tor IX promoter activity, reducing factor IX expression and resulting in hemophilia B. It has been postulated that the factor IX promoter responded to testosterone (*9*), which can increase factor IX concentrations in Leyden patients (*10*). These results suggest that the factor IX promoter contains a functional androgen-responsive element (ARE). The promoter contains a sequence resembling a consensus ARE (nucleotides −36 to −22) (Fig. 2). The −26 G to C mutation but not the −20 T to A mutation disrupts this ARE.

To test whether the ARE was functional, we linked it upstream of the herpes simplex thymidine kinase (TK) promoter and the CAT reporter gene and assayed transactivation in HeLa cells by an androgen receptor (AR)–encoding plasmid (*11*) in the presence of testosterone. When the ARE was present in one copy, the response was not significantly above background; with two copies a threefold effect was observed, and with four copies (Fig. 3A) the ARE allowed the TK promoter to be significantly (>50-fold relative to a control in the absence of AR) transactivated when both testosterone and the AR expression plasmid were present. The promoter with the −26 mutation did not support transactivation, whereas that with the −20 mutation (which lies outside the ARE) did.

When the candidate factor IX ARE was bound to a recombinant AR–protein A fusion protein (*12*), a gel-shifted complex IV was formed (Fig. 3B), which was effectively competed by both normal factor IX DNA and by DNA with the −20 mutation but not by DNA containing the −26 mutation. A potent ARE from a rat prostate binding protein gene (*12*) competed even more effectively for this complex (Fig. 3B).

These results show that the ARE in the factor IX promoter can bind AR in vitro. AR mRNA has been identified in human liver and AR protein in rat liver (*13*). The fact that the patient with a mutation at −26 in the ARE did not improve after puberty, whereas patients with mutations at −20, outside the ARE, do improve, shows that this element is important in vivo. Nevertheless, it bound AR less effectively than the well-characterized ARE from the prostatic binding protein gene and only functioned well in CAT assays when multimerized (Fig. 3). In transient transfection systems in HepG2 cells, the factor IX promoter itself supports only a small activation (less than threefold) dependent on androgen and AR (*14*). Additional upstream elements or factors may be required for the full response or the transient assay system may not faithfully reflect the in vivo situation. Although the ARE identified here is necessary, it may not be sufficient for the full response seen in Leyden patients (*10*).

In summary, the mutation in Hemophilia B Brandenburg simultaneously disrupts an LF-A1/HNF4 site and an overlapping ARE, which explains the persistent hemophilia in this patient and also why patients with nearby promoter mutations, which do not disrupt the ARE, recover after puberty. Mutations identified in hemophilia B patients in the site bound by C/EBP (or related proteins) (*4*) and now in the LF-A1/HNF4 binding site of the hepatocyte-specific factor IX promoter emphasize the function of these factors in the regulation of liver-specific genes in vivo.

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Cloning of the γ Chain of the Human IL-2 Receptor

Toshikazu Takeshita, Hironobu Asao, Kiyoshi Ohtani, Naoto Ishii, Satoru Kumaki, Nobuyuki Tanaka, Hiroshi Munakata, Masataka Nakamura, Kazuo Sugamura*

A third subunit, the γ chain, of the human interleukin-2 receptor (IL-2R) was identified, and a complementary DNA clone encoding this member of the cytokine receptor family was isolated. The γ chain is necessary for the formation of the high- and intermediate-affinity receptors, which consist of αβγ heterotrimers and βγ heterodimers, respectively. The IL-2R on murine fibroblastoid cells can be internalized after binding IL-2 only if the γ chain is present; α and β are insufficient for internalization. Thus, the γ chain is an indispensable component of the functional IL-2R.

