

DNA Rearrangement Activity during Retinoic Acid-Induced Neural Differentiation of P19 Mouse Embryonal Carcinoma Cells

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Because of the many superficial similarities between the immune system and the central nervous system, it has long been speculated that somatic DNA recombination is, like the immune system, involved in brain development and function. To examine whether or not the V(D)J recombination signals of the immune system work in an *in vitro* neural differentiation model, the P19 mouse embryonal carcinoma cell line was transfected with a reporter gene that is designed, when rearranged, to express bacterial β -galactosidase, which was previously reported to exhibit somatic DNA recombination in the transgenic mouse brain. The cloned cells were then induced into neural cells by retinoic acid treatment. This neural induction treatment resulted in the cloning of a P19 cell line that showed a high incidence of β -galactosidase-positive cells. Most of these β -galactosidase-positive cells were immunocytochemically identified as either neurons, neuroepithelial cells, or astrocytes. The 5'-end sequences of the β -galactosidase transcripts expressed in the induced cells were analyzed, and sequences were found that seemed to reflect DNA rearrangement through re-integration of the reporter gene into the host genome. However, the V(D)J recombination signals did not work in the *in vitro* model. These results suggested that DNA rearrangement activity though integration increased during neural differentiation of P19 cells.

Key words: DNA rearrangement, neural differentiation, retinoic acid, P19 embryonal carcinoma cell

Unlike germ-line cells, somatic cells of multicellular organisms do not necessarily require all of a species' DNA information, as they are fated to lose their totipotency and survive for only one generation. Some exceptions to the DNA constancy rule have been reported: chromatin diminution in nematodes [1, 2], chromosomal elimination in ciliated protozoa [3] and flies [4, 5], and DNA excision in lymphocytes of vertebrates [6]. Somatic DNA recombination plays an important role in the immune system: V(D)J recombination and

class switching of immunoglobulin genes in lymphocytes are followed by DNA excision [7]. There are many superficial similarities between the immune system and the central nervous system: extreme diversity, enormous capacity for memory, extensive developmental programmed cell death [8, 9], cytokines and their receptors [10], immunoglobulin superfamily members [11], and proteins involved in DNA rearrangement, such as Rad51, a homolog of *Escherichia coli* (*E. coli*) RecA [12], and topoisomerase II β [13]. Because of these parallels, it has long been speculated that somatic DNA recombination is, like the immune system, involved in brain development and function [14].

Recently, cadherin-related neuronal receptor/

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protocadherin (*CNR/Pcdh*) genes, which are subfamily members of the cadherin superfamily, have been shown to consist of multiple gene clusters, including multiple variable exons and several constant exons similar to the immunoglobulin and T-cell receptor gene clusters in the immune system [15]. Detailed studies have demonstrated that the diverse molecular expression of the *CNR/Pcdh* genes in neurons has been achieved through the choice of an alternative promoter by each variable exon and alternative *cis*-splicing of the transcripts, and, at a far lower level, through *trans*-splicing of the transcripts [16, 17]. Rearrangement of the gene assembly DNA, such as the deletion of genomic DNA in the immune system, has not been found in the *CNR/Pcdh* gene clusters [16, 17].

Matsuoka *et al.* reported that bacterial β -galactosidase was expressed in lymphocytes and certain regions of the brain in transgenic mice possessing a recombination reporter gene, and speculated that this expression was a result of DNA rearrangement [18]. However, using a similar approach, Kawaichi *et al.* were able to detect DNA recombination in only the lymphocytes of transgenic mice [19].

In the present study, we examined whether or not the V(D)J recombination signal of immunoglobulin works in an *in vitro* neural differentiation model, the P19 mouse embryonal carcinoma cell line [20], as Matsuoka *et al.* demonstrated in transgenic mice. We then analyzed the 5'-end sequences of the reporter gene transcripts expressed during neural differentiation.

