, with an efficiency of ~10 percent. In contrast, bypass replication efficiency with yeast DNA polymerase α was no more than 1 percent. The Rev3-Rev7 complex is the sixth eukaryotic DNA polymerase to be described, and is therefore called DNA polymerase ζ.

Mutations generated by spontaneous or mutagen-induced damage to DNA are thought to be a major contributing cause of many cancers. Many of these mutations probably arise during lesion bypass, that is, during replication past the site of DNA template damage. Bypass replication requires different protein products from those used in the replication of undamaged DNA, but the process is poorly understood. Translesion synthesis in *Escherichia coli* is usually carried out by DNA polymerase (Pol) III, the major chromosomal replicase, together with at least three accessory proteins, the products of the *recA*, *umuC*, and *umuD* genes (*1*). The Umu proteins form a complex, consisting of a dimer of UmuD′ (the proteolytically processed product of UmuD) and a monomer of UmuC, which RecA may target to the site of the DNA damage. The bound Umu-RecA complex appears to enhance the processivity of DNA Pol III, thus relieving the severe inhibition to elongation imposed by the lesion.

In the budding yeast *S. cerevisiae*, at least three genes are required for DNA damage–induced mutagenesis. The *REV1* gene (*2*) encodes a protein that has weak homology with the *E. coli* umuC protein; the *REV3* gene encodes a protein with sequence motifs characteristic of a DNA polymerase (*3*), although no activity attributable to this gene has previously been detected in cell extracts; and the *REV7* gene encodes a protein with no sequence similarities to any other protein in the National Center for Biotechnology Information sequence database (*4*). None of these genes is essential for viability, and each has a very similar mutant phenotype: *rev* deletion strains exhibit little induced mutagenesis and are slightly more sensitive than the wild type to DNA-damaging agents, but their repair and recombination functions are otherwise normal. This phenotype suggests that the proteins encoded by *REV* genes are dedicated to bypass replication.

To investigate the properties of the putative Rev3 DNA polymerase, we purified a glutathione S-transferase–Rev3 fusion protein (Gst-Rev3p), overexpressed in yeast, by affinity chromatography on glutathione-Sepharose. SDS–polyacrylamide gel electrophoresis (PAGE) analysis of the fraction obtained from this one-step purification showed a single major band of protein of ~205 kD, as expected for the Gst-Rev3 fusion (Fig. 1). Gst-Rev3p consistently showed DNA polymerase activity that was 10 to 100 times that of control preparations of Gstp alone, but this activity was unstable and difficult to study. We next investigated whether Rev3p exists as part of a multiprotein complex, which might explain its instability. We used the yeast two-hybrid system (*5*) to test for interactions between Rev1p, Rev3p, and Rev7p and to look for other proteins that might interact with the Rev proteins (*6*). A weak positive signal for interaction was observed when the LexA-Rev3 and Gal4-Rev7 fusions were paired, which suggested that Rev3p interacts with Rev7p. Compelling evidence for this interaction was obtained when the LexA-Rev3 fusion was used to screen a Gal4 fusion library of yeast cDNA for potential interacting proteins. Only two positive clones were identified in a screen of 10^6 transformants; both were in-frame fusions to Rev7, one fused at amino acid 8 and the other at amino acid 10. These results indicate that

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Rev7p and Rev3p interact and suggest that Rev7p may be a subunit of the Rev3 DNA polymerase.

To test this possibility, we introduced a second plasmid, engineered to overexpress Rev7p, into the yeast overexpressing Gst-Rev3p. When Gst-Rev3p was purified from these cells, a second major protein of ~29 kD, the expected size of Rev7p, was present in the glutathione-Sepharose fraction (Fig. 1). This 29-kD protein reacted with antibodies to Rev7p, and the 205-kD Gst-Rev3p reacted with antibodies to Rev3p and to Gstp. Thus, Rev7p apparently forms a tight complex with Rev3p that is stable during extensive washing of the glutathione-Sepharose column.

