Mutations of neither *COL4A5* alone nor *COL4A6* alone have been observed in AS-DL. It is possible that only the α6(IV) chain is critical for normal smooth muscle differentiation. If so, our failure to observe mutations of *COL4A6* alone in AS-DL might merely reflect the small sample size. Linkage studies (*21*) have shown that X-linked AS mutations are all tightly linked to markers in the Xq22 region where both *COL4A5* and *COL4A6* are located. *COL4A6* is the probable site for the ~50% of X-linked AS mutations that have not been found in *COL4A5*. Therefore, the absence of DL in these patients suggests that simultaneous mutation of both *COL4A5* and *COL4A6* is required for the development of DL.

Attention has previously been focused on the role of type IV collagen in adhesion and motility of cells during tumor invasion (*22*). Here we show that constitutional mutations in type IV collagen can result in cell proliferation in benign tumors. Mutations in other BM components, whether constitutional or acquired, may also participate in the pathogenesis of other benign tumors.

REFERENCES AND NOTES

1. R. Timpl, *Eur. J. Biochem.* **180**, 487 (1989).
2. R. J. Butkowski, J. P. M. Langeveld, J. Wieslander, J. Hamilton, B. G. Hudson, *J. Biol. Chem.* **262**, 7874 (1987); K. E. Morrison, M. Mariyama, S. T. Reeders, *Am. J. Hum. Genet.* **49**, 545 (1991); S. Gunwar, J. Saus, M. E. Noelken, B. G. Hudson, *J. Biol. Chem.* **265**, 5466 (1990); M. Mariyama, R. Kalluri, B. G. Hudson, S. T. Reeders, *ibid.* **267**, 1253 (1992); Y. Kamagata, M.-G. Mattei, Y. Ninomiya, *ibid.*, p. 23753; T. Pihlajaniemi, E.-R. Pohjolainen, J. C. Myers, *ibid.* **265**, 13758 (1990).
3. S. L. Hostikka *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1606 (1990).
4. J. Zhou, J. M. Hertz, A. Leinonen, K. Tryggvason, *J. Biol. Chem.* **267**, 12475 (1992).
5. R. J. Butkowski, J. Wieslander, M. Kleppel, A. F. Michael, A. J. Fish, *Kidney Int.* **35**, 1195 (1989); G.-Q. Shen *et al.*, *Connect. Tissue Res.* **24**, 289 (1990); M. Desjardins, F. Gros, J. Wieslander, M. C. Gubler, M. Bendayan, *Lab. Invest.* **63**, 637 (1990); J. R. Sanes, E. Engvall, R. Butkowski, D. D. Hunter, *J. Cell Biol.* **111**, 1685 (1990).
6. R. Soininen, M. Huotari, S. L. Hostikka, D. J. Prockop, K. Tryggvason, *J. Biol. Chem.* **263**, 17217 (1988); E. Pöschl, R. Pollner, K. Kühn, *EMBO J.* **7**, 2687 (1988); P. D. Burbelo, G. R. Martin, Y. Yamada, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9679 (1988).
7. M. Mariyama, K. Zheng, T. L. Yang-Feng, S. T. Reeders, *Genomics* **13**, 809 (1992); M. Mariyama and S. T. Reeders, unpublished data.
8. D. F. Barker *et al.*, *Science* **248**, 1224 (1990); J. Zhou *et al.*, *Genomics* **9**, 10 (1991); K. Tryggvason, J. Zhou, S. L. Hostikka, T. Shows, *Kidney Int.* **43**, 38 (1993); K.-O. Netzer *et al.*, *ibid.* **42**, 1336 (1992).
9. P. Cochat *et al.*, *J. Pediatr.* **113**, 339 (1988).
10. J. B. Marshall, A. A. Diaz-Arias, G. S. Bochna, K. A. Vogele, *Am. J. Gastroenterol.* **85**, 303 (1990).
11. C. Antignac *et al.*, *Kidney Int.* **42**, 1178 (1992).
12. S. Gunwar *et al.*, *J. Biol. Chem.* **266**, 15318 (1991).
13. The patients ASDL-1 and ASDL-2 are related females. ASDL-1 had persistent hematuria and esophageal and genital leiomyomas. Her brother had AS with cataracts and hearing loss and died at age 23 after an unsuccessful renal transplantation. He suffered from an undiagnosed swallowing difficulty and "narrow esophagus." Her mother also had unexplained swallowing difficulty. One sister died at age 37 from myosarcoma of the brain. Two other sisters are healthy. Her daughter, ASDL-2, developed hematuria at age 9. Ultrastructural changes in glomerular basement membrane are typical of AS. She has bilateral cataracts and DL. Patients ASDL-3, -4, and -5 have been described previously (*11*). ASDL-3 is a male with biopsy-proven AS, deafness, cataracts from age 2, and symptomatic esophageal leiomyomatosis since age 9. His mother had persistent hematuria and esophageal and genital leiomyomas. ASDL-4 is a male with AS who developed cataracts and symptomatic esophageal leiomyomatosis at age 4. His mother had hematuria and esophageal leiomyomatosis. ASDL-5 had AS, hematuria, and a grossly dilated esophagus.
14. R. L. Juliano and S. Haskill, *J. Cell Biol.* **120**, 577 (1993).
15. R. O. Hynes, *Cell* **69**, 11 (1992).
16. M. Aumailley and R. Timpl, *J. Cell Biol.* **103**, 1569 (1986).
17. M. K. Chelberg, E. C. Tsilibary, A. R. Hauser, J. B. McCarthy, *Cancer Res.* **49**, 4796 (1989).
18. T. K. Borg, K. Rubin, E. Lundgren, K. Borg, B. Öbrink, *Dev. Biol.* **104**, 86 (1984).
19. A. S. Menko and D. Boettiger, *Cell* **51**, 51 (1987).
20. P. Vandenberg *et al.*, *J. Cell Biol.* **13**, 1475 (1991).
21. H. Brunner *et al.*, *Kidney Int.* **34**, 507 (1988); F. A. Flinter, J. S. Cameron, C. Chantler, I. Houston, M. Bobrow, *Lancet* ii, 1005 (1988).
22. S. Aznavoorian, A. N. Murphy, W. G. Stetler-Stevenson, L. A. Liotta, *Cancer* **71**, 1368 (1993).
23. R. Soininen, T. Haka-Risku, D. J. Prockop, K. Tryggvason, *FEBS Lett.* **225**, 188 (1987); S. L. Hostikka, M. Kurkinen, K. Tryggvason, *ibid.* **216**, 281 (1987).
24. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