Materials and Methods

Construction of the recombination reporter gene. The EcoRI fragment (~20 kbp) of the recombination reporter gene plasmid, pkZ1, kindly provided by Dr. Sakano, H. [18], was integrated into the EcoRI site of a pSV2neo vector so that the chicken β -actin enhancer-promoter complex had the same transcriptional direction as the neomycin-resistant gene (Fig. 1A). Before the integration of the reporter gene, the BamHI site of the pSV2neo vector had been altered into a Not I site for the following linearization. The resulting reporter plasmid, pRECneo, was linearized by Not I restriction enzyme digestion and separated by 0.8% agarose gel electrophoresis. The fragment was excised and purified using glass powders included in a FlexiPrep kit (Amersham Biosciences, Piscataway, NJ, USA).

Transfection and cloning of cells. Fifteen

μ g of linearized DNA was transfected into 3×10^6 P19 mouse embryonal carcinoma cells (ATCC CRL 1825) by a calcium phosphate-mediated procedure as previously described [21]. G418 (Invitrogen, Carlsbad, CA, USA) was added to the culture medium at 400μ g/ml for selection of G418-resistant cells.

Southern blot analysis. Genomic DNA of P19 and that of the cloned cells, P19REC09, were purified using a standard phenol-chloroform extraction [22]. The genomic and pRECneo plasmid DNAs were digested with EcoRI and/or XbaI restriction enzyme. Each 10μ g of the digested genomic DNA and each 500 ng of the digested plasmid DNA was separated by 0.8% agarose gel electrophoresis and blotted onto a Hybond-N+ membrane (Amersham Biosciences) by a standard capillary method [22]. The membrane was fixed with 0.4 N NaOH. Hybridization was performed in $5 \times$ SSC, 0.1% (w/v) lauroylsarcosine, 0.02% (w/v) SDS, and 1% blocking agent (Roche Diagnostics, Basel, Switzerland) with a digoxigenin-labeled lacZ gene-specific oligonucleotide probe (Z-10, 5'-TTCCGGCACCCTTCTGGTGC-3') at 45°C. The 3' end of the Z-10 oligonucleotide was labeled with digoxigenin using digoxigenin-11-ddUTP and terminal transferase (Roche Diagnostics). The hybridized membrane was washed in $0.6 \times$ SSC and 0.02% SDS at 50°C. Non-R1 chemiluminescent detection with an alkaline phosphatase-conjugated anti-digoxigenin antibody and a CDP-Star chemiluminescent substrate was performed according to the manufacturer's instruction manual (Roche Diagnostics).

Cell culture and differentiation treatments. P19 cells were cultured on tissue culture-grade dishes in a minimal essential medium (α MEM, Cosmo Bio, Tokyo, Japan) supplemented with 10% fetal calf serum (Invitrogen) at 37°C in a 5% CO₂ atmosphere. Differentiation treatments were performed as described by Rudnicki *et al.* [20]. Briefly, for aggregation (AG) treatments, bacterial-grade culture dishes were used. For retinoic acid (RA) treatments, all-trans retinoic acid (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium at 5×10^{-7} M. For dimethyl sulfoxide (DMSO, Sigma-Aldrich) treatments, DMSO was added to the medium at 1% v/v. For retinoic acid with aggregation (RA + AG) treatments, cells were cultured on bacterial-grade culture dishes in medium containing 5×10^{-7} M RA. For dimethyl sulfoxide with aggregation (DMSO + AG) treatments, cells were cultured on bacterial-grade culture dishes in medium containing 1% v/v DMSO.

Histochemical detection of bacterial β -galactosidase activity. As described by Sanes *et al.*, cultured cells fixed with 2% formaldehyde, 0.2% glutaraldehyde were incubated for 4-16 h at 37°C in a histochemical reaction mixture containing 1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal, Sigma-Aldrich) [23]. β -galactosidase-positive, blue-stained cells were counted in a given area using a microscope. Total cell counts were obtained with a hemacytometer.

Immunocytochemistry. Following histochemical detection, immunocytochemical staining was performed. Monoclonal antibodies to nestin (clone R401, 1:500, BD Biosciences PharMingen, San Diego, CA, USA), neurofilament 200 kDa (clone NE14, 1:500, Sigma-Aldrich), and glial fibrillary acidic protein (clone G-4-A, 1:500, Roche Diagnostics) were used as primary antibodies. Biotinylated anti-mouse IgG horse serum (Vector, Burlingame, CA, USA) was the secondary antibody. Alkaline phosphatase-conjugated streptavidin (Vector) was used for labeling the biotinylated secondary antibodies, and 3-hydroxy-N-2'-biphenyl-2-naphthalenecarboxamide phosphate (Roche Diagnostics) was the alkaline phosphatase substrate.

