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27. Cyclic ADPR and 8-NH_2 were synthesized and purified on an anion-exchange column with the use of a gradient of trifluoroacetic acid (TFA) as described in (*8*) and (*23*), respectively. The residual TFA content in the purified cADPR samples after lyophilization was determined to be 0.9 to 1.7% of the weight of the sample (*15*). The TFA was removed from some preparations of 8-NH_2 by repurification on an anion-exchange column and elution with a gradient of HCl. Samples were neutralized immediately with KOH. In sea urchin egg homogenates, TFA (Na^+ salt) concentrations up to 4 mM had no effect on Ca^{2+} release. The Na^+-TFA did not release Ca^{2+} by itself nor did it inhibit cADPR from releasing Ca^{2+}. It had no effect on IP_3-induced Ca^{2+} release and did not potentiate Ca^{2+} release induced by either caffeine or divalent cation such as Sr^{2+} (*15*). In intact eggs, microinjection of 1.5 mM Na^+-TFA (intracellular concentration) alone (three out of three injected eggs) or in combination with heparin (4.7 mg/ml) (11 out of 11 injected eggs) did not cause cortical reaction or inhibit cortical reaction when the eggs were fertilized subsequently. The amounts of TFA injected in these controls were at least 80 times the residual amounts of TFA injected with the cADPR or 8-NH_2 samples.
28. This work was supported by grants from the National Institutes of Health (HD17484) to H.C.L. and from the Minnesota Medical Foundation to T.F.W.

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Colocalization of X-Linked Agammaglobulinemia and X-Linked Immunodeficiency Genes

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Mice that bear the X-linked immunodeficiency (*xid*) mutation have a B lymphocyte–specific defect resulting in an inability to make antibody responses to polysaccharide antigens. A backcross of 1114 progeny revealed the colocalization of *xid* with Bruton's agammaglobulinemia tyrosine kinase (*btk*) gene, which is implicated in the human immune deficiency, X-linked agammaglobulinemia. Mice that carry *xid* have a missense mutation that alters a highly conserved arginine near the amino-terminus of the *btk* protein, Btk. Because this region of Btk lies outside any obvious kinase domain, the *xid* mutation may define another aspect of tyrosine kinase function.

CBA/N mice, which bear the *xid* mutation, do not produce antibodies in response to immunization with polysaccharides or hapten-polysaccharide conjugates and have low serum immunoglobulin M (IgM) and IgG3 (*1*). These mice have moderately reduced numbers of B cells, and the B cells that are present have a high surface IgM to IgD ratio, an inability to form colonies in soft agar, and an absence of proliferation in response to surface Ig receptor cross-linkage. These animals do produce normal amounts of antibodies to protein antigens. Genetic characterization showed that *xid* is located between *Tabby* and *Hypophosphatemia* on the mouse X chromosome (*2*). Studies of F_1 female mice heterozygous at *xid* revealed a random pattern of X inactivation in pre-B cells, T cells, and other non-B cells. However, in the mature B cell population, a preponderance of cells have inactivated the *xid*-bearing X chromosome (*3*). Because the *xid* defect affects central aspects of B cell development and function, we set out to identify its molecular nature using a positional cloning approach (*4*).

CBA/N female (*xid/xid*) mice were mated to *Mus musculus* Skive (Skive) (+/Y), an inbred strain that has a high degree of genetic polymorphism relative to standard lab strains (*5*). Matings between the resulting F_1 female mice (+/*xid*) and CBA/N male (*xid*/Y) mice generated 1114 progeny. These "backcross" mice were immunized with trinitrophenyl (TNP)-Ficoll (*2*), and their phenotype (XID or normal) was determined by measurement of both serum IgM antibody to TNP (anti-TNP) and total serum IgG3 by enzyme-linked immunosorbant assay (ELISA) (*6*) (Fig. 1).

