On Somatic Recombination in the Central Nervous System of Transgenic Mice

Complete immunoglobulin (Ig) and T cell receptor (TCR) genes are generated by developmentally controlled DNA rearrangement referred to as V(D)J recombination or V(D)J joining (*1*). V(D)J recombination has been thought to be restricted to lymphoid cells and, until recently, there had been no evidence that recombination signal sequence (RSS)–mediated recombination of non-Ig or non-TCR genes occurred during normal vertebrate development. However, it had been speculated that this or similar types of somatic DNA rearrangement might play a role in mammalian cell differentiation (*2*). Interest in this hypothesis was refueled by the report that *RAG-1* transcripts were present, albeit in low numbers, in the central nervous system (*3*). Furthermore, it was reported that somatic recombination was detectable in the brains (*4*) of Tg mice that harbored a V(D)J recombination substrate. A second study (*5*) reported a small amount of V(D)J recombination in the brain, but this was attributed to lymphocyte contamination. We had independently constructed similar Tg mice whose initial analysis (*6*) generated data consistent with the occurrence of V(D)J recombination in the brain. However, further analysis has led us to a different interpretation of that data.

In order to prepare a Tg V(D)J recombination substrate, we constructed a plasmid pSPH-1 (*7*). This plasmid contained the transcriptional promoter and enhancer of the mouse phosphoglycerate kinase-1 gene (*pgk-1*) (*8*), a pair of RSSs derived from the Ig $V_{\kappa 21C}$ and $J_{\kappa 1}$ gene segments (*9*), and a reporter gene *lacZ* encoding bacterial β-galactosidase. The reporter gene *lacZ* was placed in an orientation opposite to that of the promoter so that RSS-mediated inversional recombination would activate its expression (Fig. 1). Cells expressing *lacZ* can be detected histochemically after staining with X-gal. Alternatively, inversional recombination can be detected more directly at the molecular level by the polymerase chain reaction (PCR) method (*10*) with the use of appropriate primers. We used both of these methods.

We generated Tg mice by injecting the pSPH1 insert into C57BL/6J zygotes (*11*). We analyzed the heterozygous progeny of five Tg lines (1-7, 1-20, 1-21, 1-28, and 1-39) for expression of β-galactosidase activity by histochemistry (*12*). Liver sections of all Tg lines were negative for β-galactosidase activity. We observed considerable enzyme activity in kidney, spleen, and thymus sections, but also in the non-Tg littermates (presumably because of endogenous β-galactosidase activity). We observed β-galactosidase activity in the brain of two of the five Tg lines, 1-7 (Fig. 2) and 1-20; we saw no such activity in non-Tg littermates. Regions of the brain in which we saw intense β-galactosidase activity in the Tg line 1-7 (Fig. 2) include the hippocampus (the dentate gyrus and the CA1 and CA3 fields), the cerebral cortex (especially the superficial layers), the superior colliculus (upper layers), nuclei of the dorsal tegmentum, and the cerebellum (especially its molecular and Purkinje cell layers). Sparse β-galactosidase activity also appeared in other sites (Fig. 2A). There was low expression in the striatum and in much of the thalamus. In Tg line 1-20, we observed a roughly similar pattern of X-gal staining, but the staining was weak. In both lines, the X-gal staining was region-specific. Cells with β-galactosidase activity appeared to be neurons in both Tg lines. The staining appeared to be limited to a small (2 to 5 μm), eccentrically located compartment of the cytoplasm (Fig. 2, C and E). Both we and Matsuoka *et al.* detected X-gal staining in the cerebral cortex and the hippocampus, but the patterns differed considerably in other regions, such as the cerebellum, where Matsuoka *et al.* saw X-gal staining in the granule and Purkinje cell layers and we saw staining primarily in the molecular cell layer.

In order to test whether RSS-mediated inversion had occurred in some tissues of the Tg mice, we analyzed genomic DNA by PCR using primers 1 and 2 (Fig. 1). If the Tg RSSs underwent an inversional V(D)J recombination, a 328-bp DNA fragment containing the joined RSSs would be generated by PCR (Fig. 1). The predicted 328-bp product was observed with the DNA isolated from the thymus of the Tg line 1-7, but not with that from non-Tg mice or from any of the other four Tg lines (1-20, 1-21, 1-28, and 1-39) (Fig. 3A). We cloned the 328-bp fragment and determined its nucleotide sequence. It contained the precisely head-to-head joined Tg RSSs. We analyzed DNA isolated from additional tissues of each Tg line, but none of the tissues derived from any Tg lines other than line 1-7 produced the 328-bp DNA fragment. In line 1-7, only spleen and thymus produced the 328-bp DNA fragment; and liver, cerebral cortex, ovary, muscle, kidney, and lung were negative (Figs. 3, B and C). We also analyzed DNA isolated from cerebral cortex and hippocampus of line 1-7, regions in which β-galactosidase–positive cells were abundant (Fig. 2) and DNA isolated from the striatum, where those cells were rare (Fig. 2). We did not detect the 328-bp DNA fragment in these tissues (Figs. 3, B and C).

We estimated the sensitivity of this PCR assay for the detection of V(D)J recombination by analyzing a fixed amount of non-Tg thymus DNA mixed with different amounts of Tg (line 1-7) thymus DNA (Fig. 3C). The 328-bp DNA fragment was detectable in DNA samples in which the Tg thymus DNA constituted only 1 part in 1000 of the total DNA. As only a fraction of the thymus cells from the Tg mice would have undergone RSS-mediated recombination, these data indicate that the sensitivity of the PCR method is at least 1 in 1000 cells. In some parts of the brain the proportion of β-galactosidase–positive cells among total nucleated cells far exceeded the sensitivity of the PCR method. For instance, we estimated their proportion in the cerebral cortex to be 1 to 10% (*13*), a figure at least one to two orders of magnitude greater than the PCR detection limit. We therefore conclude that the majority of β-galactosidase–positive cells observed did not result from RSS-mediated inversion. We also analyzed the absolute sensitivity of this PCR assay by carrying out a reconstitution experiment (*14*) in which different amounts of plasmid DNA containing *pgk-1* promoter