The DNA polymerase activity of the Gst-Rev3p:Rev7p complex was about 20 to 30 times the activity of Gst-Rev3p, as measured by the extension of a ^{32}P end-labeled primer (Fig. 2). A control fraction from cells overexpressing Gstp and Rev7p contained barely detectable amounts of DNA polymerase, and, unlike the activity of Gst-Rev3p, this activity was inhibited by aphidicolin. The polymerase activity of the Gst-Rev3p:Rev7p complex was proportional to the amount of enzyme up to 8 ng of protein sample per assay, but this activity reached a limit and then decreased as more enzyme was added. Primer extension kinetics were linear for about 10 to 20 min. Cleavage of the thrombin-sensitive site at the Gst-Rev3 junction resulted in an approximate doubling of polymerase activity, which suggested that the Gst moiety does not greatly inhibit DNA polymerase activity; the remaining studies were carried out with the uncleaved Gst-Rev3p:Rev7p complex. Because it is the sixth type of eukaryotic DNA polymerase to be described, the Rev3p:Rev7p complex is called DNA Pol ζ.

To obtain further evidence supporting the conclusion that the Gst-Rev3p:Rev7p complex is a DNA polymerase, we sedimented the glutathione-Sepharose fraction through a glycerol gradient (*7*). There was a major peak of polymerase activity at 13S coincident with both Gst-Rev3p and Rev7p. In another experiment, the glutathione-Sepharose fraction was loaded onto a Q-Sepharose column and eluted with a linear gradient of NaCl. DNA polymerase activity was eluted from the column as a single peak coincident with both Gst-Rev3p and Rev7p.

Replication past a template thymine dimer, a lesion that usually inhibits elongation, was carried out more efficiently by DNA Pol ζ than by yeast DNA Pol α. Both DNA Pol α and Pol ζ are nonprocessive enzymes and lack a 3′ to 5′ exonuclease activity (*8*). Dimer bypass replication was examined by extension of a ^{32}P end-labeled 15-nucleotide (nt) oligomer primer annealed to a 71-nt oligomer template with a TT *cis-syn* cyclobutane dimer at nucleotides 35 and 36. Comparable amounts of each enzyme were used, as judged by replication on dimer-free 71-nt oligomer templates. With smaller amounts of enzyme, most primers were apparently extended by a single polymerase binding event, because only a small fraction of primers were used (Fig. 3). On the dimer-containing template, the majority of both Pol α and Pol ζ molecules paused or stopped at nucleotide 34, just before the lesion, but with Pol ζ a substantial and approximately constant fraction (~10%) continued past the dimer at each enzyme concentration used (Fig. 3B). With yeast Pol α, however, the lesion was bypassed by no more than 1% of the enzyme molecules, even at the highest enzyme concentration studied. Because up to 0.5% of the templates may lack the dimer, actual dimer bypass by Pol α was probably even less. The apparently superior capacity of Pol ζ to carry out translesion synthesis is consistent with its suggested function in DNA damage–dependent mutagenesis, but the in vivo reaction may require additional proteins. Rev1p is essential for DNA damage–induced mutagenesis in vivo, and other subunits of Pol ζ, or associated proteins, may well remain to be identified.

Previous studies showed that ultraviolet photoproducts are absolute blocks to replication by all enzymes tested unless forcing conditions are used, such as large excesses of enzyme, long incubation periods, high deoxynucleotide triphosphate (dNTP) concentrations and bias, or the substitution of Mn^{2+} for Mg^{2+} (*9*). The modified T7 DNA polymerase Sequenase can efficiently copy past this lesion, and a small amount of bypass of a TT dimer was seen in a reaction reconstituted with *E. coli* DNA Pol III holoenzyme and RecA, UmuC, and UmuD′, which also allowed ~5% bypass of an abasic lesion (*10*). Substantial bypass of a TT dimer can be forced