Rapid amplification of cDNA 5'-ends (5' RACE) and sequencing. 5' RACE was performed as previously described [24]. Messenger RNA (mRNA) was purified from 4-day RA-treated and 4-day RA + AG-treated P19REC09 cells using a QuickPrep Micro mRNA Purification Kit (Amersham Biosciences) according to the manufacturer's protocols. Contaminating genomic DNA was digested with 1 unit per μ l DNase I (Amersham Biosciences) in a reaction buffer containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, and 2 mM MgCl₂. First-strand cDNA was reverse-transcribed from the mRNA with a lacZ-specific primer (Z-08, 5'-TG TAGC CAGCTTTTCATCAAC-3') and a reverse transcriptase, SuperScript II (Invitrogen). Poly A tails were added to the cDNA with 0.5 units per μ l of terminal deoxynucleotidyl transferase (Toyobo, Osaka, Japan). First-round polymerase chain reaction (PCR) was performed with an adapter primer for the poly A tail (ADE, 5'-GAGTCGACTCGAGAATTCTTTTTTTTTTTTTTT TTTT-3') including an EcoRI site and an inner lacZ-specific primer (Z-09, 5'-ATTGACCGTAATGGGAT AGG-3') by using ExTaq thermostable DNA polymerase (Takara Bio, Otsu, Japan) and an aliquot of the cDNA as a template under the following conditions: denaturing at 95°C, annealing at 55°C, extension at 72°C, 45

cycles. Second semi-nested PCR was carried out with the adapter primer (ADE) and another, more-inner lacZ-specific primer (ZE-06, 5'-GCGAATTCCTTGGTGTA GATGGGCGCATCGTAACCGTGC-3') containing an EcoRI site, by using an aliquot of the first PCR product as a template under the following conditions: denaturing at 95°C, annealing at 65°C, extension at 72°C, 30 cycles. The PCR products were digested with an EcoRI restriction enzyme and ligated to the EcoRI site of the λ ZIPLox phage (Invitrogen). To package the integrated λ ZIPLox phage DNA, GigaPack III Gold packaging extract was used according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). The constructed phage library was screened by a digoxigenin-labeled lacZ gene-specific oligonucleotide probe (Z-10, 5'-TTCCGG CACCGCTTCTGGTGC-3'). Digoxigenin labeling and non-radioisotopic detection were performed according to the Southern blot analysis procedure. Sequences of positive clones were analyzed by an ABI dye terminator cycle sequencing kit and an ABI sequencer model 373 (Applied Biosystems, Foster City, CA, USA).

Results

Cloning of cells. We isolated 11 G418-resistant clones out of 3×10^6 cells transfected with 15 μ g of the reporter DNA. We subjected the isolated clones to retinoic acid and aggregation treatment to induce neural cell differentiation. One clone, the P19REC09 cell, was found to have a high incidence of β -galactosidase-positive cells in the differentiated state (Fig. 4) according to histochemical X-gal staining [23], while almost no β -galactosidase-positive cells appeared in the undifferentiated state (data not shown).

Southern blot analysis. Genomic Southern blot analysis with a lacZ-specific probe confirmed that the P19REC09 cloned cell contained a full length of the reporter gene (Fig. 2). A single \sim 20-kbp fragment was identified on the lane of EcoRI-digested DNA (lane 1 in Fig. 2), and a single \sim 8-kbp fragment was found on the lane of EcoRI and XbaI digestion (lane 3 in Fig. 2). These fragments were consistent with the pRECneo reporter plasmid fragments (lanes 5 and 6 in Fig. 2, respectively) and the expected fragment sizes (Fig. 1A). Thus the cloned cell contained a full-length reporter gene.

