Ku80: Product of the *XRCC5* Gene and Its Role in DNA Repair and V(D)J Recombination

Guillermo E. Taccioli,* Tanya M. Gottlieb,* Tracy Blunt, Anne Priestley, Jocelyne Demengeot, Ryushin Mizuta, Alan R. Lehmann, Frederick W. Alt, Stephen P. Jackson, Penny A. Jeggo*†

The radiosensitive mutant *xrs-6*, derived from Chinese hamster ovary cells, is defective in DNA double-strand break repair and in ability to undergo V(D)J recombination. The human *XRCC5* DNA repair gene, which complements this mutant, is shown here through genetic and biochemical evidence to be the 80-kilodalton subunit of the Ku protein. Ku binds to free double-stranded DNA ends and is the DNA-binding component of the DNA-dependent protein kinase. Thus, the Ku protein is involved in DNA repair and in V(D)J recombination, and these results may also indicate a role for the Ku–DNA–dependent protein kinase complex in those same processes.

One of the most lethal lesions that can occur in a cell is a DNA double-strand break, because it disrupts the integrity of the DNA molecule. Mammalian cell mutants that are defective in the repair of DNA double-strand breaks (dsbs) share the common phenotype of high sensitivity to ionizing radiation and include the hamster mutants *xrs* [ionizing radiation (IR) complementation group 5], XR-1 (IR group 4), and V3, and the murine *scid* cell line containing the mutation (*1*). These mutants represent three distinct complementation groups: Human chromosomes 2, 5, and 8 correct *xrs*, XR-1, and the murine *scid* cell line, respectively (*2–5*). V3 belongs to the same complementation group as *scid* (*6*).

During T and B cell differentiation, the variable regions of the immunoglobulin and T cell receptor molecules are assembled from component V, D, and J gene segments by means of a site-specific recombination process referred to as V(D)J recombination. V(D)J recombination is initiated by a site-specific dsb between two recombining variable-region gene segments and their flanking recombination recognition sequences (RSs). The two coding ends combine to form a coding join, the ends of which frequently bear small modifications, whereas the RS ends unite to form RS joins that are normally recombined precisely (*7*). In addition to radiosensitivity, all the mutants mentioned above show an impaired ability to carry out V(D)J recombination (*6*, *8–10*). Other radiosensitive cell lines that can rejoin dsbs (such as ataxia-telangiectasia cells) have normal abilities to carry out V(D)J recombination (*8*, *10*, *11*). Therefore, a defect in V(D)J recombination is not characteristic of all radiosensitive mutants but is associated specifically with defects in dsb rejoining. These results suggest a mechanistic overlap between these two processes and indicate that the site-specific dsbs introduced during V(D)J recombination may be processed, at least initially, by a mechanism similar to that operating on dsbs induced by radiation.

Ionizing-radiation group 5 includes six *xrs* mutants derived from Chinese hamster ovary (CHO) cells, as well as a single cell line, XR-V15B, derived from the hamster cell line V79 (*12*). To clone the gene that is defective in these mutants (designated *XRCC5*), we have pursued a positional cloning approach and have localized a complementing gene to human chromosome region 2q33-35 (*3*, *13*). The gene encoding the 80-kD subunit of the Ku protein also localizes to 2q33-35 (*14*). Ku is an abundant nuclear protein identified originally as an autoantigen recognized by sera from various autoimmune patients and is a heterodimer composed of two subunits with molecular weights of 70 and 80 kD (*15*). The Ku protein binds to free double-stranded DNA ends, which suggests a possible role in DNA repair and recombination (*16*). Our data show that Ku80 is the product of the *XRCC5* gene.