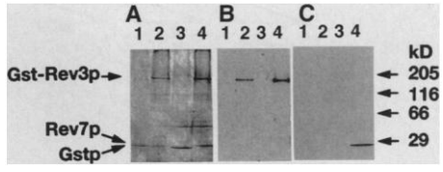

Fig. 1. SDS-PAGE analysis of glutathione-Sepharose fractions. Yeast strain Sc334 (*17*) containing either pGST (*18*) or pGST-REV3 (*19*) with or without pREV7 (*20*) was grown in synthetic dextrose medium selective for the plasmid(s). Cultures (2×10^7 cells/ml) were resuspended in 1% yeast extract, 2% Bacto Peptone, 1% galactose, and 0.5% glucose (+ 0.1 mM $CuSO_4$ when pREV7 was present), grown for an additional 3 hours, washed with cold water, and stored in 10% glycerol at –70°C. Cells (5 g) were suspended in 40 ml of 25 mM K_2HPO_4, 5 mM dithiothreitol (DTT), 1 mM EDTA, 1 M NaCl, 5% glycerol, 5% ethylene glycol, and 0.03% silicon antifoam, and the cells were disrupted by shaking at 0°C with glass beads. Triton X-100 was added to the extract (pH ~7) to a final concentration of 1%, the extract was clarified twice by centrifugation, and the supernatant was mixed gently for 12 hours at 4°C with 4 ml of glutathione-Sepharose 4B (Pharmacia) equilibrated in 1 M NaCl in buffer B [50 mM KPO_4 (pH 7.2), 1 mM DTT, 0.5 mM EDTA, 10% glycerol, and 1% Triton X-100]. The slurry was packed into a column, and the resin was washed with 400 ml of buffer B containing 0.5 M NaCl. Protein was eluted with buffer B containing 0.5 M NaCl and 20 mM glutathione, and pooled fractions were concentrated to 2 ml by dialysis in buffer B containing 0.5 M NaCl and 50% glycerol. A 10-μl sample of the glutathione-Sepharose fraction prepared from cells carrying plasmids to overproduce Gstp (lane 1), Gst-Rev3p (lane 2), Gstp + Rev7p (lane 3), or Gst-Rev3p + Rev7p (lane 4) was analyzed by 5 to 20% SDS-PAGE. The gel was stained with silver (**A**) or transferred to nitrocellulose and probed with antibodies to Rev3p (**B**) or to Rev7p (**C**) (*21*).

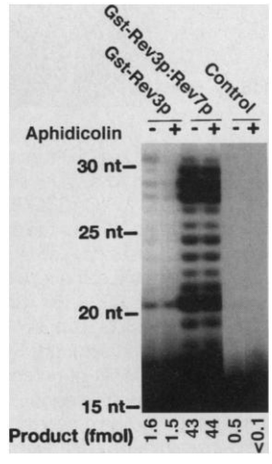

Fig. 2. DNA polymerase activity of Pol ζ. Reactions (20 μl) contained 25 mM KPO_4 (pH 7.4), 1 mM DTT, acetylated BSA (0.1 mg/ml), 10% glycerol, 6 mM $MgCl_2$, 50 μM each of deoxyadenosine triphosphate (dATP), dGTP, and dTTP, 10 μM dCTP, 10 nM 5′-labeled 15-nt oligomer primer (5′-ACGACGTTGTAAAAC-3′) annealed to a 30-nt oligomer template (5′-AATTCACTGGCCGTCGTTTTACAACGTCGT-3′), and 200 μM aphidicolin where indicated. Reactions were initiated with enzyme (glutathione-Sepharose fraction); after incubation at 30°C for 10 min, reactions were terminated with 20 μl of 20 mM EDTA and 95% formamide, and the reaction products were resolved on a 12% denaturing polyacrylamide gel and visualized by autoradiography. The gel was analyzed with a PhosphorImager (Molecular Dynamics), and the fraction of total radioactivity present as 16- to 30-nt products was used to calculate femtomoles of product in the 20-μl reaction. Reactions contained 3 ng (*22*) of Gst-Rev3p, 4 ng of Gst-Rev3p:Rev7p, or an equivalent volume (1 ng of Gstp) of the control fraction from cells expressing Gstp and Rev7p.

by high concentrations and long incubation times (up to 90 min) with *E. coli* DNA Pol I (*11*) or with calf DNA Pol δ and proliferating cell nuclear antigen, a eukaryotic processivity factor (*12*). However, neither of these enzymes gave detectable amounts of bypass in short incubations with enzyme sufficient only to elongate 20% or less of the primers (*9*, *11*, *12*), the conditions used in the Pol ζ experiments. Bypass in experiments under forcing conditions is also qualitatively different from bypass by Pol ζ; additional prominent gel bands, representing stall sites opposite the 3′ thymine and opposite the 5′ thymine of the dimer, are observed successively with increasing amounts of enzyme or time of incubation, and bypass products are first seen only at higher enzyme concentrations or longer incubation times. These stall sites are not seen with Pol ζ, which also gives a constant amount of bypass at all enzyme concentrations in 5-min reactions. The superior ability of Pol ζ, relative to Pol α, to efficiently extend an abnormal primer terminus of the kind formed after nucleotide insertion opposite a lesion was also evident in experiments with dimer-free templates and primers that formed 3′-terminal mismatches (*13*).