The lymphokine IL-2 affects the growth and differentiation of T cells, B cells, natural killer cells, glioma cells, and cells of the monocyte lineage after specifically interacting with IL-2Rs (*1*, *2*). The IL-2R contains at least two distinct subunits, called α and β chains, and exists in three different isoforms: high affinity for IL-2 (the dissociation constant K_d is $\sim 10^{-11}$ M), intermediate affinity ($K_d = \sim 10^{-9}$ M), and low affinity ($K_d = \sim 10^{-8}$ M) (*1*). The high- and intermediate-affinity receptors, which are thought to consist of the α and β heterodimers and the β chain alone, respectively, are effective in IL-2–mediated signal transduction, but the low-affinity receptor, which consists of the α chain alone, is ineffective; therefore, the β chain but not the α chain is indispensable (*3–5*). Several lines of evidence implicate a third lymphoid-specific component that is required for the formation of the high- and intermediate-affinity receptors. (i) The β chain, when expressed on lymphoid

T. Takeshita, H. Asao, K. Ohtani, N. Ishii, S. Kumaki, N. Tanaka, M. Nakamura, K. Sugamura, Department of Microbiology, Tohoku University School of Medicine, Sendai 980, Japan.
H. Munakata, Second Department of Biochemistry, Tohoku University School of Medicine, Sendai 980, Japan.

*To whom correspondence should be addressed.

cell lines but not on fibroblastoid cell lines, can bind to IL-2 and can transduce growth signals (*4–7*). (ii) The number of β chain molecules expressed on lymphoid cell surfaces does not determine the number of intermediate-affinity IL-2 binding sites (*8, 9*). (iii) Coexpression of the α and β chains in fibroblastoid cell lines results in receptors with greater affinities than the intermediate-affinity receptors, but these higher affinity receptors cannot respond to IL-2 functionally (*7*). We have detected a p64 component, tentatively named the γ chain of IL-2R, that associates with the β chain in an IL-2–dependent manner in immunoprecipitates with the monoclonal antibody (MAb) TU11, which is specific for human IL-2R β (*9, 10*). We now have cloned the γ chain. It is a member of the cytokine receptor family, participates in the formation of the high- and intermediate-affinity IL-2Rs, and allows the formation of IL-2Rs that can internalize IL-2.

We purified the γ chain molecule to determine its amino acid sequence. As a source of the γ chain, a subline of MOLTβ cells was chosen that produced the most γ chains, as assessed by immunoprecipitation. MOLTβ is a stable transfectant of the human T cell line MOLT4 into which the human IL-2R β cDNA had been introduced; MOLTβ expresses the intermediate-affinity IL-2R (*9*). The γ chain was detectable [61 to 71 kD; isoelectric point (pI) 4.2 to 4.6] in the immunoprecipitates of the β chain when IL-2 was added to the cells (Fig. 1). To purify the γ chain, we subjected the MOLTβ cell lysate to an immunoaffinity column conjugated with MAb TU11 (*11*). The molecules bound to the column were eluted, separated by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), and transferred onto Immobilon membranes (*12*). The membrane pieces corresponding to the IL-2R γ spot were collected and subjected to amino acid sequencing. The NH_2-terminal 22–amino acid residues of the γ chain molecules were determined to be (Leu or Ile)-(Asn or Cys)-(Thr or Phe)-(Thr or Phe)-Ile-Leu-Thr-Pro-Asn-Gly-Asn-Glu-(Asp or Arg)-(Thr or Ala)-(any amino acid)-Ala-(Asp or Gly)-Phe-Phe-Leu-Ser-Arg.

To isolate a cDNA clone coding for the γ chain gene, we first used the reverse transcriptase polymerase chain reaction (RT-PCR) with a pair of primers and then prepared polyadenylated [poly(A)$^+$] RNA from MOLTβ cells (*13*). A 54-bp fragment was generated and sequenced. The amino acid sequence deduced from the nucleotide sequence of this fragment included the amino acid sequence directly determined with the purified γ chain. With the 54-bp fragment as a probe, cDNA clone pIL-2Rγ1 was identified from a randomly primed cDNA library from MOLTβ cells (*14*). The pIL-2Rγ1 cDNA contained an open reading frame (ORF) that included the sequence for the directly determined NH_2-terminus except that we determined that the last two residues were Thr-Thr by nucleotide sequencing. No in-frame stop codon was present; thus, pIL-2Rγ1 carries an incomplete cDNA. We used this cDNA clone as a probe to screen an oligo(dT)-primed cDNA library and cloned pIL-2Rγ2 from this library with an ORF that ended at an in-frame stop codon. No complete homology with any other published nucleotide sequences was seen.