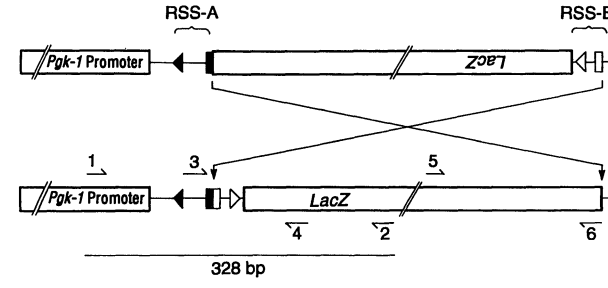

Fig. 1. Schematic representations of the Tg V(D)J recombination substrate (top) and the predicted product (bottom) of a V(D)J recombination event. (Top) In the substrate the bacterial β-galactosidase gene, *lacZ*, is oriented inversely to the *pgk-1* promoter. Recombination signal sequences (RSS-A and RSS-B) flank *lacZ* and are comprised of heptamer (rectangle), nonamer (triangle), and spacer elements. (Bottom) V(D)J recombination of the Tg substrate is expected to join the two RSSs precisely and to invert *lacZ*, thereby activating its transcription by the *pgk-1* promoter. Oligonucleotide primers used for PCR amplification (small arrows) are numbered. PCR amplification of the predicted product of a V(D)J recombination event with primers 1 and 2 results in the 328-bp product indicated at bottom.

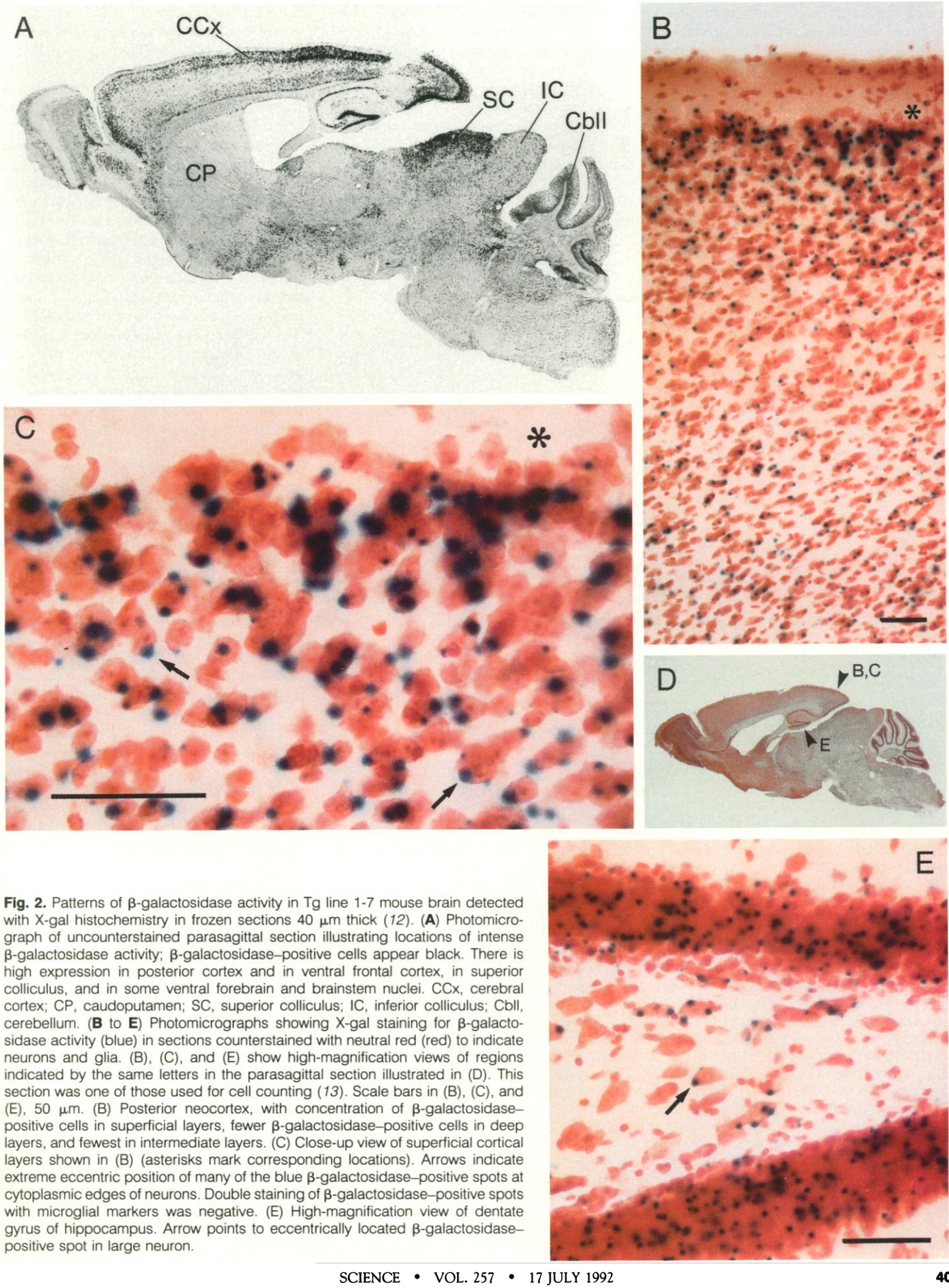

Fig. 2. Patterns of β-galactosidase activity in Tg line 1-7 mouse brain detected with X-gal histochemistry in frozen sections 40 μm thick (*12*). (**A**) Photomicrograph of uncounterstained parasagittal section illustrating locations of intense β-galactosidase activity; β-galactosidase–positive cells appear black. There is high expression in posterior cortex and in ventral frontal cortex, in superior colliculus, and in some ventral forebrain and brainstem nuclei. CCx, cerebral cortex; CP, caudoputamen; SC, superior colliculus; IC, inferior colliculus; CbII, cerebellum. (**B** to **E**) Photomicrographs showing X-gal staining for β-galactosidase activity (blue) in sections counterstained with neutral red (red) to indicate neurons and glia. (B), (C), and (E) show high-magnification views of regions indicated by the same letters in the parasagittal section illustrated in (D). This section was one of those used for cell counting (*13*). Scale bars in (B), (C), and (E), 50 μm. (B) Posterior neocortex, with concentration of β-galactosidase–positive cells in superficial layers, fewer β-galactosidase–positive cells in deep layers, and fewest in intermediate layers. (C) Close-up view of superficial cortical layers shown in (B) (asterisks mark corresponding locations). Arrows indicate extreme eccentric position of many of the blue β-galactosidase–positive spots at cytoplasmic edges of neurons. Double staining of β-galactosidase–positive spots with microglial markers was negative. (E) High-magnification view of dentate gyrus of hippocampus. Arrow points to eccentrically located β-galactosidase–positive spot in large neuron.