Our strategy for cloning *XRCC5* involved generating a series of hamster-human hybrid cell lines derived from XR-V15B and from *xrs-6* mutants containing different regions of human chromosome 2 (*13*). In some of these, radiation resistance was restored (complementing) and in others it was not (noncomplementing). One specific marker, TNP-1, which maps to 2q33-35, was retained in all complementing hybrids and was absent in noncomplementing hybrids, which suggests that it is tightly linked to *XRCC5* (*13*, *17*). In this study, we examined both complementing and noncomplementing hybrids containing small regions of chromosome 2, including the hybrid D2-X-38, which has a single fragment of approximately 3 Mbp mapping to the region 2q33-35 (Fig. 1). (*17*).

To determine whether the human gene encoding Ku80 might be mediating the complementation in our hybrids, we examined their genomic DNA by Southern (DNA) hybridization using human Ku80 complementary DNA (cDNA) as a probe (Fig. 2A). Although some cross-hybridization to hamster sequences was evident in all hybrids, human-specific bands were detected only in the complementing hybrids. To examine a wider range of hybrids, primers from a small part of the human Ku80 3′ untranslated region (UTR), which amplified Ku80 sequences from genomic DNA, were used in polymerase chain reaction (PCR) assays. No product was produced with these primers in the presence of hamster DNA. Out of 20 hybrids examined, including subclones derived from D2, the Ku80 gene cosegregated with TNP-1 in all cases. We also tested for the presence of the Ku80 protein in whole-cell extracts of complementing and noncomplementing hybrids by protein immunoblotting with antibody S10B1, which recognizes human but not rodent Ku80 (Fig. 2B). The p80 subunit of

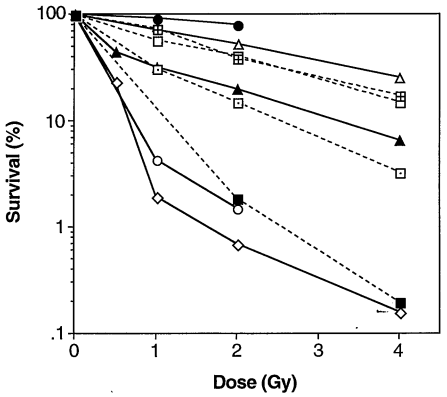

Fig. 1. Cell survival after ionizing radiation of hybrids and transfectants. Ionizing radiation was done as described (*2*). Plasmid DNA (5 μg; vector alone or carrying the Ku80 coding sequence) was transfected into *xrs-6* cells by the polybrene method (*28*). Stable transfectants were selected with hygromycin (600 μg/ml). Filled circles, CHO-K1; open triangles, H22; filled triangles, D2-X-38; open circles, *xrs-6*; open diamonds, D2-X-38D; open boxes, open boxes with crosses, and open boxes with dots, three transfectants expressing Ku80; filled boxes, cells transfected with p220LTR.

G. E. Taccioli, J. Demengeot, R. Mizuta, F. W. Alt, Howard Hughes Medical Institute, Children's Hospital, and Department of Genetics, Harvard University Medical School, Boston, MA 02115, USA.
T. M. Gottlieb and S. P. Jackson, Wellcome/CRC Institute and Department of Zoology, Cambridge University, Tennis Court Road, Cambridge CB2 1QR, UK.
T. Blunt, A. Priestley, A. R. Lehmann, P. A. Jeggo, Medical Research Council, Cell Mutation Unit, University of Sussex, Brighton, BN1 9RR, UK.

*Joint first authors. This work represents an equal contribution from the three participating laboratories.
†To whom correspondence should be addressed.

Ku was detected only in the complementing hybrids.