Because of the large number of backcross animals, we designed a highly focused strategy for mapping mouse X chromosome DNA markers relative to *xid*. We first analyzed a small, independently generated set of backcross mice using polymorphic markers that span the X chromosome. This analysis confirmed the previous mapping of *xid* (*2*). We therefore identified polymorphisms between CBA/N and Skive at three loci located in this region (DXCrc47, DXWas17, and DXSmh43) (*7*) and developed polymerase chain reaction (PCR)–based assays to facilitate rapid analysis (*8*). Alleles at each of these loci were determined with the use of genomic DNA that was isolated from backcross males (Fig. 2A). This analysis showed that *xid* lies between DXWas17 and DXSmh43. The 46 male backcross DNAs that were recombinant between these two loci were analyzed at several loci containing simple sequence repeats (SSRs): DXMit3, DXMit4, DXMit9, and DXNds2 (Fig. 2A) (*9, 10*).

We used a more restricted analysis to identify informative recombinants among the backcross females. All females were analyzed at three loci: *xid*, DXWas17, and DXMit4 (Fig. 2A). The 20 backcross females found to be recombinant between DXWas17 and DXMit4 were analyzed at DXMit3, DXMit9, and DXNds2 (Fig. 2A). The resultant map (Fig. 2C) is in good agreement with and extends the current map of the mouse X chromosome (*7*).

The X chromosome marker DXMit3 did not recombine with *xid* in any of the 30 backcross animals recombinant between DXWas17 and DXNds2, placing DXMit3 within 0.27 centimorgan (cM) of *xid* at the 95% confidence limits. Thus, DXMit3 defines an excellent entry point for the identification of candidate genes in the *xid* region, as had been our original goal. Our mapping results suggested that *xid* lies in the region of the mouse genome that is homologous to the region of the human genome containing the X-linked agammaglobulinemia (XLA) mutation (*7*). The defective gene in XLA, named *btk* (formerly *atk* or BPK), encodes a cyto-

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plasmic tyrosine kinase (*11, 12*). Because *btk* is expressed in B cell progenitors and in mature B cells but not T cells (*11, 12*), and because *xid* mutations and XLA share some phenotypes (*1*), we mapped the *btk* gene in our backcross mice.

Using a human *btk* cDNA as a probe, we isolated a cDNA clone of the mouse homolog of *btk* and determined its sequence (*13*). Fragments of *btk* cDNA were amplified from RNA isolated from CBA/N B cells by reverse transcription and PCR (*14*). Sequence analysis of these fragments revealed a mutation that resulted in the loss of a Hha I restriction site in the coding sequence of *btk* (below). Restriction analysis of PCR-amplified products from Skive genomic DNA showed that this site was present in the Skive *btk* gene (Fig. 2B). We assayed the informative subset of backcross animals for the presence or absence of this Hha I polymorphism. We observed complete cosegregation of the Skive allele of *btk* and immunocompetence. Thus, *xid*, DXMit3, and *btk* are all located in the same nonrecombinant interval defined by the 1114 backcross progeny we analyzed.

Based on the genetic proximity of DXMit3 and *btk*, large genomic clones, such as those contained in yeast artificial chromosome (YAC) libraries, ought to contain both sequences. We used primers that amplified DXMit3 to isolate eight unique YAC clones, two from a mouse genomic library that contains clones with an average insert size of 250 kb (*15*) and six from a similar library in which the average insert size is 680 kb (*16*). Using a PCR assay (*8*), we found that all eight YAC clones contained the *btk* gene, confirming physical linkage of DXMit3 and *btk*.

The cytosine to thymidine mutation that resulted in the Hha I restriction site polymorphism also converted Arg^{28} of Btk (*11, 12*) to Cys^{28} (Fig. 3). To determine whether this mutation existed in the isogenic, immunocompetent strain, CBA/CaHN (*17*), and to investigate whether any other mutations existed in the coding sequence, we did a side by side analysis of the sequence of the *btk* cDNA from CBA/N and CBA/CaHN B cells (*14*). The cytosine to thymidine transition was the only difference between CBA/N and CBA/CaHN *btk* cDNA within the entire 1977-nucleotide coding region.