and *lacZ* sequences in a direct orientation were mixed with a fixed, bulk amount of Tg cerebral cortex DNA. The results suggested that our PCR conditions would detect as few as one to ten recombination events among 10^5 cells. These results confirmed that, if V(D)J recombination occurs in the nonlymphoid tissues that we examined, it is rare.

If not an RSS-mediated inversion, what mechanism allows β-galactosidase to be expressed in the brain? To answer this question, we synthesized cDNA of *lacZ* mRNA isolated from the brain of Tg line 1-7, cloned the amplified cDNA (*15*), and determined the nucleotide sequences of the clones. Among nine randomly selected clones (Fig. 4), one (1-2) produced sequences that started at RSS-B (Fig. 1). This cDNA did not indicate whether the reporter *lacZ* gene was rearranged. On the other hand, the sequence of each of the remaining eight cDNA clones began farther upstream, and six of these began within the *pgk-1* promoter. The promoter was used in the orientation opposite to the conventional one, and no RSS-A sequence was present on these cDNA's adjacent to the RSS-B sequences, which would be expected if the cDNA had been derived from mRNAs transcribed from the inverted *lacZ* gene (Fig. 1). It is likely that these cDNA sequences were derived from mRNAs that were transcribed from the unrearranged *lacZ* gene by using, in the backward orientation, the *pgk-1* promoter of the adjacently inserted plasmid copy (Fig. 4). The Tg line 1-7 contains about ten such tandemly integrated copies of the plasmid. Bidirectional activity of promoters of some eukaryotic housekeeping genes, including the human *pgk-1* promoter, has been reported (*16*), but the cell type and tissue-specific regulation of the "backward" transcription has been unknown. The patterns of Tg β-galactosidase (*lacZ*) expression that we found in the brains of the Tg mice appear to reflect, at least in part, such regulation.

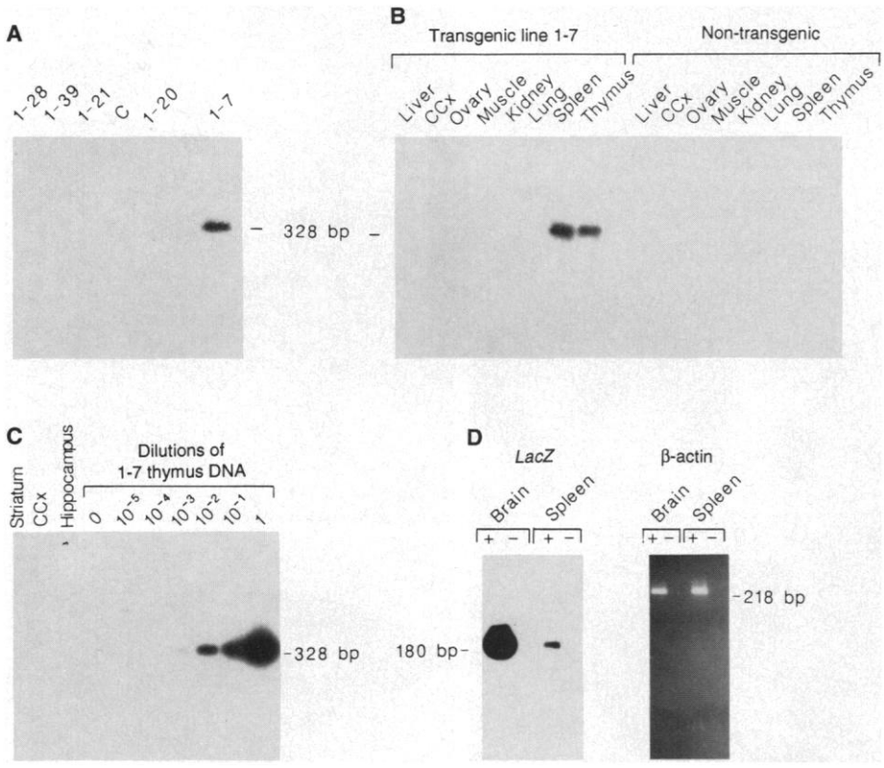

Fig. 3. (**A**) V(D)J recombination of the Tg substrate detected in the thymuses of mice from Tg line 1-7 but not in the thymuses of mice from other Tg lines (1-28, 1-39, 1-21, 1-20) or in the thymus of non-Tg control mice (C). One microgram of genomic DNA was digested with the restriction enzyme Eco RI, precipitated by ethanol, and then subjected to 30 cycles of PCR (*24*) with primers 1 and 2 (Fig. 1). Ten percent of the PCR products was then separated on a 2% agarose gel and examined by DNA (Southern) blotting. ^{32}P-labeled oligonucleotide 29, a 32-bp oligonucleotide that includes RSS-B, was used as a hybridization probe. (**B**) V(D)J recombination of the Tg substrate, which is limited to the lymphoid organs in mice from Tg line 1-7. Assays performed as in (A). CCx, cerebral cortex. (**C**) Sensitivity of the PCR assay. To establish the detection limit of this assay, thymus DNA from strain 1-7 Tg mice was mixed with thymus DNA from non-Tg littermates. The proportion of the Tg DNA in each sample is indicated. Each sample consisted of 1 μg of DNA. Assays were performed as described in (A) except that 40 cycles of PCR were performed instead of 30. A very faint band was visible in the 10^{-5} dilution sample which appeared at very low dilutions of transgene thymus DNA; we presume it is a PCR artifact. CCx, cerebral cortex. (**D**) Abundant transciption of the *lacZ* transgene in the brain. Poly(A)$^+$–positive RNA was isolated (*25*) from mice that had been perfused with 1% sodium nitrate in phosphate-buffered saline and then with deoxyribonuclease I (*26*). One microgram of poly(A)$^+$ RNA was reverse-transcribed (+), or the reverse transcriptase was omitted (−), to establish that PCR products were derived from RNA and were not contaminating genomic DNA. Samples were subjected to 30 cycles of PCR (*24*) with primers 5 and 6 (Fig. 1), and then 10% of the PCR products was separated and examined by Southern blotting as in (A). ^{32}P-labeled oligonucleotide 13 (5′-GTCCAAACTCATCAATGTATCTT-3′) was used as a hybridization probe. PCR analysis was also performed with β-actin–specific primers 11 (5′-GGATGCAGAAGGAGAT TACT-3′) and 12 (5′-AAAACGCAGCTCAGTAACAG-3′) under the same conditions in order to establish that equivalent amounts of cDNA were present in the two samples (right). An agarose gel stained with ethidium bromide is illustrated.