The combined nucleotide sequences of the two clones, which had identical overlapping sequences, contained an ORF of 369 amino acid residues (Fig. 2A). The nucleotide context surrounding the initiator ATG had consensus sequences for translation initiation (*15*). Analysis of the hydrophobicity of the deduced amino acid sequence predicted that a hydrophobic stretch of the NH_2-terminal initial 22

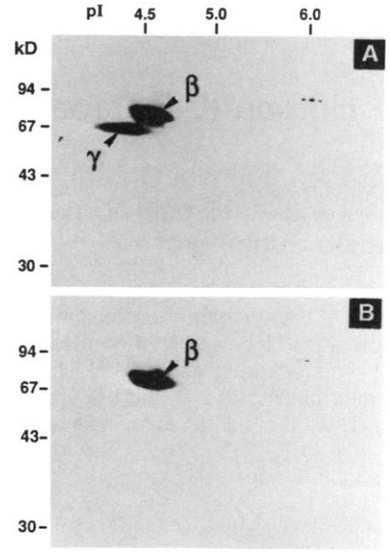

Fig. 1. IL-2–dependent association of the γ chain molecules with IL-2R. MOLTβ cells were surface-labeled with $Na^{125}I$ with iodination reagent (IODOGEN, Pierce Chemical, Rockford, Illinois). They were treated with 30 nM IL-2 (**A**) or left untreated (**B**), and then all cultures were incubated for 1 hour on ice and solubilized in 2 ml of lysis buffer [25 mM tris-HCl (pH 7.5), 140 mM NaCl, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, and 0.5% Nonidet P-40]. The lysates were immunoprecipitated with MAb TU11 (*11*), and the immunoprecipitates were separated by 2-D PAGE as described (*9*). Arrowheads indicate spots corresponding to the β and γ chains, as labeled. Molecular size standards are indicated on the left, and pI's are indicated at the top.

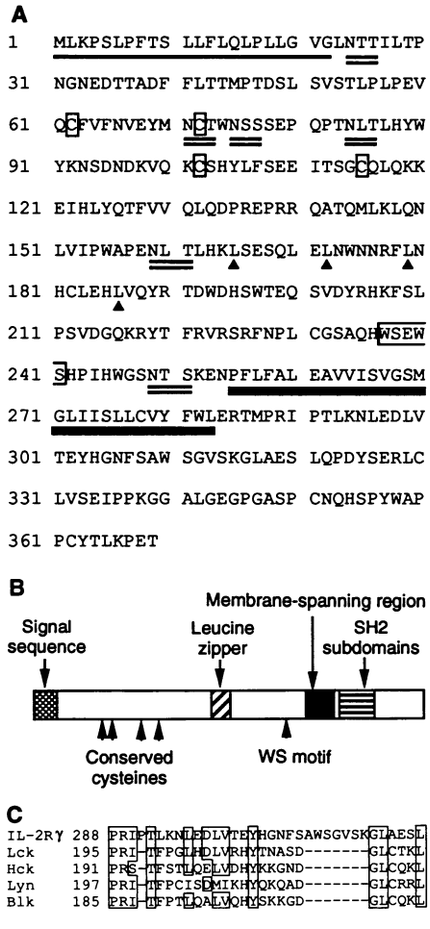

Fig. 2. Deduced amino acid sequence and schematic structure of the human IL-2R γ chain cDNA. (**A**) Deduced amino acid sequence of IL-2R γ cDNA (*30*). A predicted signal sequence is underlined once with a thin line. The thick underline indicates a predicted transmembrane region. The four cysteine residues and WS motif are boxed. Four leucine residues similar to the leucine zipper structure are indicated by closed triangles below the amino acid sequence. Six potential N-glycosylation sites are double underlined. The nucleotide sequence has been deposited at DNA Data Bank of Japan and GenBank/European Molecular Biology Laboratory and is available through accession number D11086. (**B**) Schematic representation of the IL-2R γ cDNA and its product. The possible signal sequence, cysteine-rich region, WS motif, leucine zipper, transmembrane region, and SH2 subdomain regions are shown. (**C**) Sequence alignment of the SH2 subdomains of human IL-2R γ and Src-related kinases (*30*). The intracytoplasmic amino acid sequence (positions 288 to 321) of IL-2 γ and the SH2 subdomains of human Lck, Hck, Lyn, and Blk of the Src tyrosine kinase family were aligned (*18*). Amino acid positions of the first residues shown are indicated at the left. Amino acids residues that are identical to those of the γ chain are boxed. Dashes indicate gaps.