To examine the processivity of DNA Pol ζ, we compared primer extension reactions on the 7.3-kilobase circular single-stranded M13 DNA and the 30-nt oligomer templates (Fig. 4). Although the reactions with M13 DNA contained 33 times as much enzyme as did those with the 30-nt oligomer template, fewer primers were extended, presumably because of nonproductive binding to the large regions of single-stranded DNA. The preference of Pol ζ for short templates was about 10 times that of Pol α. About half of the Pol ζ and Pol α molecules dissociated from the M13 template after adding only 1 to 3 nt to the 15-nt oligomer primer, but some large products (up to ~200 nt) were formed (*14*). Because only a small fraction of the primer molecules were extended in these reactions, most of these products probably resulted from a single interaction between the polymerase and the primer-template. Like other DNA polymerases, Pol ζ and Pol α stop or pause at preferred sites along the DNA template, though not always at the same ones. In addition, Pol ζ, unlike Pol α, stops 2 and 3 nt from the end of both the 71-nt and 30-nt oligomer templates (Figs. 3 and 4), neither of which are pause sites for Pol ζ on M13 DNA.

DNA Pol ζ exhibits a unique spectrum of sensitivity to inhibitors (*15*). It is insensitive to aphidicolin (200 μM, >90% activity), as are Pol β (*16*) and Pol γ (*17*), but it is also insensitive to dideoxynucleotide triphosphates (ddNTPs) (100 μM, >90%

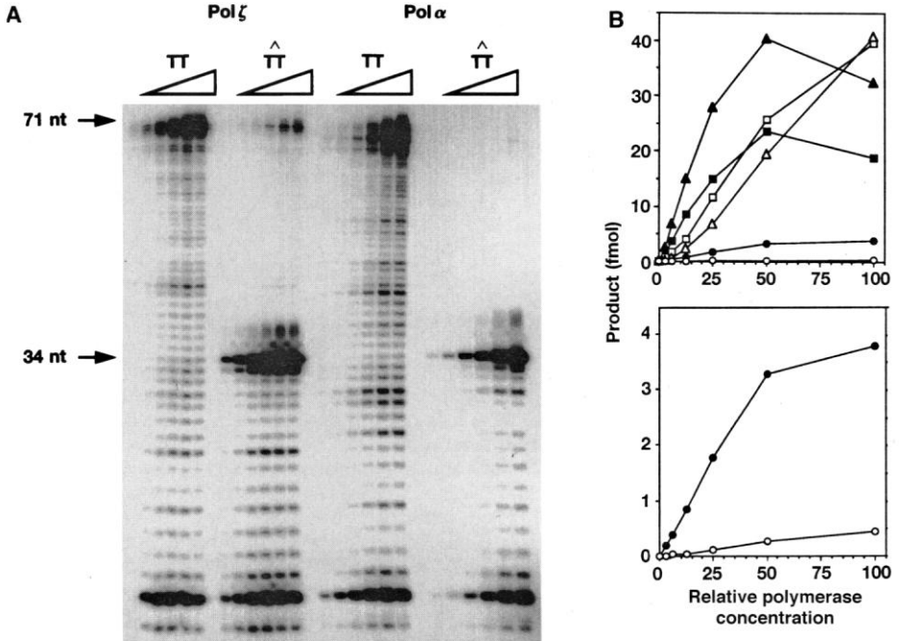

Fig. 3. Translesion DNA synthesis by DNA Pol ζ and DNA Pol α. (**A**) DNA polymerase reactions were carried out as in Fig. 2, except that 50 mM tris-HCl (pH 7.4) was substituted for KPO_4, incubation was for 5 min at 37°C, dCTP was at 50 μM, and the primer-template was 5 nM 5′-labeled 15-nt oligomer primer annealed to a 71-nt oligomer template [5′-ACAGGAAACAGCTATGACCATGATTCAGTGGC-AAG***TT***GGAGAATTCACTGGCCGTCGTTTTACAACGTCGT-3′ with or without a *cis-syn* TT dimer at positions 35 and 36 from the 3′ end (shown in bold italic)]. TT, control lanes; T̂T, dimer lanes. Reactions were carried out under yellow light to eliminate the possibility of photorepair by contaminating photolyase, although the same results were obtained in experiments done under normal light. Pol ζ reactions contained 0.75, 1.5, 3, 6, 12, or 24 ng of Gst-Rev3p:Rev7p, and Pol α reactions contained 0.025, 0.045, 0.09, 0.18, 0.36, or 0.72 ng of the catalytic subunit of yeast Pol α. (**B**) Products were quantitated as in Fig. 2. Open symbols, Pol α (0.72 ng = 100); closed symbols, Pol ζ (24 ng = 100). □ and ■, products of 39 to 71 nt on control templates; △ and ▲, products of 33 to 34 nt on dimer-containing templates (stopped at lesion). The DNA in the broad band between 36 and 37 nt is actually 34 nt long, as shown by isolation and reelectrophoresis; ○ and ●, products of 39 to 71 nt on dimer-containing template (synthesis past dimer). In the lower panel, the ordinate is expanded by a factor of 10.