The lack of V(D)J recombination of the Tg substrate in the brain may have resulted from either a lack of recombinase activity or an inaccessibility of the substrate. The latter condition seems to be correlated with the absence of transcripts (*17*). Our ability to clone the cDNA of *lacZ* mRNA from the brain of the Tg line 1-7 suggested that the Tg substrate is accessible in this organ, but there nevertheless remained the possibility that the level of *lacZ* transcription was insufficient for V(D)J recombination to occur. We therefore used a PCR assay with a relatively low number of reaction cycles (that is, 30) in order to compare the levels of *lacZ* transcription in the brain and the spleen of Tg line 1-7. We found the transgene to be transcribed more strongly in the brain than in the spleen (Fig. 3D). These data reinforce the argument that the lack of V(D)J recombination in the brain does not result from the inaccessibility of substrate, although we cannot rule out the possibility that the transgene is inaccessible in restricted regions or during developmental stages of the nervous system where or when V(D)J recombinase is available.

Although we focus on a single transgenic line in this study, we believe our conclusions are valid because in this line V(D)J recombination does take place in the lymphoid organs, and therefore the transgene present in this line is fully capable of undergoing V(D)J recombination. We, of course, do not rule out the possibility that a small fraction of the β-galactosidase–positive cells did undergo V(D)J recombination at a frequency that was smaller than the detection limit of the PCR assay. However, this does not change our conclusion that evidence for somatic V(D)J recombination in the brain, if any indeed occurs, is yet to be obtained.

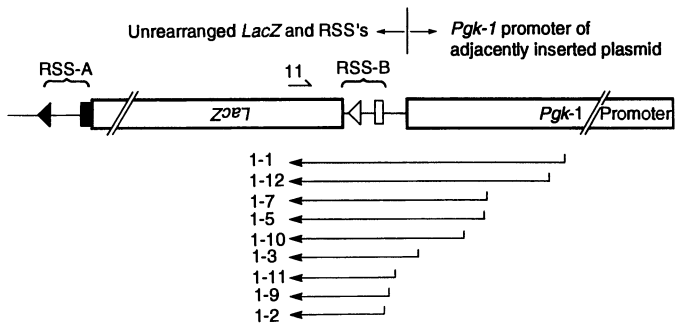

Fig. 4. β-galactosidase mRNA detected in the brains of Tg mice from line 1-7 that are not derived from a recombined transgene. Complementary DNA of mRNA from the brains of line 1-7 Tg mice was amplified by using anchored PCR (*16*). PCR products were then cloned into the vector Bluescript and sequenced as described. The *pgk-1* promoter of an adjacent transgene was present at the 5′ end of all clones that extended beyond the 5′ end of the *lacZ* gene and the adjacent RSS-B. This *pgk-1* promoter, however, is inverted relative to the *lacZ* gene. Individual PCR products are depicted by arrows and are numbered.

This conclusion is at variance with the interpretations drawn by Matsuoka *et al.* (*4*), who used Tg mice that were constructed independently but with a similar strategy. They also found abundant and region-specific expression of *lacZ* in the brain. However, in contrast to our findings with PCR, their PCR assay detected sequences that were apparently produced by inversional recombination of the reporter gene that took place 9 to 138 bp away from the head of the RSSs. Matsuoka *et al.* concluded that "somatic gene rearrangement may be involved in neonatal development" (*18*). Although it is possible that our conclusion differs from that of Matsuoka *et al.* because of variations in the experimental protocols, including differences in the composition of the Tg plasmids, we believe it is more likely that the different conclusions arise from different interpretations of data.

First, in light of our analysis of β-galactosidase cDNA clones, we concluded that the β-galactosidase expression observed in the brains of our Tg mice is most probably due to backward transcription from the promoter of an adjacent transgene rather than RSS-mediated inversion. We suspect that the same may be true for the Tg mice reported by Matsuoka *et al.* because the arrangement of various sequence motifs in the chicken cytoplasmic β-actin promoter that Matsuoka *et al.* used is similar to that in the chicken skeletal α-actin promoter, a demonstrated bidirectional promoter (*19*).

Second, we did not detect any evidence of V(D)J recombination with brain DNA using PCR. In contrast, Matsuoka *et al.* cloned PCR products that they interpret as having been derived from "imprecise" V(D)J recombination that had occurred at sites 9 to 138 bp away from the heads of the RSSs. There are at least two other interpretations of this finding. Recombination may have occurred in vitro during PCR amplification by "PCR mediated recombination" (*20*). The two parental sequences involved in each recombination event reported by Matsuoka *et al.* carry 10-, 3-, and 2-bp homologies, respectively, at the recombination sites. Short homology is expected at the site of PCR recombination, whereas it is more an exception than a rule in the noncoding joints of V(D)J recombination. In one published case 12 nucleotides (*21*), and in another study 7 and 5 nucleotides (*22*), have been shown to be sufficient for PCR recombination. If PCR recombination accounted for the sequences that Matsuoka *et al.* observed, the apparent tissue specificity of the PCR products (*5*, figure 2) may have resulted from sample-to-sample variation, which is commonly encountered with PCR artifacts. Another possibility is that the observed joints resulted from rare illegitimate recombination events that took place among the 15 copies of the integrated plasmid that would be unrelated to developmentally meaningful somatic recombination. Transgenes are generally unstable genetic elements, and short stretches of homology of one to five nucleotides at the junction are generally observed in illegitimate recombination (*23*).