amino acids is a signal peptide. This assumption agreed with the results of direct amino acid sequencing. Therefore, the mature form of the protein backbone of the γ chain consists of 347 amino acid residues with a calculated molecular mass of 39,918 daltons. The hydrophobicity plot also predicted a single membrane-spanning region at amino acid residues 255 to 283 (Fig. 2B). The predicted extracellular domain included six potential N-linked glycosylation sites and the consensus sequences of the cytokine receptor family—the four conserved cysteines (positions 62, 72, 102, and 115) and the Trp-Ser-X-Trp-Ser WS motif (positions 237 to 241; X is an unconserved amino acid residue; W, Trp; S, Ser)—in the proximity of the transmembrane region (*16*). A computer search showed that the deduced amino acid sequence of the γ chain shared partial homology in the cysteine-rich region and in the WS motif, with sequences of members of the cytokine receptor family, such as subunits of receptors for interleukin (IL)-4, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Four leucine residues with the six–amino acid interval (positions 165 to 186) appeared between the cysteine-rich region and the WS motif. This configuration is reminiscent of the leucine zipper structure. Computer simulation according to the Garnier's method (*17*) predicted an α helix structure for two regions (positions 163 to 172 and positions 179 to 189) within the tentative leucine zipper domain; thus, this region may actually compose the leucine zipper structure.

The cytoplasmic domain of 86 amino acid residues was considerably shorter than that of the β chain. It may, however, have a role in signal transduction because sequences from positions 288 to 321 appeared to be homologous to the Src homology region 2 (SH2), which can bind to phosphotyrosine residues of some phosphoproteins. Among the five highly conserved subdomains of SH2 (*18*), the last two subdomains are identified in the sequence of the γ chain (Fig. 2C). Nevertheless, no consensus sequence for tyrosine kinases was seen in the cytoplasmic domain.

Expression of the γ chain gene in various cells was analyzed by Northern (RNA) blot. Human lymphoid cell lines MOLTβ, MOLT4, Jurkat, MT-1 and MT-2 (T cell lines both infected with human T cell leukemia virus type–1), and Raji (an Epstein-Barr virus–infected B cell line) expressed the IL-2R γ mRNA (Fig. 3). Normal human peripheral blood leukocytes (PBLs) also expressed the IL-2R γ mRNA whether or not the cells were stimulated with phytohemagglutinin (PHA). In these cells, the ~1.8-kb message was dominant; a second species of ~3.6 kb was present in MOLTβ, MOLT4, Jurkat, and PHA-stimulated PBLs (PHA-PBLs). The IL-2R γ mRNA was absent in human nonlymphoid cell lines such as promonocytic THP-1, epitheloid HeLa, and hepatocytic HepG-2.

To gain insight into the function of the γ chain, we studied the effect of the γ chain on the formation of the three IL-2R isoforms that differed in their IL-2 binding affinities. The IL-2R γ cDNA was introduced with or without IL-2R α or β cDNA into sublines derived from murine fibroblastoid cell line L929 (Fig. 4). The binding of IL-2 to the transfected cell lines was then analyzed by Scatchard plots (Fig. 4). The LCT-4 cell line, which is an IL-2R α transfectant clone of L929, expressed 4600 low-affinity IL-2R sites per cell (K_d = 12 nM) but did not express any high- or intermediate-affinity receptors (*19*). The Lβ-1 cell line, which expresses only the β chain, did not show any appreciable IL-2 binding. The γ chain by itself in L cells as the Lγ-4 cell line could not bind IL-2. However, co-introduction into L cells of