During the course of this study, it was reported that IR group 5 mutants lack a DNA-end–binding activity (*18*). We therefore investigated whole-cell extracts from *xrs*-6 mutants and from parental CHO-K1 cells for DNA-binding proteins, using the electrophoretic mobility shift assay (EMSA). When compared to the extract from CHO-K1 cells, the extract from *xrs*-6 mutants is missing a band corresponding to a specific DNA-binding protein (band K) (Fig. 3A). Band K is competed away effectively by the addition of oligonucleotides but not of circular DNA, which indicates that this protein, like Ku, interacts with DNA ends (*19*). The similarly shifted band present in the human cell extract (HeLa cell line, Fig. 3A) migrates with a slightly different mobility, presumably reflecting differences between the human and hamster proteins. Electrophoretic mobility shift assays with extracts from complementing hybrids D2-X-38 and H22 showed restoration of band K (Fig. 3A) that had the mobility of the human rather than the hamster band, which supports the contention that it results from expression of the human gene encoding Ku80. The intensity of this band is fainter in hybrid D2-X-38 than in H22, which is consistent with the instability of the former hybrid and its corresponding lower radioresistance (Fig. 1 and Fig. 3A).

To determine whether band K indeed contains the Ku protein, we incubated extracts with monoclonal antibodies (mAbs) to either the 70- or 80-kD subunit of Ku before carrying out EMSA. A mAb to Ku80, S10B1, does not recognize rodent Ku80 efficiently. When extracts from a complemented hybrid, H22, were treated with this mAb, the band K was removed, which showed that it contained human Ku80 (Fig. 3B). The mAb N3H10 recognizes the 70-kD subunit of both human and hamster proteins. Band K was depleted from all extracts after treatment with N3H10 (Fig. 3A). In contrast, boiled N3H10, boiled S10B1, and other antibodies did not affect the presence of band K (Fig. 3B) (*19*). Other gel shift complexes (such as those containing Sp1) remained intact in the presence of N3H10 (*19*). Taken together, these results verify that the DNA-end–binding protein that was lacking in *xrs*-6 cells and restored in complementing hybrids contained both the 70- and 80-kD subunits of the heterodimeric Ku complex.

The mapping and biochemical data suggested that Ku80 might be the product of the *XRCC5* gene. To prove this, we introduced the gene encoding Ku80 into *xrs*-6 cells by DNA transfection and assayed for complementation of the defect in V(D)J recombination and for radiosensitivity. To assay for V(D)J recombination in these nonlymphoid cell lines, we used a transient DNA transfection assay (*20*) in which the recombination activating genes *RAG1* and *RAG2* (*21*) were cotransfected into the mutant cells (*10*, *22*) with either a control vector or with one carrying the Ku80 open reading frame (ORF) (*23*). The presence of the Ku80 ORF did not affect either the frequency or accuracy of join formation in CHO-K1 cells (Table 1). The *xrs*-6 cells containing the control vector alone showed the decreased frequency of RS and coding joins and increased frequency of incorrect joins that are characteristic of *xrs*-6 cells (Table 1). The *xrs*-6 cells expressing Ku80 regained both the frequency and accuracy of RS join formation in amounts approaching those of wild-type cells (Table 1). The abnormal RS joins analyzed from *xrs*-6 cells transfected with Ku80 had deletions of less than two nucleotides, which is in sharp contrast with the large deletions observed in junctions from *xrs*-6 cells (*10*) or from *xrs*-6 cells transfected with vector alone. The frequency of coding join formation was increased fivefold in *xrs*-6 cells transfected with Ku80, which represents partial recov-