25. We thank J. Gruen, L. Ollitervo, M. Mariyama, H. Lemmink, and L. Kluytmans for scientific discussion and technical assistance and P. Cochat and B. Roussel for referring patients. Supported by the Sigrid Juselius Foundation, the Academy of Finland, National Institutes of Health, and the Howard Hughes Medical Institute.

22 April 1993; accepted 7 July 1993

Identification of a Structural Glycoprotein of an RNA Virus as a Ribonuclease

Rainer Schneider, Gabriele Unger, Robert Stark, Elisabeth Schneider-Scherzer, Heinz-Jürgen Thiel

One of the three structural glycoproteins of classical swine fever virus (CSFV) is E0, a disulfide-bonded homodimer that induces virus-neutralizing antibodies and occurs in a virion-bound as well as a secreted form. E0 was shown to be similar to a family of fungal and plant ribonucleases. Purified E0 from CSFV-infected cells was a potent ribonuclease specific for uridine and inhibitable by zinc ions.

Members of the genus *Pestivirus*, family Flaviviridae, are causative agents of severe animal diseases. The positive strand RNA genome of pestiviruses encodes one large polypeptide that is processed to the mature viral proteins (*1*, *2*). The CSFV structural glycoprotein E0 forms a disulfide-bonded homodimer with an apparent molecular size of 97 kD (*3*). Each monomer consists of 227 amino acids that correspond to amino acids 268 to 494 of the CSFV polyprotein (*2*, *4*). Accordingly, about one-half of the mature viral protein consists of carbohydrate. The amino acid sequence of E0 lacks a transmembrane anchor, and the glycoprotein is secreted from infected cells (*4*). E0 is also localized at the surface of the virion (*5*); linkage to the virion occurs by an as yet unknown mechanism.