Because the mutation in the *btk* gene of XID is located in a region of the protein that lies outside the known functional domains of tyrosine kinases, we determined whether this arginine was evolutionarily conserved. We amplified by PCR this region of *btk* from genomic DNA of BALB/c, C57BL/6, and *Mus spretus* mice (*8*). Using Hha I, we found that the *btk* gene of each strain contains Arg^{28}. We also amplified

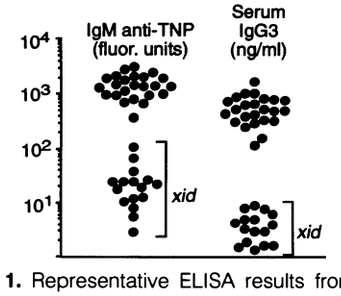

Fig. 1. Representative ELISA results from 40 backcross animals. Eight-week-old backcross animals were injected intraperitoneally with 10 μg of trinitrophenyl (TNP)-Ficoll. One week later, serum was collected and analyzed by ELISA (*6*). Brackets indicate mice typed as expressing the XID phenotype.

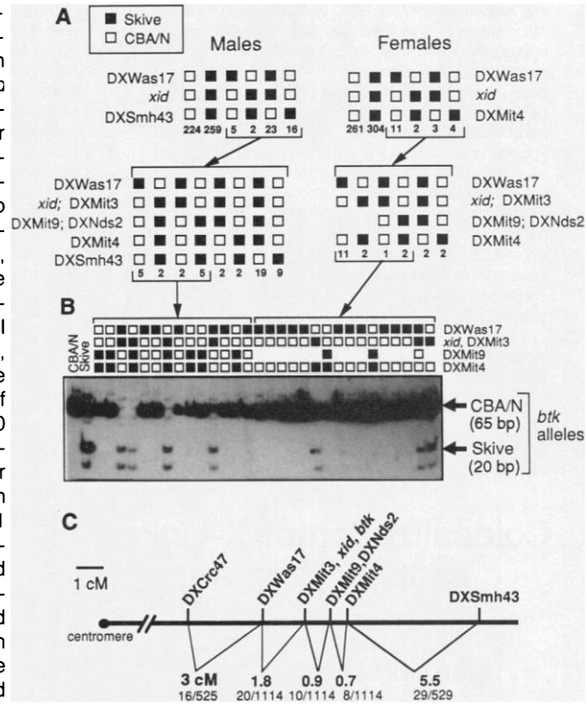

Fig. 2. Mapping results from backcross analysis. (**A**) Haplotype results. Number of individuals of each haplotype are indicated. The *xid* alleles were determined as described in Fig. 1. Alleles at other loci were determined by PCR, dot-blotting and allele-specific oligonucleotide hybridization (*8*) (used to analyze males at DXCrc47, DXWas17, and DXSmh43), or PCR, then denaturing polyacrylamide electrophoresis (*9*) (used to analyze females at DXWas17 plus all animals at DXMit3, DXMit4, DXMit9, and DXNds2). DXCrc47 haplotype data are not shown. (**B**) Analysis of segregation of *btk* alleles in the 30 backcross mice recombinant between DXWas17 and DXMit9. After amplification of genomic DNA from individual mice with primers MXL1 and MXL2 (*8*), samples were digested with Hha I and Rsa I and then electrophoresed on a nondenaturing 15% acrylamide gel and electroblotted onto GeneScreen Plus (Du Pont). Oligonucleotide MXL23 (*8*) was end-labeled and hybridized to the resulting blot. The Skive allele (containing an Hha I site) is predicted to yield a 20–base pair (bp) fragment, whereas the CBA/N allele (lacking an Hha I site) should yield a 65-bp fragment. This was confirmed by the controls shown in the far left lanes. That the two alleles do not give signals of equal intensity is due to the inefficient binding of the 20-bp fragment to the membrane. The origin of the Skive-specific band migrating below the 20-bp fragment is unknown. Individual haplotypes at closely linked loci are indicated above the corresponding lanes. (**C**) Map of the *xid* region of the mouse X chromosome, showing genetic distances determined by the above haplotype analysis. We determined genetic distances by dividing the number of recombinants by the number of meiotic events analyzed, shown below each interval, respectively.