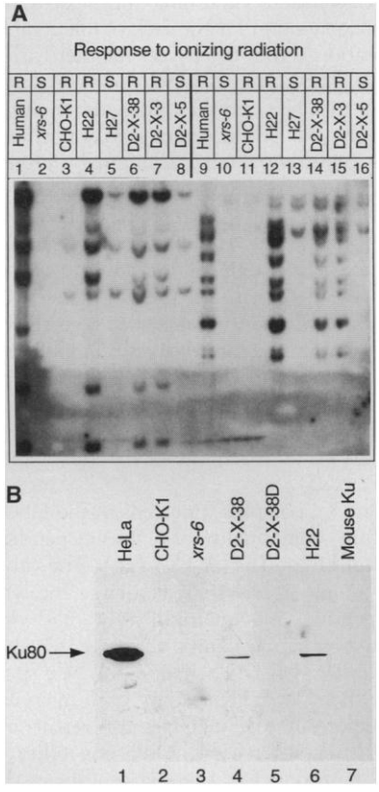

Fig. 2. (**A**) Southern hybridization analysis of hybrid clones. DNA samples were digested by Hind III (lanes 1 through 8) or Eco RI (lanes 9 through 16) and analyzed by Southern hybridization by means of standard protocols. The probe was the Ku80 coding sequence derived by PCR from cDNA encoding Ku80 and radiolabeled by nick translation. The derivation of hybrids is given in the text and in (*17*). R, greater than 20% survival at 2 grays (Gy); S, less than 5% survival at 2 Gy. (**B**) Protein immunoblot analysis of hybrids by means of an antibody to Ku80. Analyses were done as described previously (*26*). Samples of whole-cell extracts (40 μg) were electrophoresed on an 8% SDS-polyacrylamide gel, then analyzed by protein immunoblotting with a mAb (S10B1) specific for the human Ku80 subunit (*29*). Extracts used were HeLa (lane 1), CHO-K1 (lane 2); *xrs-6* (lane 3); complementing hybrid D2-X-38 (lane 4); noncomplementing hybrid D2-X-38D (lane 5); complementing microcell hybrid H22 (lane 6); and purified mouse Ku (lane 7).

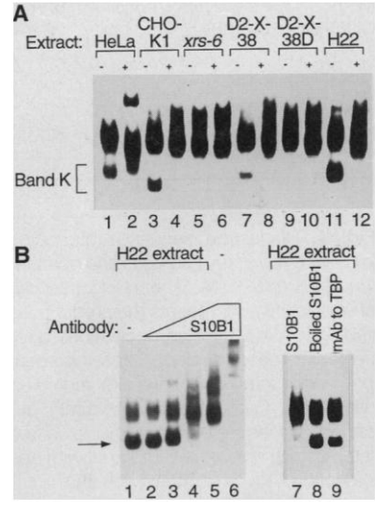

Fig. 3. The DNA-binding protein absent in *xrs-6* cells and restored in complementing hybrids contains both subunits of Ku. (**A**) Extracts were prepared by a modification of the method of Scholer *et al.* (*30*). Extracts (10 μg) were preincubated at 4°C for 10 min either in the absence (odd-numbered lanes) or presence (even-numbered lanes) of 0.5 μg of antibody N3H10 that recognized the 70-kD subunit of Ku. Next, 20 fmol of [α-^{32}P]-labeled double-stranded oligonucleotide M1/M2 (*26*) was added and, after a further incubation at 30°C for 10 min, DNA-protein complexes were resolved on a 4% native polyacrylamide gel. Extracts used were from: HeLa (lanes 1 and 2); CHO-K1 (lanes 3 and 4); *xrs-6* (lanes 5 and 6); D2-X-38 (lanes 7 and 8); D2-X-38D (lanes 9 and 10); and H22 (lanes 11 and 12). The protein-DNA complex observed in extracts from parental but not mutant cells is indicated by a bracket (band K). (**B**) Assays were done as above with 10 μg of extract derived from hybrid H22. Preincubations were in the presence of the following: no antibody (lane 1); 0.02 μl, 0.1 μl, 0.5 μl, or 2.5 μl of mAb to Ku80 S10B1 (lanes 2 through 5, respectively); 0.5 μl S10B1 (lane 7); 0.5 μl boiled S10B1 (lane 8); and 0.5 μl mAb to TBP (lane 9). Preincubation reaction 6 contained 2.5 μl of S10B1 alone. Antibodies to Ku70 (N3H10) and Ku80 were as described (*29*) and the antibody to TBP was MBP-6 (*31*). Heat treatment of antibodies was accomplished by boiling for 5 min and taking the supernatant after a 1-min centrifugation.

ery toward the amounts seen in CHO-K1 cells. The analysis of the coding sequence junctions in complemented cells showed a dramatic change, with deletions ranging from 3 to 12 nucleotides, which is close to the normal range (*10*) (Fig. 4). In contrast, we did not recover any coding joins from noncomplemented *xrs-6* cells. We transiently introduced the p220LTR vector carrying the Ku80 coding sequence into *xrs-6* cells and obtained similar complementation to that observed with the pcDNA1/AmpKu80 vector. As controls, we also introduced the Ku70 coding sequence into *xrs-6* cells and the pcDNA1/AmpKu80 vector into XR-1 cells; in neither case was the defect in the mutant corrected (Table 1).

