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(DJ) 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 pSPH1-1 (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 4C1C and J 4A1 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, 1-28, 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 supracallosal (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 representation 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.](http://science.sciencemag.org/content/257/5077/404.f1)

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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; Cbl, 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 non-lymphoid 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 unarranged lacZ gene by using, in the backward orientation, the pgk-1 promoter of the adjacent insert 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.

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.
The 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 inversionsal 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 neuronal 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 results 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.
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 number of neurons per unit volume, a Biocon- 
computer (Les Ulis, France) was used to determine the numbers of neutral red–positive cells visible in cortical samples in parasagittal sections. Neutral red–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 = 0.015 cubic micrometers. Counts were made for frontal (n = 2), mid-anteposterior (n = 2), and posterior (n = 1) cortex in two parasagittal sections. Figure 2D illustrates one such section, in which the longitudinal axis 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 
numerous products were derived, cells RNA, and not from 
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 1 μl of 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 be more than one neutral red– 
spots associated with them (Fig. 2, and some neutral red–stained cells may have overlapped 
each other. However, even if we had reduced our estimates by a factor 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 
amplification (Fig. 3A and B), serial dilutions of plasmid p11, which contains directly oriented 
pgk-1 and lacZ sequences, were added to 1 μg of 
cerebral RNA. The equivalent of approximately 1000 copies of plasmid p11 for 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(DJ) recombi-
nated DNA. Because of the similarity in size and 
structure of the 328- and 266-bp PCR products, 
reversely amplification (reverse transcriptase) 
was performed to demonstrate that subsequent 
transcripts were the result of amplification of plasmid p11 and not of contaminating genomic DNA, and the cDNA product was precipitated by ethanol and then 
tailed with terminal deoxynuclease 
transferase.

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21. 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’--ATCTTG- 
CACGTCCTAAAAGCGC-3’), 2. (5’--ATCTTG- 
TCAGTGCAGTATCG-3’), 3. (5’--GACCA- 
GAGTGGATGACTAC-3’), 4. (5’--AGTGTC-
CCCGCTTTTATAC-3’), 5. (5’--TCTCCTG- TCCGATT-3’), 6. (5’--ACACATGAACTACATT-
GATG-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 recombi-
nation in the brain might not be as strict as in V(DJ) 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 crho-
mental staining of the brain’s vasculature.

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. Recombi-
nation 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 recombi-
nation seen in lymphocytes. 

Abeliovich et al. point out the possibil-
ity 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 bet-
ween 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 se-
cence was conserved at the joint, a short homology (1 to 6 bp) was present at the junction. Because circular DNA is abun-
dant 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 recombi-
nation machinery in the brain, we have mutated the RSSs in the substrate and are making transgenic mice. One of the recombi-
nation 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 trans-
genic mice is affected by RAG gene muta-
tions.

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