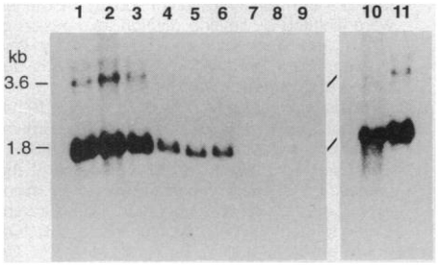

Fig. 3. IL-2R γ mRNA expression in various cell types. Total RNA (20 μg) was separated by electrophoresis, blotted onto nitrocellulose, and hybridized with the ^{32}P-labeled 0.9-kb Eco RI fragment of pIL-2Rγ1 as described (*31*). Lane 1, MOLTβ; lane 2, MOLT4; lane 3, Jurkat; lane 4, MT-1; lane 5, MT-2; lane 6, Raji; lane 7, THP-1; lane 8, HeLa; lane 9, HepG-2; lane 10, PBLs; and lane 11, PHA-stimulated PBLs. Molecular sizes are indicated at the left in kilobases.

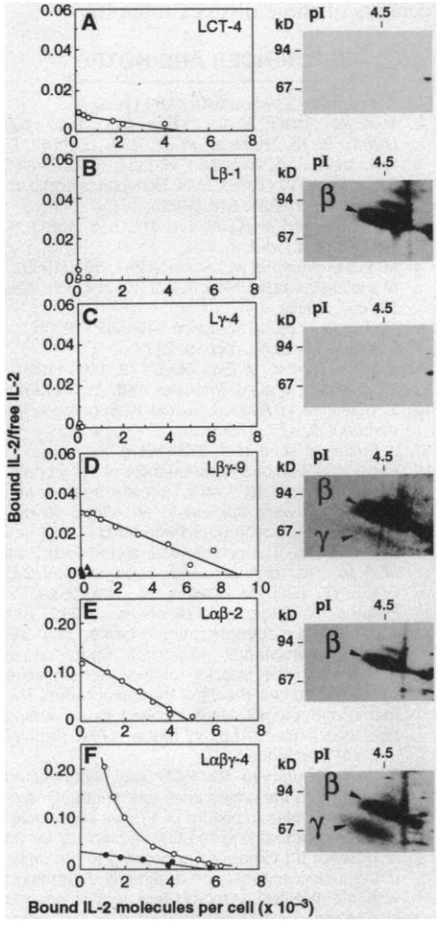

Fig. 4. IL-2 binding and IL-2–dependent association of the γ chain in the transfectants. Six sublines, LCT-4 (**A**), Lβ-1 (**B**), Lγ-4 (**C**), Lβγ-9 (**D**), Laβ-2 (**E**), and Laβγ-4 (**F**), of the murine fibroblastoid L929 cell line were established by transfection with various combinations of expression vectors for the human IL-2R α, β, or γ chains (*32*) and used for examination of their ability to bind to IL-2. The ^{125}I-labeled IL-2 binding was analyzed by Scatchard plots. The IL-2 binding was similarly assayed in the presence of MAb TU27 (▲) (*33*), MAb H-31 (●) (*34*), or control MAb PAR3 (○) as described (*33*) (left-hand panels). The transfectants were also examined for association of the γ chain with the β chain (right-hand panels). The cells were labeled with [^{35}S]methionine, treated with 30 nM IL-2, and immunoprecipitated with MAb TU11. The immunoprecipitates were analyzed by 2-D PAGE as described in Fig. 1. Axes are labeled as in Fig. 1.

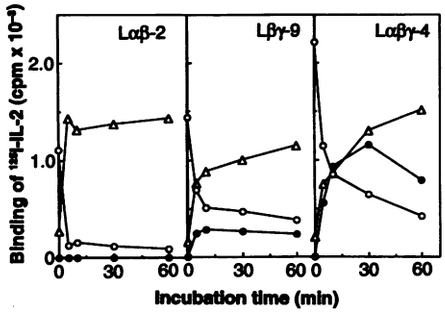

Fig. 5. Internalization of IL-2 in the transfectants. Laβ-2, Lβγ-9, and Laβγ-4 transfectants were examined for receptor-mediated internalization of IL-2 as described (*20*). The cells were incubated with 10 nM ^{125}I-labeled IL-2 at 0°C for 1 hour, washed, and further incubated at 37°C for the times indicated. They were then centrifuged and treated with chilled 0.2 M glycine buffer (pH 2.8) for 10 min. Radioactivities associated with unbound IL-2 (△), bound to cells but removed by acid wash (○), and bound to cells even after acid wash (●), were determined separately. We considered acid wash–resistant radioactivity to be internalized IL-2 molecules.