To analyze complementation of the radiosensitivity characteristic of *xrs-6* cells, the p220LTR vector alone or carrying the Ku80 ORF was introduced into *xrs-6* cells by DNA transfection, and stable transfectants were selected by use of the *hyg* marker (*23*). Three clones, carrying Ku80 sequences and expressing Ku80 protein (shown by protein immunoblotting), were partially corrected for radiosensitivity similar to that of the complemented hamster-human hybrids (Fig. 1). Eleven clones from transfections with p220LTR vector alone showed the radiosensitivity of *xrs-6* cells (data for one clone are shown in Fig. 1). To verify that complementation of the defects in V(D)J recombination and radiosensitivity result from the same genetic event, we analyzed two clones that were stably transfected with Ku80 for V(D)J recombination and observed concomitant correction of this defective phenotype. Similar correction of both defects was also achieved with stable transformants obtained with the pcDNA1/AmpKu80 vector. Therefore, we conclude that expression of the Ku80 ORF results in correction of the radiosensitivity of *xrs-6* cells and the defects in V(D)J recombination.

Our results demonstrate that Ku80 is the product of the *XRCC5* gene and that the Ku protein is necessary for the processing of dsbs induced by DNA-damaging agents and of site-specific dsbs introduced during V(D)J recombination. Ku most likely serves the same function in both processes. Once bound to DNA, the Ku protein can translocate along the DNA, coating it with evenly spaced multimeric complexes (*16*). During V(D)J recombination, the extent of exonuclease digestion appears to be finely controlled. One possibility, therefore, is that the Ku protein complex acts to prevent nuclease digestion of dsbs [either radiation-induced or introduced during V(D)J recombination]. A precedent for a protective role for a double-stranded DNA-end–binding activity against exonuclease activity is seen in the gam protein of phage Mu (*24*). Another possibility, not mutually exclusive with the first, is that Ku helps to promote normal dsb rejoining or recombination processes, possibly by holding the DNA ends in a required conformation or by activating downstream activities.

Neither the defect in V(D)J recombination nor radiosensitivity was restored to parental levels. A similar amount of radioresistance, representing partial recovery, was observed in all complementing hamster-human hybrid cells bearing chromosome 2, as well as in transfectants expressing the Ku80 ORF. One explanation for the lack of full complementation is that the hybrid hamster Ku70–human Ku80 complex is not completely functional in DNA-end–binding activity. Alternatively, the hybrid complex may not function effectively in additional protein interactions required to execute V(D)J recombination and dsb repair. At least two additional proteins, the products of the genes that are defective in XR-1 and in the *scid* cell line, are required for these processes (*1*, *8*–*10*). Ku70 does not appear to be defective in either of these; first, because complementing genes map to chromosomes 5 and 8, respectively (*5*), while Ku70 maps to chromosome 22 (*14*); and second, because both appear to be proficient in DNA-end binding (*18*, *19*, *25*).

The Ku complex functions as the DNA-binding component of DNA-dependent protein kinase (DNA-PK) (*26*). The other component of DNA-PK is a large catalytic polypeptide of approximately 350 kD (p350), which becomes activated when it associates with DNA-bound Ku. We speculate that DNA-PK activity itself may play an important role in DNA dsb repair and V(D)J recombination. One possibility is that the product of the *scid* gene or of the gene defective in XR-1 could be either p350 itself or another DNA-PK–associated polypeptide. Thus far, it has been an enigma why the catalytic subunit of the DNA-PK complex is so large. Although other possibilities exist, it may be that this huge polypeptide provides a framework around which other repair and recombination enzymes are recruited to the complex. DNA-PK phosphorylates a number of potentially relevant factors in vitro, including transcription factors, such as Sp1 and p53, and topoisomerase II, which can nick hairpin structures (*26*, *27*).