In summary, we suggest that it is premature to conclude whether or not developmentally meaningful somatic recombination occurs in the brain. However, a positive and interesting finding that emerged from our study (and possibly also from that of Matsuoka *et al.*) is that backward transcription in the brain can occur in a highly region- and neuron-specific manner. The physiological role of backward transcription is unknown, but in light of its remarkable tissue or organ specificity, it is possible that backward transcription may participate in the regulation of genes associated with a bidirectional promoter, including genes in the central nervous system.

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7. Plasmid pSPH1 was constructed as follows. Oligonucleotides 29 (5′-TCGAGCACAGTGCTCCAGGGCTAACAAAAACC-3′) and 30 (5′-AGCTTGGTTTTTGTTCAGCCCTGGAGCACTGTG-3′), which constitute RSS-A, and oligonucleotides 27 (5′-GATCCACAGTGGTAGTACTCCACTGTCTGGCTGTACAAAAACC-3′) and 28 (5′-TCGAGGGTTTTTGTACACCCAGACAGTGGAGTACTACCACAGTG-3′), which constitute RSS-B, were annealed and then ligated to a Bam HI–Hind III fragment of plasmid pCH110 (Pharmacia, Piscataway, NJ) that contains *lacZ*. The ligation product was ligated into the Sal I site of pUC19 to form the plasmid pAA14. A Sma I–Pst I fragment of pAA14 was treated with T4 polymerase and then ligated into Bam HI–Pst I–digested and Klenow enzyme–treated plasmid pKJ (containing the murine *pgk-1* promoter, a gift from M. Rudnicki and M. McBurney) to form the plasmid pK14, in which *lacZ* was inserted in an orientation opposite to the *pgk-1* promoter, and the plasmid p11, in which *lacZ* was inserted in the same orientation as the *pgk-1* promoter. An Eco RI fragment of pK14 that contains the *pgk-1* promoter was subsequently ligated into the Eco RI site of pAA14 to form pSPHI.
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12. C. Walsh and C. L. Cepko, *Science* **241**, 1342 (1988). Mice were perfused with 2% paraformaldehyde in PIPES buffer under deep sodium pentobarbitol anesthesia (anesthesia procedure according to guidelines of the Massachusetts Institute of Technology Division of Comparative Medicine), and their brains were postfixed for up to 8 hours at 4°C and soaked overnight in phosphate buffer containing 30% sucrose and 2 mM $MgCl_2$ at 4°C. Parasagittal or transverse sections were cut at 30 or 40 μm on a sliding microtome and were stained for β-galactosidase with X-gal. Selected sections were counterstained with neutral red.

13. The percentage of β-galactosidase–positive cells in the cerebral cortex of the transgenic line 1-7 mice was estimated as follows. First, to establish the total number of cells per unit volume, a Biocom computer (Les Ulis, France) was used to determine the numbers of neutral red–positive cells visible in cortical samples in parasagittal sections 40 μm thick. Stained cells were counted in rectangles (100 by 150 μm) stacked to cover the full depth of the cortex. Values were calculated as cells per 0.1 mm by 0.15 mm by 0.25 mm = 0.006 cubic millimeters. Counts were made for frontal (n = 2), mid-anteroposterior (n = 2), and posterior (n = 1) cortex in two parasagittal sections. Figure 2D illustrates one such section, in which counts were made of the cortex illustrated in B and C (lettered arrowhead in D). Blue (β-galactosidase–positive) spots (Fig. 2) were counted in the same sections and sample sites. For numbers of neutral red cells, we obtained values of 333,000 to 833,000 cells per cubic millimeter. In order to compare our estimates to figures in the literature, we used the estimate of three neurons to one glial cell for rat (*27*) and, multiplying by 0.75, obtained an estimate of 250,000 to 625,500 neurons per cubic millimeter. These values are slightly higher but in the same order of magnitude as those in the literature for mouse neocortex (87,000 to 214,000 per cubic millimeter) (*28*). We estimate that roughly 10% of the cortical cells were β-galactosidase–positive. Some neurons appeared to have more than one X-gal–stained spot associated with them (Fig. 2), and some neutral red–stained cells may have overlapped each other. However, even if we had reduced our estimate by an order of magnitude to take into account these potential sources of error, we still would have obtained a value of 1% of the cortical cells being β-galactosidase–positive.
14. To estimate the absolute sensitivity of the PCR assay used (Fig. 3, A and B), serial dilutions of plasmid p11 (*8*), which contains directly oriented *pgk-1* and *lacZ* sequences, were added to 1 μg of cerebral DNA, the equivalent of approximately 10^5 genomes. PCR amplification of plasmid p11 with primers 1 and 2 resulted in a 266-bp product that was slightly smaller than the 328-bp product expected after amplification of a V(D)J recombined Tg plasmid. Because of the similarity in size and structure of the 328-bp and 266-bp PCR products, it is likely that the sensitivity of this PCR assay is equivalent for the plasmid p11 and the V(D)J recombined Tg plasmid.
15. M. Frohman, in *PCR Protocols*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds. (Academic Press, San Diego, CA, 1990), pp. 28–38. Briefly, 1 μg of poly(A)$^+$ mRNA was primed with oligonucleotide 7 (5′-TTGCGAGTTTACGT-3′) and reverse-transcribed (or reverse transcriptase was omitted to demonstrate that subsequent products were derived from RNA and not from contaminating genomic DNA), and the cDNA product was precipitated by ethanol and then tailed with terminal deoxynucleotide transferase. The tailed cDNA was subjected to PCR amplification with oligonucleotides 8 (5′-GACTCGAGTCGAGTCGACATCGATTTTTTTTTTTTTTT-3′), 9 (5′-GACTCGAGTCGACATCG-3′), and 10 (5′-GGGCATGCGTGTCCCAGCCTGTTTA-3′). PCR parameters for five cycles were 60 s at 94°C, 90 s at 45°C, and 15 s at 72°C. For the subsequent 40 cycles, parameters were as described in (*25*). To clone the amplified cDNAs, an additional 30 cycles of PCR were performed on 1% of the initial reaction products, as described (*25*), with the use of oligonucleotides 9 and 11 (5′-GTTCGAGGGGAAAATAG-3′).
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24. PCR cycle parameters consisted of 60 s at 94°C, 60 s at 50°C, and 90 s at 72°C. Autofluorography was performed on the BioImage Analyzer (Fuji Film). Sequences of oligonucleotide primers used in this assay were as follows. 1, (5′-ATTCTGCACGCTTCAAAAAGCGCA-3′); 2, (5′-CATTGTTCAGAAGGCATCAGTCG-3′); 3, (5′-ACACCCAGACAGTGGAGTACTAC-3′); 4, (5′-AGTGTCCCAGCCTGTTTATCTAC-3′); 5, (5′-TCTCTGTTCTCGCTATT-3′); 6, (5′-AGACATGATAAGATACATTGATG-3′).
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Response: Abeliovich *et al.* make valuable comments about our recent paper (*1*). A common question addressed in our studies and in theirs is whether somatic DNA rearrangement is occurring during development in the brain, as it is in the immune system. With the *lacZ* gene as a reporter, both groups examined rearrangement activity in the transgenic mouse.