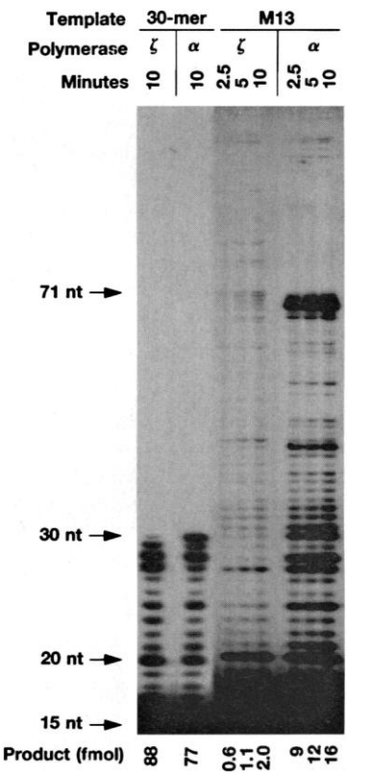

Fig. 4. Processivity of Pol ζ and Pol α. DNA polymerase reactions were carried out as described in Fig. 2, except that 50 μM dCTP and 10 nM 15-nt oligomer primer annealed either to the 30-nt oligomer (30-mer) or to single-stranded M13 DNA (7250 nt) were used as indicated. The 30-nt and 71-nt oligomer sequences (Fig. 3) are present in M13 DNA. Products were quantitated as in Fig. 2. Reactions with the 30-nt oligomer template had 6 ng of Pol ζ or 0.6 ng of Pol α, whereas reactions with the M13 template had 200 ng of Pol ζ or 20 ng of Pol α.

activity), which inhibit Pol β and Pol γ. It is moderately sensitive to butylphenyl-guanosine triphosphate (10 μM, 72% activity; 100 μM, 14% activity). Other properties include a broad pH optimum for activity around pH 7.4 and only moderate sensitivity to salt (73% maximal activity at 0.15 M NaCl). The enzyme was relatively inactive in assays with activated salmon sperm DNA or primed homopolymers such as poly(dA):oligo(dT) or poly(dT):oligo(rA). The ratio of activity with these polymer templates relative to that obtained with the oligonucleotide template-primers is only 2 to 5% of the ratio obtained with yeast DNA Pol α. Pol ζ may be unusually sensitive to inhibition from nonproductive template binding, as was also suggested by its low activity on the M13 DNA template (Fig. 4). The existence of a nonessential DNA polymerase that is responsible for mutagenesis may have implications for the treatment of cancer and may provide opportunities for novel therapeutic strategies. Inhibition of a human homolog of *REV3*, if it exists, may be useful in patients who have been exposed to mutagenic agents or have an inherited predisposition to cancer.

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6. The two-hybrid pairwise test for protein interactions and the library screen for interacting proteins were carried out as described [S. M. Hollenberg, R. Sternglanz, P. F. Cheng, H. Weintraub, *Mol. Cell. Biol.* **15**, 3813 (1995)].
7. J. R. Nelson, C. W. Lawrence, D. C. Hinkle, data not shown.
8. Proofreading 3′ to 5′ exonuclease activity was assayed as described in Fig. 2, except that dNTPs were omitted and the 5′-labeled primer contained a single 3′-terminal nucleotide mismatch. No shortening of the primer was detected (<1 fmol) with up to 90 ng of Pol ζ or 3 ng of Pol α, nor was shortening observed with several other substrates (including two additional primers with mismatched ends, completely complementary primers, and single-stranded primers in the absence of template). In contrast, incubation of 200 fmol of one of these mismatched substrates with 0.1, 1, and 10 ng of Pol I Klenow fragment (which contains a 3′ to 5′ exonuclease) respectively shortened 2.5%, 25%, and 89% of the primers by 1 to 2 nt.
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14. At each time and with both enzymes, 55 to 60% of the products were 16- to 18-nt oligomers, ~25% of the products were 19- to 32-nt oligomers, and 15 to 20% of the products were 33- to 200-nt oligomers.
15. DNA polymerase activity was determined as described in Fig. 2, except that the concentration of the competing nucleotide [deoxycytidine triphosphate (dCTP) for aphidicolin, deoxyguanosine triphosphate (dGTP) for butylphenyl-dGTP, deoxythymidine triphosphate (dTTP) for ddTTP, and dCTP for ddCTP) was lowered to 10 μM. Activity values are relative to activity without inhibitor.
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19. The plasmid pGST-REV3 was created by ligating a 4515-bp Eco RI–Sal I fragment containing *REV3* into pGST.
20. The plasmid pREV7 was constructed by ligating a 430-bp fragment containing the copper metallothionine gene promoter and a 747-bp fragment containing *REV7* into YEplac181, which contains the 2μ replication origin and *LEU2* for selection.
21. Rabbit polyclonal antibodies were generated against an SDS-PAGE–purified Rev3-TrpE fusion or Rev7p expressed in *E. coli*. Immunoblots were developed with goat antibody to horseradish peroxidase–conjugated rabbit secondary antibodies (Bio-Rad) and chemiluminescence (DuPont NEN).
22. The concentration of Gst-Rev3p was estimated by scanning a Coomassie blue–stained SDS–polyacrylamide gel and comparing the band intensities to the intensities of known amounts of bovine serum albumin (BSA).