Comparative sequence analysis, including extensive searches for degenerate functional motifs in the deduced primary structure of E0, led to the identification of sequence features characteristic of a family of fungal and plant ribonucleases (RNases). Significant sequence similarity was shared by two regions in the E0 amino acid sequence and the most conserved regions of the extracellular RNases T2 of

R. Schneider and E. Schneider-Scherzer, Department of Biochemistry, Faculty of Natural Sciences, University of Innsbruck, A-6020 Innsbruck, Austria.
G. Unger, R. Stark, H.-J. Thiel, Federal Research Centre for Virus Diseases of Animals, D-72076 Tübingen, Germany.

Table 1. Hydrolysis of various nucleic acids by purified E0 as measured by perchloric acid precipitation (*22*) at 37°C. A value of 100% corresponds to 0.015 A_{260} units min^{-1} ml^{-1}. The relative activities determined for two E0 preparations are separated by slashes. Abbreviations: ds, double-stranded; ss, single-stranded.

Substrate	Relative activity (%)
Yeast RNA	100/100
Escherichia coli ribosomal RNA	51/47
Escherichia coli tRNA	48/53
PolyU	1755/1673
PolyA	2/2
PolyC	0/0
PolyG	0/0
ds DNA	0/0
ss DNA	0/0

Aspergillus oryzae (6), Rh of *Rhizopus niveus* (7), and plant RNases of the self-incompatibility type, such as the stylar glycoprotein of winged tobacco (8) (Fig. 1). Although the sequence similarities were limited to two small regions of E0, they included all of the proposed catalytically important residues (6) [residues 28 to 40 and 71 to 89 of CSFV and bovine viral diarrhea virus (BVDV) (Fig. 1) include His^{70} and His^{132} of T2 RNase]. In addition, the conserved amino acids showed similar spacing in E0 and the RNase family, respectively. These regions of similarity are also highly conserved between E0 and the respective protein of other pestiviruses, that is, gp48 of BVDV (9). These similarities prompted us to investigate whether E0 exhibits RNase activity.

Immunoaffinity purification of E0 from CSFV-infected cells resulted in a homogeneous protein preparation (Fig. 2). Incubation of this preparation with yeast total RNA resulted in a pronounced degradation of the nucleic acids (Table 1). The determined specific activity of 353 absorbance units at 260 nm (A_{260} units) min^{-1} mg^{-1} is within the range reported for the fungal and plant RNases (170 to 3900 A_{260} units min^{-1} mg^{-1}) (*10*). To investigate the possibility that a minor contamination in the E0 preparation was responsible for the measured RNase activity, we applied the same purification procedure to mock-infected cells. The resulting preparation contained no detectable protein, and RNase activity could not be demonstrated. Further evidence for the RNase activity of E0 was obtained by the development of a renaturation procedure for the RNase activity after SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Under reducing and nonreducing conditions the E0 preparation had apparent molecular sizes of 44 or 48 kD and 97 kD, respectively; the latter represents the homodimeric form of the glycoprotein. Renaturation of the protein by removal of detergent, and subsequent measurement of RNase activity in the sliced lanes revealed that the RNase activity resided only in the slices that contained reduced or nonreduced E0 protein (Fig. 2). In both cases, residual activity was additionally detected at the position corresponding to the other molecular forms of E0; this is probably due to incomplete dimerization or reduction, respectively. Thus, even under reducing and nonreducing conditions RNase activity comigrates exclusively with E0. The RNase activity predicted from the amino acid sequence is therefore an intrinsic property of E0.

During optimization of the renaturation procedure we noted that pretreatment of E0 with up to 1 M dithiothreitol did not affect the RNase activity. Thus, E0 RNase catalysis is apparently not dependent on the existence of disulfide-bonded dimers. RNase activity was optimal at pH 6.0 to 6.5 and at 55°C and exhibited 90% activity at 65°C. It did not degrade single- or double-stranded DNA, showed a pronounced substrate specificity for uridine, and reduced activities for RNA substrates with high amounts of secondary structure (Table 1). The enzymatic activity was not affected by Ca^{2+}, Mg^{2+}, or EDTA (1 mM) and showed a 50% inhibition by Mn^{2+} at 250 μM. A 50% inhibition could also be achieved by Zn^{2+} concentrations as low as 15 μM. Since this is within the range of physiological Zn^{2+} levels (*11*), inhibition of E0 RNase activity might be possible in vivo.