expression vectors for both γ and β, yielding the Lβγ-9 cell line, resulted in induction of an IL-2R of intermediate affinity (K_d = 4.6 nM; 8800 sites per cell). The intermediate-affinity binding was abolished by addition of MAb TU27, which is specific for human IL-2R β, indicating that association of the γ chain with the β chain is necessary for IL-2 binding. The IL-2R of Lαβ-2 cells, which contains only α and β chains (K_d = 600 pM; 4800 sites per cell), was converted to the high-affinity (K_d = 77 pM; 1700 sites per cell) IL-2R by introduction of the γ chain (Lαβγ-4), and the high affinity of IL-2 binding in Lαβγ-4 cells was lowered to intermediate-affinity binding in the presence of MAb H-31 (specific for human IL-2R α). Thus, the high-affinity IL-2R may be a heterotrimer. IL-2–dependent physical association of the γ chain with the β chain was seen only with Lαβγ-4 and Lβγ-9 cells but not with LCT-4, Lαβ-2, Lβ-1, and Lγ-4 cells (Fig. 4). This coimmunoprecipitation pattern of the γ chain with the β chain is consistent with data from lymphoid cell lines (*9, 10*).

These results show that the γ chain is functionally involved in the formation of IL-2R complexes; that the intermediate-affinity IL-2R consists of two subunits, the β and γ chains; and that the high-affinity IL-2R consists of three subunits, the α, β, and γ chains. The γ chain appears to have no effect on the low-affinity IL-2R, which consists of IL-2R α alone, because only the low-affinity IL-2R was detected on MT-1 cells that express the α chain and IL-2R γ mRNA (Fig. 3) (*20*).

We further investigated the biological significance of the γ chain in receptor-mediated internalization of IL-2 (Fig. 5). IL-2 that bound to Lαβγ-4 and Lβγ-9 cells was rapidly internalized after incubation at 37°C. Lαβγ-4 with the high-affinity receptors internalized IL-2 more efficiently than Lβγ-9 with the intermediate-affinity receptors. However, Lαβ-2 cells, which have no γ chain but do have IL-2–binding receptors with greater affinities for IL-2 than the intermediate-affinity receptors, rarely internalized IL-2. These results suggest that the γ chain is required for the receptor-mediated internalization of IL-2.

There is evidence that IL-2R requires another component besides the α and β chains to form high- and intermediate-affinity IL-2Rs (*4, 8, 9, 21*). We reported a molecule, γ chain, associated with the β chain of IL-2R (*9, 10*). We have now cloned a cDNA of the γ chain gene and implicate it in complex formation of the functional IL-2R. Other cytokine receptors, such as those for IL-3 (*22, 23*), IL-5 (*24, 25*), IL-6 (*26, 27*), and GM-CSF (*28, 29*), also contain two α and β subunits, (*16*). Because the β chain of IL-2R is also a member of the cytokine receptor family but the α chain is not, the heterodimers that consist of β and γ subunits of IL-2R may correspond to the αβ heterodimers of the other cytokine receptors. However, the α chain of IL-2R seems functionally similar to the α chains of the other cytokine receptors in that even on fibroblastoid cells the α chain has ligand-binding ability without the other subunits (*22, 24, 26, 28*). Thus, this high affinity is unusual among the cytokine receptors because it consists of three distinct subunits.