Differentiation treatments. The incidence of β -galactosidase-positive cells was analyzed in the time course of induction treatments (Fig. 3). In the treatment

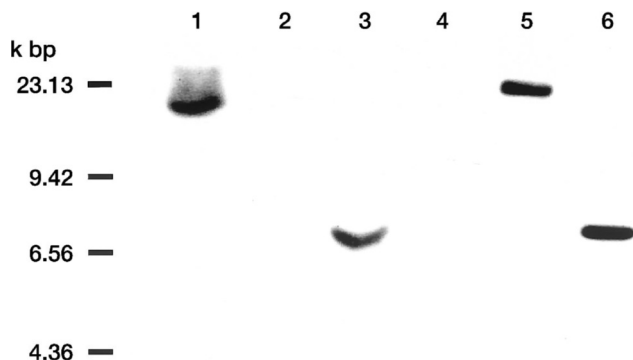


Fig. 2 Southern blot analysis. Each 10 μ g of genomic DNA was separated by 0.8% agarose gel electrophoresis and blotted onto a nylon membrane. Detection was performed with a bacterial lacZ-specific probe. Genomic DNA from the cloned P19 cell with the reporter gene was digested with EcoRI (lane 1) or EcoRI and XbaI (lane 3). Genomic DNA from the P19 cell line without the reporter gene was also digested with EcoRI (lane 2) or EcoRI and XbaI (lane 4). The reporter gene plasmid was digested with EcoRI (lane 5) or EcoRI and XbaI (lane 6).

of RA without aggregation (RA) for neuroepithelial cell induction, the incidence of β -galactosidase-positive cells was 201.7 ± 24.2 at day 2, 534.9 ± 15.9 at day 4, and 149.0 ± 8.1 at day 7 (per 10^5 total cells, mean \pm SEM, $n = 5$). In the treatment of RA with aggregation (RA + AG) for induction of neurons and glia, the incidence was 102.2 ± 18.2 at day 2, 194.8 ± 18.3 at day 4, and 19.2 ± 2.3 at day 7. There were significant increases at all days of RA treatment and at days 2 and 4 of RA + AG treatment *vs.* no induction treatment (0.8 ± 0.05 , one-way ANOVA, $P < 0.05$). On the other hand, there were no significant differences at day 7 of RA + AG treatment for the neural cell induction or at any day of the other treatments for the induction of non-neural cells: aggregation only (AG), DMSO without aggregation (DMSO), and DMSO with aggregation (DMSO + AG). Treatment by retinoic acids with or without aggregation significantly increased the incidence of β -galactosidase-positive cells.

Immunocytochemistry. To clarify whether β -galactosidase-positive cells are neural cells or not, X-gal histochemical and immunocytochemical double-staining studies were performed (Fig. 4). Histochemically detected blue-stained β -galactosidase-positive cells induced by treatment of RA (Fig. 4A and D) or RA + AG (Fig. 4B, C, E, F) were further stained with antibodies

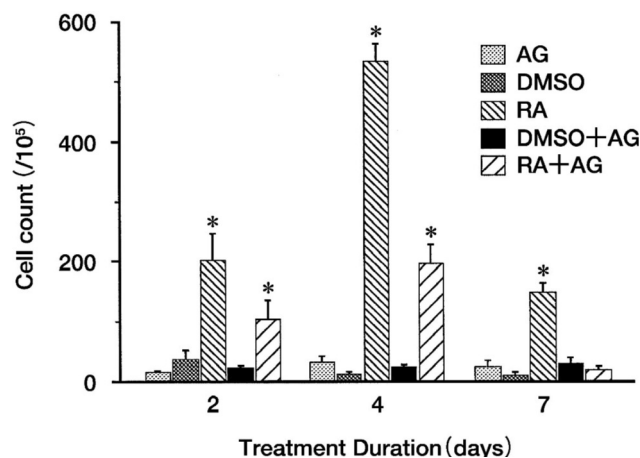


Fig. 3 Effects of differentiation treatments on the incidences of β -galactosidase-positive cells and their time course. Abbreviations refer to treatment types. AG, aggregation; DMSO, dimethylsulfoxide; RA, retinoic acid; DMSO + AG, dimethylsulfoxide with aggregation; RA + AG, retinoic acid with aggregation. Each column represents the mean \pm SEM ($n = 5$). The asterisks denote significant differences *vs.* control of undifferentiated cells ($P < 0.05$, one-way ANOVA).

to nestin (Fig. 4D), neurofilament 200kD (Fig. 4E), or glial fibrillary acidic protein (Fig. 4F); these are markers of neuroepithelial cells, neurons, and astrocytes, respectively. The neural markers stained almost no β -galactosidase-positive cells induced by the other treatments (AG, DMSO, DMSO + AG) (data not shown). Most β -galactosidase-positive cells consisted of neuroepithelial cells, neurons, and astrocytes, in addition to a trace portion that was non-neural (Fig. 4B and E).