Our results raise several questions. First, how does the virus avoid degradation of its own RNA genome while expressing a potent RNase? Initial speculation may center on specific protective modifications of the viral RNA. However, escape from genome destruction could simply be due to the continuous separation of RNase and viral RNA. Pestiviruses probably enter the cell either by fusion with the plasma membrane or by receptor-mediated endocytosis. In either case the viral RNA may not be exposed to the structural surface glycoprotein E0. The intracellular localization of newly synthesized E0 should be limited to the lumen of endoplasmic reticulum and Golgi apparatus because an internal signal sequence most likely mediates membrane translocation of the nascent polypeptide chain (*4*). It remains to be determined whether viral RNA is accessible to its own RNase.

A second question concerns the possible function of an RNase residing in a structural protein of an RNA virus. The function of RNases likely involves a modulatory or cytotoxic action mediated by the specific degradation of cellular RNA components. In the case of E0, an effect on distant noninfected cells seems proba-

Box A

```
CS   28 SLHGIWPEkiCkG
BV   28 SLHGIWPEkiCtG
RH   60 TLHGLWPDk-CsG
T2   68 TIHGLWPDn-CdG
St   51 TIHGLWPDn----
           *
```

Box B

```
CS   71 LqrHEWNKHGwCnwynIDP
BV   71 LqrHEWNKHGwCnwynIEP
RH  117 FwsHEWsKHGtC-vstyDP
T2  124 FweHEWNKHGtC-intIEP
St  105 FwkdEYvKHGtC-------
                  *
```

Fig. 1. Sequence similarities between E0 of CSFV and fungal and plant RNases. Alignments of the most conserved regions (homology boxes A and B) within the family of known RNases and corresponding regions in E0 are shown. These similarities were found by visual inspection and by application of the program FIND of the GCG program package (*19*) with highly degenerate sets of consensus sequences (*20, 21*) of various protein families, using the NBRF database. Residues identical to all RNases of this type are shown in uppercase bold letters; similar residues or residues not conserved in all these RNases are shown in uppercase letters. Dashes indicate gaps inserted to maximize the homology. CS, CSFV E0; BV, BVDV gp48; RH, RNase Rh of *Rhizopus niveus*; T2, RNase T2 of *Aspergillus oryzae*; St, stylar glycoprotein of winged tobacco. Asterisks indicate residues reported to be involved in catalysis (*6*). Single-letter abbreviations for the amino acid residues are as follows: C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

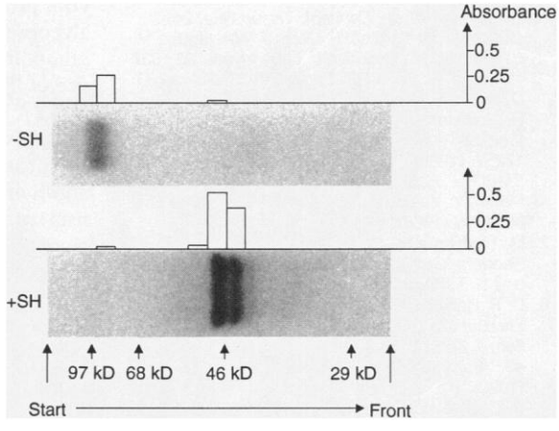

Fig. 2. Identification of E0 RNase activity after SDS-PAGE. Immunoaffinity-purified E0 was separated by electrophoresis in reduced (+SH) or nonreduced (−SH) form. After SDS-PAGE, lanes were either stained with Coomassie blue or sliced and renatured by removal of SDS through several washing steps with renaturing buffer [40 mM tris-acetate (pH 6.5), 5 mM DTT, 0.5 mM EDTA]. RNase activities were measured by a modification of the perchloric acid precipitation method (*22*). The gel slices were homogenized and incubated with 80 μg of polyU in renaturing buffer (final volume, 100 μl) with shaking for 2 hours at 55°C. The incubation mixtures were then centrifuged to remove the homogenized gel, and an equal volume of ice-cold 1.2 M perchloric acid containing 20 mM lanthanum sulfate was added to the supernatant. After 15 min on ice, the reaction mixture was centrifuged at 15000*g* for 15 min at 4°C. The supernatant was then diluted threefold and its absorbance measured at 260 nm. The absorbance values (given in A_{260} units per slice per 2 hours) are shown in histograms above the respective Coomassie-stained lanes. Background levels of RNase activity (<0.01 A_{260} units per slice per 2 hours) are not indicated.