Our recombination substrate contained the *lacZ* gene in reverse orientation with respect to a ubiquitous promoter from the β-actin gene, such that rearrangement results in β-galactosidase (β-gal) expression (Fig. 1A). In our construct, sufficient space was maintained in front of the *lacZ* gene to allow for flexibility of the recombination site, because the site specificity of recombination in the brain might not be as strict as in V(D)J joining in lymphocytes. In the transgenic mice, we found that not only lymphatic tissues but certain areas of the brain were stained blue with X-gal, a chromogenic substrate of β-gal (*1*).

To determine whether our substrate was actually rearranging, we amplified DNA sequences surrounding the recombination junctions by PCR. We detected a discrete 400-bp band in spleen and thymus. This band was of the size expected for a typical V(D)J type signal joint, in which a 12-bp RSS and a 23-bp RSS are joined in head-to-head fashion. Rearranged transgenes were not found in DNA from the tail or kidney. Brain samples produced multiple bands, each of which differed in size from the 400-bp band. In contrast to the signal joint found in the lymphatic tissues, the recombination breakpoints we found in brain were not adjacent to RSSs. Recombination junctions were located at various distances away from RSSs. In the brain, the joining detected in the reporter gene thus is imprecise in site specificity and is probably distinct from the V-(D)-J type of recombination seen in lymphocytes.

Abeliovich *et al.* point out the possibility of PCR artifacts in our experiment. In vitro recombination mediated by PCR may need to be considered for recombinants that contain more than several identical residues at recombination sites on both substrates. However, the possibility of in vitro recombination is unlikely for recombinants that contain only a few nucleotides shared between the two substrates. Among 11 clones analyzed, three recombinants contained only one and another three contained only two shared nucleotide residues. Similar junctional structures have been found at recombination breakpoints in circular DNA we have isolated from the mouse brain (*2*). Although no characteristic sequence was conserved at the joint, a short homology (1 to 6 bp) was present at the junction. Because circular DNA is abundant in the brain right after birth, it is possible that recombination in the reporter gene may reflect an increased illegitimate-type recombination activity in the brain.

The reporter gene, which was originally designed to detect V(D)J type joining, may not contain all of the appropriate signals required for recombination in the brain. If this is the case, the recombination detected in the reporter gene may not necessarily reflect the normal joining event in the brain. To address the question of whether or not RSSs are recognized by the recombination machinery in the brain, we have mutated the RSSs in the substrate and are making transgenic mice. One of the recombination activator genes, *RAG-1*, is known to be transcribed in the mouse brain (*3*). Because *RAG-1* is essential for the activation of recombination in lymphocytes, it may play a role in somatic DNA changes in the brain. We are studying whether the staining pattern of the brain of our transgenic mice is affected by *RAG* gene mutations.

In addition to DNA inversion (Fig. 1A), read-through transcription should be considered as a possible alternative mechanism for activating the reporter gene. We considered three possibilities for the read-through of *lacZ* (Fig. 1, B to D). One is the

so-called promoter trap, where the transgene is activated by a nearby promoter in the chromosome (Fig. 1B). To examine this possibility, we analyzed two transgenic founder mice (Nos. 1 and 2) by staining with X-gal. In both mouse founders, we saw β-gal expression in the brain as well as in lymphocytes. Similar regions in the brain were stained in both mouse No. 1 and mouse No. 2 (Fig. 2). Detection of β-gal–positive cells in the brains of two independent founder mice indicates that *lacZ* expression was not an accidental event resulting from transcriptional activity near the integration site of the transgene.

Because multiple copies of transgenes are usually integrated tandemly in a chromosome, read-through activation from the neighboring substrate should also be considered (Fig. 1C). If two substrates were integrated in a tail-to-tail fashion, the inverted *lacZ* gene could be activated by the promoter in the adjacent substrate. In our construct, however, RNA polymerase would have to read through multiple transcriptional termination regions to activate *lacZ*. Furthermore, if read-through transcription did occur, β-gal expression would be expected to appear in many tissues in the transgenic mouse. Because we found X-gal–stained cells only in limited areas in the brain and the spinal cord, in addition to lymphoid tissues, the possibility of nonspecific read-through seems to be unlikely. Furthermore, if read-through transcription causes our blue staining, we would expect that most cells of a particular type would be stained. This is not the case.

Abeliovich *et al.* propose the interesting possibility of backward transcription to explain the activation of the unrearranged reporter. They found that when two substrates were integrated in a tandem orientation, *lacZ* could be activated by read-through transcription initiated backward from the neighboring promoter. While this may occur in the substrate designed by Abeliovich *et al.*, the possibility seems unlikely in our substrate. The neighboring promoter is 13 kb away from the *lacZ* gene in our construct (Fig. 1D), while the distance in the construct of Abeliovich *et al.* is only about 120 bp. Furthermore, even if *lacZ* were transcribed in our construct, a long stretch of the 5′ sequence would have to be processed properly for translation. In this model, one must also explain the tissue-specific and region-specific expression of the reporter gene, unless one assumes that backward transcription is activated within restricted areas in the brain. In any case, analysis of the *lacZ* transcript is an informative way to examine the possibility of backward transcription.