Our demonstration that Ku80 is the product of the *XRCC5* gene will aid in examining the mechanism of DNA dsb repair and V(D)J recombination. Characterization of the role of Ku and potentially of DNA-PK and other associated factors should ultimately provide further insights

Table 1. Analysis of signal and coding join formation in CHO cell lines. Abbreviations are as follows: wt, wild-type; NA, not applicable; ND, not determined (*22*).

Cell line	Expression vector	pJH200 (RS sequences)			pJH290 (Coding sequences)	
		Amp^RCam^R/Amp^R	Percent	Correct joins (%)	Amp^RCam^R/Amp^R	Percent
CHO-K1 (wt)	pcDNA1/Amp	64/10950	0.58	100	59/17475	0.34
Mock	pcDNA1/Amp	0/10480*	<0.009	NA	0/17880*	<0.005
CHO-K1	Ku80	259/43575	0.59	100	321/91500	0.35
xrs-6	pcDNA1/Amp	69/128250	0.05	0	29/135750	0.02
xrs-6	Ku80	186/39750	0.46	91	128/11550	0.11
xrs-6	Ku70	60/832505	0.07	0	ND	
XR-1	Ku80	63/1222500	0.005	10	ND	

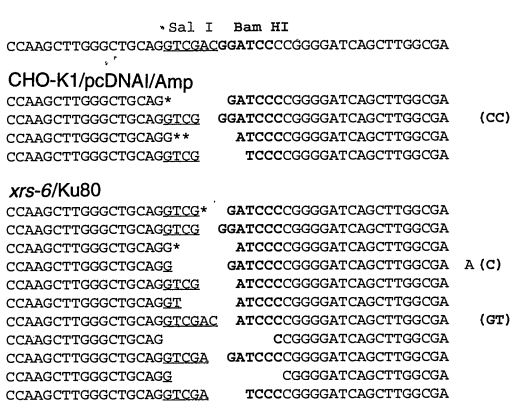

Fig. 4. Nucleotide sequence of coding joins recovered from parental and complemented *xrs-6* cells. The nucleotide sequence shown represents the product of a coding join in which no nucleotides have been deleted or added. Gaps represent lost sequences, and asterisks represent bases that cannot be unequivocally assigned. Bases inserted at the joins are listed at the right of the figure and, with one exception, are consistent with the presence of P elements only (*32*). No coding joins were recovered from *xrs-6* cells.

into the mechanisms involved in these processes.