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12. For purification of a large quantity of the γ chain, lysates of 4 × 10^{10} MOLTβ cells treated with 30 nM IL-2 were applied to an Affigel 10 column (Bio-Rad) conjugated with MAb TU11 (1-ml bed volume). The protein was eluted with 2 ml of 8 M urea, concentrated, separated by 2-D PAGE [T. Ishii, M. Kohno, M. Nakamura, Y. Hinuma, K. Sugamura, *Biochem. J.* **242**, 211 (1987)], and electrophoretically blotted onto Immobilon membranes (Millipore). Spots corresponding to the position of the γ chain were excised from two sheets of the membranes. The membrane pieces were applied to an amino acid sequencer (Applied Model 477A Protein/Peptide Sequencer).
13. A pair of primers for PCR was synthesized according to the amino acid sequences. One of the primers was a mixture of 17-mer oligonucleotides encompassing the first nucleotide for Ile at position 5 (numbered according to the order of the amino acid sequence directly determined with the purified γ molecules) to the second nucleotide for Gly at position 10. The other primer was a mixture of 22-mer oligonucleotides that consisted of a 7-mer sequence containing the Bam HI recognition site at the 3′ end, followed by antisense 15-mer sequences derived from the second nucleotide for Arg at position 22 to the first nucleotide for Phe at position 18. Randomly primed cDNA was synthesized with oligo(dT)-selected RNA from MOLTβ cells and subjected to 30 cycles of PCR amplification with the mixed primers. Primer-directed extension was repeatedly carried out by serial cycles of denaturation (1 min at 94°C), annealing (2 min at 50°C), and synthesis (3 min at 37°C). One-fifth of the product was again subjected to another 30 cycles of PCR amplification. All fragments were separated on a 7% nondenaturing polyacrylamide gel, and a 54-bp fragment was isolated and sequenced by the dideoxy chain termination method with the pUC19 vector.
14. Using poly(A)$^+$ RNA from MOLTβ cells, we separately synthesized oligo(dT)-primed and random primer–directed cDNAs using λZAPII (Stratagene). Two cDNA libraries containing 4.5 × 10^5 and 4.0 × 10^5 independent clones, respectively, were produced. The ^{32}P-labeled 54-bp oligonucleotide fragment generated by RT-PCR was used as a probe on the randomly primed library, and two clones were positively cloned. One of them (pIL-2Rγ1), which contained a longer insert, was sequenced and found to have incomplete cDNA. We used this insert as a probe to screen the oligo(dT)-directed cDNA library. Three positive cDNA clones were isolated, and one of them (pIL-2Rγ2) was sequenced.
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30. Single-letter abbreviations for the amino acid residues are: 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.
31. S. Miura *et al.*, *Mol. Cell. Biol.* **11**, 1313 (1991).
32. Expression vectors for human IL-2R α (pSRA4) and β (pSRB5) chains have been described (*9*). We constructed a human IL-2R γ chain expression vector by insertion of the 5′ Xba I–Nco I fragment (0.9 kb) from pIL-2Rγ1 and the 3′ Nco I–Xba I fragment (0.7 kb) from pIL-2Rγ2 into the Xba I site of pcDSRα [Y. Takebe *et al.*, *Mol. Cell. Biol.* **8**, 466 (1988)] to yield pSRG1. We transfected L929 cells by electroporation with pSV2neo, which carries the neomycin resistance gene, and either pSRB5 or pSRG1, as described [K. Ohtani *et al.*, *Nucleic Acids Res.* **17**, 1589 (1989)], to establish the Lβ-1 or Lγ-4 transfectant, respectively. We transfected LCT-4 with pSRB5 and pSV2neo, to generate Lαβ-2. We further transfected Lβ-1 and Lαβ-2 with pSRG1 and pHygJM109, which carries the hygromycin resistance gene, to yield Lβγ-9 and Lαβγ-4, respectively. These transfectants were selected in medium with G418 (Gibco) at a final concentration of 500 μg/ml or with hygromycin B (Sigma) at a final concentration of 350 μg/ml. Clones were isolated by limiting dilution, expression of the α and β chains was examined by flow cytometry, and mRNA for the γ chain was detected by Northern blot.
33. T. Takeshita *et al.*, *J. Exp. Med.* **169**, 1323 (1989).
34. Y. Tanaka, H. Tozawa, M. Hayami, K. Sugamura, Y. Hinuma, *Microbiol. Immunol.* **29**, 959 (1985).

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