At this point, the recombination we observed in the brain appears to be distinct from V(D)J joining, although the involvement of the *RAG-1* gene is yet to be studied. Because the reporter gene system does not identify rearranging genes in the brain, a new approach should be taken when one tries to identify them. For deletion-type recombination, isolation of circular DNA has been instrumental in the study of antigen receptor gene rearrangement in lymphocytes (*4*). Characterization of brain circular DNA may allow us to identify the rearranging genes in the central nervous system. If rearranging genes are identified, they will give us a new insight into the potential roles of recombination in the brain.

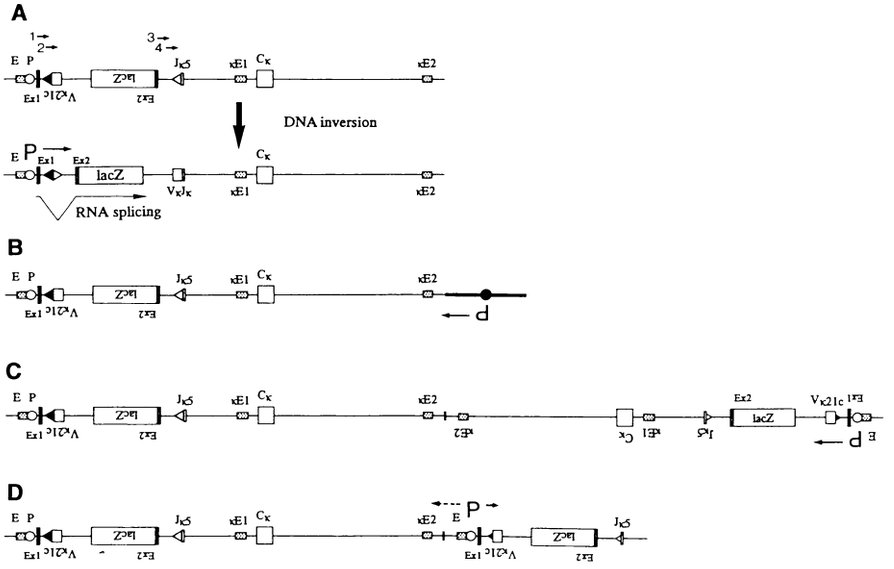

Fig. 1. Activation of the reporter gene in a transgenic recombination substrate. (**A**) DNA inversion relocating *lacZ* is shown activating β-gal expression. In the substrate, the bacterial *lacZ* gene was placed in an inverse orientation relative to the transcriptional direction of the β-actin enhancer-promoter (EP) complex. The *lacZ* gene was flanked by the mouse Ig V_κ and J_κ recombination signals (triangles). Sufficient space was maintained in front of the *lacZ* gene to allow for flexibility of the recombination site. Positions of PCR primers are shown by horizontal arrows (5′ → 3′). (**B**) Read-through transcription of the reporter gene from a nearby promoter in the chromosome (P). This phenomenon is the so-called promoter trap. (**C**) Read-through transcription from the neighboring substrate. If two substrates are integrated in a tail-to-tail fashion, the inverted *lacZ* gene could be activated by the promoter in the adjacent substrate. (**D**) Backward transcription may be another possible case of read-through transcription from the neighboring substrate. If two substrates are integrated in a tandem orientation, *lacZ* might be activated by transcription initiated backward (<- - -) from the neighboring promoter.

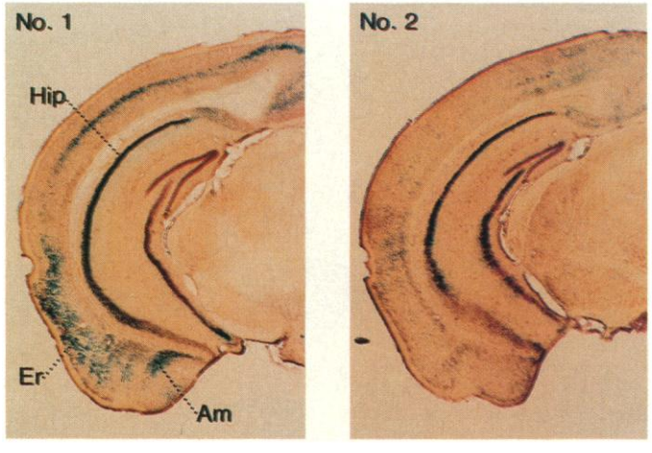

Fig. 2. β-gal activity detected in transgenic mouse brains. Two transgenic founder mice (Nos. 1 and 2) were analyzed. Coronal sections were stained with X-gal and neutral red. Blue-stained regions contain cells expressing β-gal. β-gal–positive cells were found in the entorhinal cortex (Er), hippocampus (Hip), and amygdala (Am).

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Unraveling the Structure of IL-2

Interleukin-2 (IL-2) is the prototype member of an emerging class of pleiotropic cytokines that have extraordinary immunologic and therapeutic promise (*1*). An influential guide for much of the research (*2*) on IL-2 structure and function has been a paper by Brandhuber *et al.* (*3*). However, their x-ray structure of human IL-2 at 3 Å resolution appears to be at odds with experimental results garnered from deletion, mutagenic, and binding studies of IL-2. An alternative protein folding for IL-2, presented here, is closely similar to that of the recently elucidated structures of granulocyte-macrophage colony-stimulating factor (GM-CSF) (*4*) and interleukin-4 (IL-4) (*5*).

Schrader *et al.* have suggested that IL-2 and GM-CSF are structurally homologous proteins that share a low but significant degree of sequence identity (*6*). However, the x-ray structures appear topologically unrelated. Although both IL-2 and GM-CSF show a compact core bundle of four antiparallel α helices, the IL-2 x-ray fold features short hairpin loops between helices (*3*) (Fig. 1A) and is classified as a left-handed, type 0 bundle (*7*). The GM-CSF structure has two long crossover connections between the first and second and third and fourth helices (*4*) (Fig. 1B). Aside from IL-4 (*5*), the latter folding topology [a left-handed, type 2 bundle (*7*)] is observed only in two other cytokine structures, growth hormone and interferon-β (*8*).