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17. XR-V15B/H22 (D2) is a complementing hybrid that contains two closely located but independent regions of chromosome 2, mapping to 2q36 (including the marker GNT1) and to 2q33-35 (including the marker TNP-1 (*13*). Subclones derived from D2 segregated the markers GNT1 and TNP-1 independently, and in every subclone examined (more than 20), radioresistance and ability to carry out V(D)J recombination cosegregated with the TNP-1 marker. D2-X-3 is a complementing subclone retaining both fragments. D2-X-5 is noncomplementing and contains only the GNT1 fragment. D2-X-38 is complementing and contains only the TNP-1 fragment. D2-X-38D is a noncomplementing derivative of D2-X-38 and does not contain detectable human sequences. Microcell transfer hybrids H22 and H27 were isolated and characterized previously (*13*). H22, a complementing hybrid, contains the distal third of chromosome 2, including GNT1 and TNP-1; H27, a noncomplementing hybrid, contains three small fragments, including GNT1 but excluding TNP-1. Other microsatellite markers present in D2, D2-X-3, and D2-X-38 are D2S301, D2S164, and D2S137. Markers analyzed and absent in all D2 hybrids are D2S143, D2S128, D2S334, D2S371, D2S317, D2S155, D2S154, D2S157, D2S153, D2S173, D2S163, D2S120, and those listed in (*13*). Primer sequences for the Ku80 3′ UTR are 5′-ACATCACAAGGGCTGCAACTGTCA-3′ and 5′-TCGCTGTGATGCTGGGAGTTCTAA-3′, and PCR conditions were as described for D2S137 (*13*).
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22. The V(D)J recombination assay was done as described (*10*). Substrates pJH200 and pJH290 allowed the analysis of RS and coding joins, respectively (*20*). The percentage of V(D)J recombination was measured by the ratio of rearranged substrate (based on gain of chloramphenicol resistance, Cam^R) to the total amount of recovered substrate (based on ampicillin resistance, Amp^R). The accuracy of join formation and the junction modifications were analyzed by specific restriction endonuclease digestion or by direct DNA sequencing as described (*10*) clones were analyzed. A minimum of 20 clones were analyzed, and 100 for *xrs-6* samples containing Ku80. The results of complementation of *xrs-6* with Ku80 were reproducible in three independent experiments.
23. A 2200–Base pair sequence containing the Ku80 ORF was cloned into the expression vectors pcDNA1/Amp and p220LTR, where it was under the control of the cytomegalovirus and Rous sarcoma virus long terminal repeat (LTR) promoters, respectively. The p220LTR vector also contained a hygromycin gene (*hyg*), which is a dominant selectable marker in mammalian cells.
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Thrombocytopenia in c-*mpl*–Deficient Mice

Austin L. Gurney, Karen Carver-Moore, Frederic J. de Sauvage, Mark W. Moore*

Thrombopoietin (TPO) is a cytokine that is involved in the regulation of platelet production. The receptor for TPO is c-Mpl. To further investigate the role and specificity of this receptor in regulating megakaryocytopoiesis, c-*mpl*–deficient mice were generated by gene targeting. The c-$mpl^{-/-}$ mice had an 85 percent decrease in their number of platelets and megakaryocytes but had normal amounts of other hematopoietic cell types. These mice also had increased concentrations of circulating TPO. These results show that c-*mpl* specifically regulates megakaryocytopoiesis and thrombopoiesis through activation by its ligand TPO.

The proto-oncogene c-*mpl* is a member of the cytokine receptor superfamily with sequence similarity to the erythropoietin receptor and to the granulocyte colony-stimulating factor (G-CSF) receptor (*1*, *2*). Expression of c-*mpl* in normal mice appears to be restricted to hematopoietic tissue, primitive hematopoietic stem cells, megakaryocytes, and platelets, although low levels of expression have been detected in endothelial cells (*2*, *3*). Antisense oligodeoxynucleotides to c-*mpl* selectively inhibit in vitro megakaryocytic colony formation without affecting the growth of erythroid or granulomacrophage colonies, which suggests that c-Mpl and its putative ligand may function in regulating megakaryocytopoiesis (*3*). This hypothesis has been reinforced by the recent purification and cloning of the ligand for c-Mpl (*4*, *5*). This ligand has an NH_2-terminal domain homologous to erythropoietin and a COOH-terminal glycosylated domain unrelated to any known protein. In vitro and in vivo experiments with recombinant Mpl ligand indicate that it has both megakaryocyte colony-stimulating activity and thrombopoietin activity and therefore corresponds to the long-sought platelet growth factor thrombopoietin (TPO) (*4*, *6*, *7*).

To substantiate the involvement of

A. L. Gurney and F. J. de Sauvage, Department of Molecular Biology, Genentech, South San Francisco, CA 94080, USA.
K. Carver-Moore and M. W. Moore, Department of Cell Genetics, Genentech, South San Francisco, CA 94080, USA.

*To whom correspondence should be addressed.