ble, because it is secreted in significant amounts from the infected cells (*4*) and can also easily be demonstrated in the sera of infected animals (*12*). Secreted members of the RNase superfamily are the focus of growing attention as a result of their involvement in diverse biological processes, including organogenesis, cancer, and immunomodulation (*13–15*). Cytotoxic action of E0 against the host immune system would be in agreement with known properties of other RNases, such as pancreatic-type bovine seminal RNase (*13, 16, 17*) and the S-RNases of plants (*10*). In vivo studies on the biological functions of E0 will require either defined E0 mutants of CSFV or large amounts of active protein from heterologous expression systems.

The structural E0 protein is apparently important for virus growth for several reasons: (i) E0 represents a well-conserved structural glycoprotein within the *Pestivirus* genus; (ii) immunization with E0 alone mediates a protective immune response against lethal CSF (*18*); and (iii) E0 has retained its intrinsic catalytic activity during evolution despite its large divergence from classical RNases. Because RNases are among the best studied proteins with respect to their structure and catalytic mechanistics, the E0 RNase activity should serve as a convenient target for the development of antiviral drugs. The unexpected finding that a viral glycoprotein is a potent RNase will thus not only further our understanding on the pathogenesis of diseases caused by mammalian viruses but may also provide us with the means to combat them.

REFERENCES AND NOTES

1. M. S. Collett, R. Larson, C. Gold, D. K. Anderson, A. F. Purchio, *Virology* **165**, 191 (1988).
2. G. Meyers, R. Rümenapf, H.-J. Thiel, *ibid.* **171**, 555 (1989).
3. H.-J. Thiel, R. Stark, E. Weiland, T. Rümenapf, G. Meyers, *J. Virol.* **65**, 4705 (1991).
4. T. Rümenapf, G. Unger, J. H. Strauss, H.-J. Thiel, *ibid.* **67**, 3288 (1993).
5. E. Weiland, R. Ahl, R. Stark, F. Weiland, H.-J. Thiel, *ibid.* **66**, 3677 (1992).
6. Y. Kawata, F. Sakiyama, H. Tamaoki, *Eur. J. Biochem.* **176**, 683 (1988).
7. H. Horiuchi *et al.*, *J. Biochem.* **103**, 408 (1988).
8. M. A. Anderson *et al.*, *Nature* **321**, 38 (1986).
9. M. S. Collett, R. Larson, S. K. Belzer, E. Retzel, *Virology* **165**, 200 (1988).
10. B. A. McClure, J. E. Gray, M. A. Anderson, A. E. Clarke, *Nature* **347**, 757 (1990).
11. T. J. B. Simons, *J. Membr. Biol.* **123**, 63 (1991).
12. R. Stark and H.-J. Thiel, unpublished data.
13. S. A. Benner and R. K. Allemann, *Trends Biochem. Sci.* **14**, 396 (1989).
14. G. D'Alessio, A. DiDonato, A. Parente, R. Piccoli, *ibid.* **16**, 104 (1991).
15. G. D'Alessio, *Trends Cell Biol.* **3**, 106 (1993).
16. M. Tamburini *et al.*, *Eur. J. Biochem.* **190**, 145 (1990).
17. S. Vescia, D. Tramontano, G. Augusti-Tocco, G. D'Alessio, *Cancer Res.* **40**, 3740 (1980).
18. Preliminary results from immunizations of pigs with recombinant E0 show a protective influence in lethal CSF.
19. J. Devereux, P. Haeberli, O. Smithies, *Nucleic Acids Res.* **12**, 387 (1984).
20. R. Schneider and M. Schweiger, *Oncogene* **6**, 1807 (1991).
21. R. Schneider, *Trends Biochem. Sci.* **17**, 468 (1992).
22. P. B. Brown and T. H. D. Ho, *Plant Physiol.* **82**, 801 (1986).