23. We thank R. Bambara, M. Goodman, and R. Woodgate for comments; P. Ingles for initial work on the project; E. DiMuzio, M. Langer, and W. Sun for help with plasmid constructions; and M. Liskay and members of his lab for their help with the yeast two-hybrid screen. Supported by NIH grants GM21858 and GM29686.

4 December 1995; accepted 11 April 1996

A Quasi-Monoclonal Mouse

Marilia Cascalho, Audrey Ma, Steven Lee, Linda Masat, Matthias Wabl

As a model for studying the generation of antibody diversity, a gene-targeted mouse was produced that is hemizygous for a rearranged V(D)J segment at the immunoglobulin (Ig) heavy chain locus, the other allele being nonfunctional. The mouse also has no functional kappa light chain allele. The heavy chain, when paired with any lambda light chain, is specific for the hapten (4-hydroxy-3-nitrophenyl) acetyl (NP). The primary repertoire of this quasi-monoclonal mouse is monospecific, but somatic hypermutation and secondary rearrangements change the specificity of 20 percent of the antigen receptors on B cells. The serum concentrations of the Ig isotypes are similar to those in nontransgenic littermates, but less than half of the serum IgM binds to NP, and none of the other isotypes do. Thus, neither network interactions nor random activation of a small fraction of the B cell population can account for serum Ig concentrations.

The large diversity of antigen receptors on B cells hampers their study in intact mice. For several reasons discussed below, it is probably not possible to generate a truly monoclonal mouse—that is, a mouse in which every B cell expresses Ig molecules consisting of the same heavy (H) and light (L) chains—with a functional immune system. To study the generation of antibody diversity, we have therefore combined targeted gene replacement with mouse breeding to generate a mouse that is almost monoclonal. We call this animal the quasi-monoclonal (QM) mouse.

The extensively studied hapten NP induces an immune response that is restricted in the idiotype (*1*). Antibodies produced during primary responses are almost exclusively of the λ type. In particular, an Ig molecule consisting of a 17.2.25 heavy chain (*1*, *2*) combined with a λ light chain is specific for NP. In a conventional transgenic mouse line with a 17.2.25 transgene, μ_H17.2.25 combined with a λ1 light chain was shown to be specific for NP (*3*). In embryonic stem (ES) cells, we replaced the stretch of genomic DNA containing the J_H gene segments (*4*) with the V_HDJ_H 17.2.25 segment (Fig. 1A). Southern (DNA) blots were probed with μ and V_H 17.2.25 sequences to confirm that the introduced rearranged V(D)J segment was linked to C_μ and, hence, likely to be functional. Replacement mice obtained from the targeted ES lines were crossed to double "knockout" mice unable to express Ig heavy and κ chains because the J stretch had been deleted at both loci (*5*, *6*). The QM mice are those with the genotype V_HDJ_H 17.2.25/H^-, κ^-/κ^-, λ^+/λ^+ (Fig. 1B); they mimic the situation with a normal B lymphocyte, in which only one heavy and one light chain allele are expressed.

We used flow cytometry with the B220 and CD43 markers to analyze B cell development in spleen, bone marrow, and peripheral blood of QM mice. No gross abnormality was detected in these mice, with the exception

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