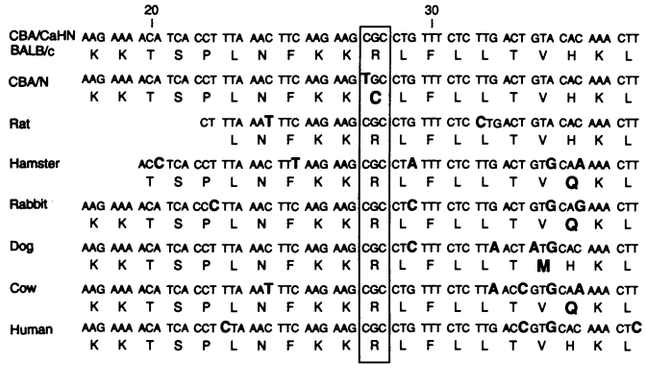

Fig. 3. Comparison of *btk* sequences from various mouse strains and from various mammalian species. A fragment of the *btk* gene was amplified by PCR from genomic DNA with primers MXL1 and MXL2 (*8*). Sequences of PCR products were determined by cycle sequencing of these products using the MXL1 primer (*8, 14*). BALB/c sequence is from (*13*). Differences from the "wild type" mouse sequence are denoted by enlarged letters and are highlighted in bold. Single-letter abbreviations for the amino acid residues are as follows: K, lysine; T, threonine; S, serine; P, proline; L, leucine; N, asparagine; F, phenylalanine; R, arginine; V, valine; H, histidine; C, cysteine; M, methionine; and Q, glutamine.

the same region from six additional mammalian species and did limited sequence analysis on the resultant products (Fig. 3). The Arg^{28} and all but two amino acids were conserved among these species, despite the occurrence of many silent mutations.

Although the mutation in the *btk* gene of XID mice is not predicted to cause alterations in *btk* transcript size, abundance, or inducibility in XID B lymphocytes, other undetected mutations located in the intronic or flanking regions of the *btk* gene might do so. To address this possibility, we isolated resting B cells from the spleens of adult CBA/N and CBA/CaHN mice, stimulated them with interleukin-4 (IL-4) and lipopolysaccharide (LPS) for 0, 4, or 21 hours, and then harvested total RNA. Northern (RNA) blot analysis of these preparations showed that a single 2.8-kb transcript could be detected in both CBA/CaHN and CBA/N B cells (Fig. 4A). Densitometric analysis showed that relative to β-actin mRNA, there was no significant difference in *btk* mRNA levels in resting or stimulated CBA/N cells in comparison to resting or stimulated CBA/CaHN B cells.

To determine whether the mutation at Arg^{28} affected autophosphorylation activity of Btk protein, we precipitated Btk from lysates of unstimulated CBA/N and CBA/CaHN B cells with antiserum to Btk (*12, 18*). Half of the precipitated material was incubated with adenosine triphosphate (ATP) in a buffer appropriate for tyrosine phosphorylation (*19*). Protein immunoblot analysis of these samples revealed that Btk was present in both CBA/N and CBA/CaHN B cells and that Btk from both sources could autophosphorylate (Fig. 4B).

Until it can be shown that the unmutated *btk* gene can complement the *xid* mutation, the possibility that XID is caused by a mutation in an unidentified gene that lies very near *btk* cannot be formally excluded, although the data presented here suggest that the Arg to Cys mutation in the *btk* gene of CBA/N mice is responsible for the XID phenotype. Several inherited diseases are caused by Arg to Cys mutations that alter enzymatic activity (*20*), ligand-receptor interactions (*21*), or cytoskeletal structure (*22*). XID also shares distinctive features with human XLA. In both these immune deficiencies, defects are restricted to the B lineage and alter B lymphocyte ontogeny (*1, 3, 23*). The XLA alleles that have been characterized bear mutations in the kinase domain (*11*) and exhibit a more severe immune deficiency than XID. However, the single existing allele of XID exhibits a higher degree of expressivity when present in thymectomized mice (*24*), with the *nu* (athymic) mutation (*25*), or in C3H mouse strains (*26*). In particular, young adult *nu/nu xid* mutant mice display profound depletion of lymphocytes from spleen and lymph nodes and a striking hypogammaglobulinemia (*25*), features commonly observed in XLA (*23*).