23. Supported by the Bundesminister für Forschung und Technologie and Intervet BV (project 0319028A).

8 April 1993; accepted 6 July 1993

Lack of N Regions in Antigen Receptor Variable Region Genes of TdT-Deficient Lymphocytes

Toshihisa Komori, Ami Okada, Valerie Stewart, Frederick W. Alt

During the assembly of immunoglobulin and T cell receptor variable region genes from variable (V), diversity (D), and joining (J) segments, the germline-encoded repertoire is further diversified by processes that include the template-independent addition of nucleotides (N regions) at gene segment junctions. Terminal deoxynucleotidyl transferase (TdT)–deficient lymphocytes had no N regions in their variable region genes, which shows that TdT is responsible for N region addition. In addition, certain variable region genes appeared at increased frequency in TdT-deficient thymocytes, which indicates that N region addition also influences repertoire development by alleviating sequence-specific constraints imposed on the joining of particular V, D, and J segments.

Immunoglobulin (Ig) and T cell receptor (TCR) variable region genes are created by the assembly of V, D, and J segments [V(D)J recombination] in developing lymphocytes. V(D)J recombinase activity is targeted by conserved recognition sequences (RS's) that flank each germline gene segment. The reaction involves recognition of RS's, introduction of double-strand breaks at RS-coding junctions; potential loss, addition, or both of nucleotides at coding junctions; and polymerization-ligation activities to complete joining (*1, 2*). The initiation of the reaction is probably mediated by one or two tissue-specific components, whereas most other events are carried out by more generally expressed activities (*2*).

Variable region diversity is created both by combinatorial assortment of V, D, and J segments as well as by the loss or addition of nucleotides at their junctions. Additions fall into two categories: template-dependent (P nucleotides) (*3, 4*) and template-independent (N regions) (*5*). N region addition has been hypothesized to be effected by TdT (*5*), which can add deoxynucleotides to available 3′ ends (*6*). In support of this notion, TdT is found in immature lymphocytes (*7*) and leukemic cells (*8*), but not in nonlymphoid cells. Likewise, the abundance of N regions in V(D)J junctions in adult as compared to fetal lymphocytes also correlates with substantial TdT expression in precursors of the former but not the latter populations (*4, 9–11*). N region addition to V(D)J junctions in cell lines also has been correlated with TdT expression (*12–14*). However, some cell lines that lacked readily detectable TdT activity were found to add N regions (*13, 14*), leading to speculation that N regions also may be added by other mechanisms (*15*).

To unequivocally evaluate the role of TdT in V(D)J recombination and repertoire development, we used gene-targeted mutation to generate chimeric mice in which all mature lymphocytes develop from precursors lacking TdT expression. We prepared a targeting vector that eliminated the TdT promoter and first exon and allowed for both positive and negative selection (*16*) (Fig. 1A). We generated 43 independent TdT knockout ($TdT^{+/-}$) clones of the CCE line of embryonic stem (ES) cells (Fig. 1B; representative data are shown). We then selected with increased G418 (*17*) and obtained independent homozygous TdT knockout clones ($TdT^{-/-}$) from four different $TdT^{+/-}$ clones (Fig. 1B; representative clones are shown).

All mature lymphocytes in chimeras formed by injection of ES cells into RAG-2–deficient blastocysts derive from the injected ES cells [RAG-2–deficient blastocyst complementation (*18*)]. Complementation of RAG-2–deficient blastocysts with either $TdT^{-/-}$ ES cells or $TdT^{+/-}$ ES cells generated chimeras with substantial numbers of ES cell–derived B and T cells in primary and peripheral lymphoid organs (Fig. 1, B and C). However, we did not detect either

T. Komori and V. Stewart, Howard Hughes Medical Institute, Children's Hospital, Boston, MA 02115.
A. Okada, Department of Genetics and Center for Blood Research, Harvard University Medical School, Boston, MA 02115.
F. W. Alt, Howard Hughes Medical Institute, Children's Hospital, Boston, MA 02115, and Department of Genetics and Center for Blood Research, Harvard University Medical School, Boston, MA 02115.