I have compared available GM-CSF and IL-4 sequences and structures with IL-2 (Fig. 2) to extend the findings of Schrader *et al.* (*6*). Except for the COOH-terminal helices, my findings show a significant lack of spatial correspondence between sequential helices in the IL-2 x-ray fold proposed by Brandhuber *et al.* and those of GM-CSF and IL-4. I set aside the x-ray helix assignment for IL-2 (*3*) and used six IL-2 sequences to derive a consensus secondary structure of five α helices and two short β strands (*9*) (Fig. 2). Four of the predicted amphipathic α helices and both β strands in the IL-2 model are naturally aligned with similar elements in GM-CSF and IL-4 (Fig. 2). [The IL-2 helix predictions for the most part match the segments located by Cohen *et al.* (*10*) in their early model of human IL-2 based on a right-handed, type 0 bundle.] The largely helical nature of IL-2 is supported by circular dichroism spectra that also distinguish the presence of some β structure (*10*), in further analogy with GM-CSF and IL-4 (*11*). The structure-based alignment of the three cytokines also matches the critical Cys^{58}-Cys^{105} disulfide bridge in human IL-2 (*3*), with analogous Cys^{54}-Cys^{96} and Cys^{46}-Cys^{99} links in the human GM-CSF and IL-4 structures (*4, 5*), respectively (Fig. 2A). Similar helices and loops are also encoded by like exons in IL-2, GM-CSF, and IL-4 genes (Fig. 2) (*1*).

A tertiary model of the human IL-2 structure, similar to GM-CSF/IL-4, that incorporates the predicted α and β segments can be parsimoniously accommodated by the existing x-ray scaffold if a few changes are made (compare Fig. 1, A and B): (i) the four core helices of the x-ray bundle—2-2′, 3, 4, and 6—are replaced by model helices B, C, A, and D while retaining only the 2′-3 (now B-C) loop; (ii) the long A-B crossover loop of the model includes a short helical segment (spatially equivalent to x-ray helix 5) and a β strand which now forms a link to the "downward" chain (Fig. 1B) of a meandering loop that connects extracore x-ray helix 1 to 2. There is no counterpart to x-ray helix 1; (iii) the long C-D crossover loop of the model traces a chain across the space occupied by x-ray helix 1, through the "upward" part of the loose x-ray 1-2 loop to form a second short β strand that leads to COOH-terminal model helix D; (iv) the model requires a relocation of the Cys^{58}-Cys^{105} disulfide from the "top" of the helix bundle (Fig. 1A) to form a new link between model helix B and the C-D loop (Fig. 1B).

By implication, the major helical features of the IL-2 x-ray fold appear to be correct; however, approximately 87% of the protein chain may have to be retraced and several loops rebuilt in order to link the core helices in the new topology. Difficulty in interpreting the electron density map at medium resolution is the likely cause of these errors (*12*). The backbone course of the proposed long loops was perhaps ambiguous because density was poor, a salt bridge or bulky side chain could have mimicked the disulfide link, and a correct sequence tracing through the core helices might have been impeded by unresolved side chains. Indeed, of the several "tether" points located by the x-ray analysis to guide the chain tracing (*3*), only the heavy atom-aided positioning of Cys^{125} in COOH-terminal helix 6 (D in Fig. 1B) remains fully consistent with the new IL-2 topology. Similar mistracings of the structures of *ras* p21 and HIV-1 protease (*13*) have occurred recently.

Among structure modification techniques, fine deletion mapping analyses of GM-CSF have proved effective in outlining structurally important regions of the chain before the determination of the GM-CSF x-ray fold (Fig. 2) (*4, 14*). An analogous, exhaustive structure mapping of mouse IL-2 (*15*) should prove equally diagnostic of the helix boundaries in IL-2. These results are more supportive of the model IL-2 secondary structure (Fig. 2): the x-ray determined helices appear too short (helix 1), lie in apparently nonessential (loop-like) regions (helices 2-2′ and 5), or are out of register with the critical zones (helices 3 and 4).

Another line of inquiry that is distinctly at odds with the x-ray findings concerns the mapping of receptor-binding epitopes in the folded IL-2 molecule. The cellular IL-2

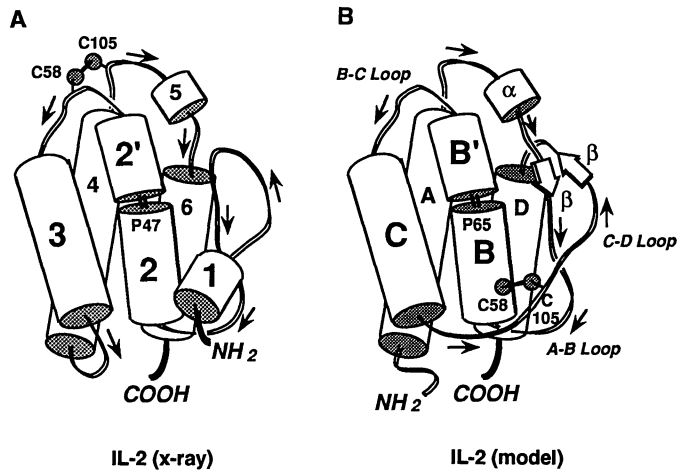

Fig. 1. Comparison of x-ray–derived and model folds of IL-2. (**A**) Schematic drawing of the IL-2 x-ray helix bundle (*3*). Cylindrical helices are marked 1 to 6. Loops are drawn as loose ribbons; Pro^{47} (P47) is marked. The disulfide is noted by linked spheres. In the GM-CSF/IL-4–like IL-2 model (**B**), the chain through the core helices is retraced and reconnected, the disulfide bridge is relocated, the existence of a small β sheet is proposed (*4, 5*); Pro^{65} (P65) is marked. Only helix D remains fully equivalent in sequence to x-ray helix 6.