The *xid* mutation lies in the unique region of *btk* (*11, 12, 27*). Though little is known about the function of the NH_2-terminal unique domains of tyrosine kinases, they presumably mediate specific intermolecular interactions. Unique domain splice variants are common in nonreceptor kinases (*28, 29*), and some are expressed in a tissue-specific manner (*28*). Mutation of the NH_2-terminal glycine in Src prevents both myristylation and morphological transformation (*30*). Missense point mutations elsewhere in the unique domain of Src also affect functional differences (*31*). Thus, it is possible that the mutation interferes with the ability of Btk to interact with regulatory or substrate molecules that are important components of a signaling pathway. The *xid* mutation could alter intramolecular interactions between the unique region and other functional domains of Btk or interfere with posttranslational modifications of Btk such as phosphorylation. The *xid* mutation might also affect a potential nuclear localization signal in Btk (*32*). If Btk is localized in a subcellular compartment in which disulfide bonds can form, the Cys^{28} residue could be involved in such linkages. Two other recently identified nonreceptor kinases, Tec and Itk (*33*), share several features with Btk including a high degree of homology in their NH_2-terminal unique domains (*11, 12*) and have been suggested to comprise a new family of kinases (*28*). The *xid* mutation is in the unique region of one of these proteins and will likely help in elucidation of functions of this domain.

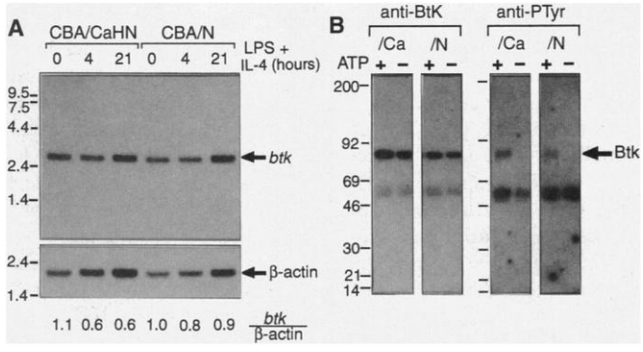

Fig. 4. Analysis of the expression of *btk* in XID (CBA/N) and normal (CBA/CaHN) mice. (**A**) Analysis of *btk* mRNA in resting and stimulated B cells. Total RNA was isolated from resting splenic B cells (*18*) that had been cultured with LPS (20 μg/ml) and mouse IL-4 (1000 U/ml) for 0, 4, or 21 hours. The RNA samples were then subjected to northern blot analysis. The blot in the upper panel was probed with a ^{32}P-labeled fragment of a mouse *btk* cDNA clone containing nucleotides −127 through 1316 relative to the initiation codon (*13*). After removal of the *btk* probe, this blot was reprobed with a ^{32}P-labeled fragment of mouse β-actin cDNA, and the results are shown in the lower panel. Molecular size standards (in kilobases) are shown on the left. The ratio of *btk* mRNA to β-actin mRNA, as determined by densitometric scanning, is shown below the blots. (**B**) Analysis of Btk protein and its ability to autophosphorylate. Btk was immunoprecipitated from 5×10^7 resting B cells isolated from CBA/CaHN (/Ca) or CBA/N (/N) animals with a rabbit antiserum to Btk (anti-Btk) (*12, 18*). Half of the immunoprecipitated material was resuspended in kinase buffer and incubated for 10 min with ATP (*19*). Each of these four samples was divided again, electrophoresed on 4 to 20% SDS-polyacrylamide gels, and blotted onto nitrocellulose. The blots were probed with either anti-Btk (left two panels) or monoclonal antibody to phosphotyrosine 4G10 (anti-PTyr) (Upstate Biotechnology) (right two panels), washed, and then probed with ^{125}I-labeled protein A (Amersham) (*18*). The 50- to 55-kD band represents the heavy chain of immunoglobulin. Molecular weight standards (in kilodaltons) are shown on the left.

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However, because of the proximity of markers used in this analysis, it is unlikely that more than one such event occurred over the region of interest among the entire set of backcross animals (male and female). Such undetected recombination events could not affect the order of loci that we determined or significantly alter the genetic distances we determined.
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19. Immunoprecipitated material was washed twice with phosphate-buffered saline containing 0.5% NP-40 and 1 mM phenylmethylsulfonyl fluoride (wash buffer) and resuspended in 20 μl of kinase buffer [50 mM Hepes, 0.1 mM EDTA, 0.01% Brij35, bovine serum albumin (0.1 mg/ml), 10 mM dithiothreitol, 150 mM NaCl, 0.1 mM orthovanadate, and 10 mM NaF] to which 10 μl of 0.3 mM ATP and 48 mM $MgCl_2$ were added to initiate the reaction. After 20 min at room temperature, samples were washed twice with wash buffer and eluted in SDS-containing sample buffer.
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Mutation of Unique Region of Bruton's Tyrosine Kinase in Immunodeficient XID Mice

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The cytoplasmic tyrosine kinase, Bruton's tyrosine kinase (Btk, formerly bpk or atk), is crucial for B cell development. Loss of kinase activity results in the human immunodeficiency, X-linked agammaglobulinemia, characterized by a failure to produce B cells. In the murine X-linked immunodeficiency (XID), B cells are present but respond abnormally to activating signals. The Btk gene, *btk*, was mapped to the *xid* region of the mouse X chromosome by interspecific backcross analysis. A single conserved residue within the amino terminal unique region of Btk was mutated in XID mice. This change in *xid* probably interferes with normal B cell signaling mediated by Btk protein interactions.

B cell development is characterized by an orderly expression of cell surface markers and responses to specific activation signals (*1*). Tyrosine kinases are important in the signaling pathways regulating these events (*2*). Btk, expressed in B and myeloid cells, and several homologous genes constitute a unique tyrosine kinase subfamily distinct from the Src subfamily (*3*, *4*). The Btk family members have a long, highly conserved NH_2-terminal unique region and lack a negative regulatory tyrosine. Despite its critical role in B cell development, the specific functions of Btk in B cell signaling are not yet known.

The *xid* gene defect results in failure of B cells to become phenotypically and functionally diverse (*5*, *6*). B cells from XID mice do not respond to thymus-independent type 2 antigens, have abnormal responses to a variety of activation signals (*5–7*), and have a surface phenotype suggestive of disordered maturation (*8*). These findings suggest that B cells with the *xid* mutation lack essential signals for B cell activation and maturation. Despite characterization of these cellular defects, the underlying genetic change remains unknown.

In contrast to the *xid* mutation, X-linked agammaglobulinemia (XLA) results in production of few B cells and severe humoral immunodeficiency. Despite this distinction, XLA and XID have several common features. First, the surface phenotypes of XLA and XID B cells are similar, suggesting that both result in abnormal B cell activation (*6*, *9*). Second, both disorders appear to result from an intrinsic B lineage defect, as evidenced by nonrandom X chromosome inactivation limited to B cells (*10*). And finally, *xid* maps to a region of the X chromosome that shares homology with the human XLA locus at Xq21.3-Xq22 (*11*, *12*). For these reasons, we evaluated the possible role of Btk in XID.

The murine chromosomal location of *btk* was determined by interspecific backcross analysis using a mapping panel typed for over 1100 loci (*13*). Southern (DNA) blot analysis using a *btk* unique region probe identified three *Mus spretus*–specific Sph I restriction fragment length polymorphisms. These were used to follow the segregation of the *btk* locus in backcross mice (Fig. 1). The *btk* locus was mapped to the distal portion of the X chromosome linked to *pgk-1*, *DXPas2*, and *plp*. The gene order and recombination frequencies expressed in centimorgans (cM ± SE) are centromere, *pgk-1* (1.7 ± 1.7), *DXPas2* (3.6 ± 2.1), *btk* (3.3 ± 1.9), and *plp* (Fig. 1). The *xid* locus is distal to *Pgk-1* in the interval surrounding *DXPas2* (in Fig. 1) (*12*). These two data

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