Sequence analysis. To determine the transcript sequence involved in the expression of bacterial β -galactosidase activity, we analyzed the 5' ends using the 5' RACE method. As summarized in Fig. 5, sequence analysis of 54 randomly selected clones showed 25 different sequences of 5' ends. Of these sequences, 17, indicated by arrowheads, included the start codon of artificial β -galactosidase from *E. coli* and were expected to express functional enzymes, while the other 8, indicated by open arrowheads, were from downstream of the start codon, suggesting that they failed to gain enzyme activity. Two of the 17 transcripts with the start codon (exons 1a and 1b in Fig. 5) were found to have parts of the chicken β -actin first intron in the reporter gene as first exons, using cryptic GT-AG splicing signals; 2 others (exons 1c and 1d in Fig. 5) had unknown short sequences that did not obey the GT-AG splicing rule. Of the 25

transcripts, 20 contained one guanine residue at each 5' end, which strongly suggested that these were not derived from artificially truncated mRNAs [25].

Discussion

The P19 cell is well established as an *in vitro* model of neural differentiation. It is known that the number of RA-induced neuroepithelial cells peaks at day 3 after RA treatment and declines by day 5; it is also known that RA + AG-induced neurons become overwhelmed by highly proliferative, non-neural fibroblast-like cells after day 5, and that RA + AG-induced astrocytes appear after day 10 [20, 26]. Thus the time course of RA and RA + AG-induced β -galactosidase-positive cells (Fig. 3) corresponds with that of RA-induced neuroepithelial cells and RA + AG-induced neurons and neuroepithelial cells, respectively. This was confirmed by immunocytochemical analysis (Fig. 4).

Analysis of the 5'-end sequences of the reporter gene transcripts revealed that DNA rearrangement through re-integration of the reporter gene into the host genomic DNA might result in the expression of β -galactosidase activity, but the V(D)J recombination signals of the reporter gene did not work in the P19 neuronal differentiation model, in contrast to the previous findings in transgenic mice reported by Matsuoka *et al.* [18]. Because the *in vivo* brain contains an enormous number of neurons, the fragments rearranged at or around the V(D)J recombination signals in the transgenic mice brain might exist as a result of DNA rearrangement that increases during brain development but is not specific to the recombination signals. Assumed modes of bacterial β -galactosidase mRNA expression are shown in Fig. 1B to H. The accurate (Fig. 1B) and ambiguous (Fig. 1C) inversions in transgenic mice previously reported by Matsuoka *et al.* [18] were not detected in this study. These inversions may not be involved in the expression of the reporter gene observed in the induced neural cells. The 2 transcripts with unknown sequences (exons 1c and 1d in Fig. 5) may have been generated by integration in an exon of a certain gene expressed in the induced cells (Fig. 1E), because they did not obey the GT-AG splicing rules, unlike the 2 transcripts with chicken introns (exons 1a and 1b), as shown in Fig. 5. Their sequences were too short to confirm the rearranged sites in genomic DNA. Three modes (Fig. 1F, G, H) can result in the other 23 transcripts: 2 transcripts with the chicken

introns (exons 1a and 1b in Fig. 5) and the other 21 transcribed from upstream or downstream of the start codon of β -galactosidase in the reporter gene, as indicated by the closed and open arrows (Fig. 5). Because the ability of enhancers to activate transcription is relatively unrelated to their position, orientation or, to a lesser degree, the distance from the target promoter [27], and because expression of multiple transcripts has been observed, the most probable mode of expression is integration in regions controlled by an enhancer activated in the induced cells (Fig. 1F). Integration in the vicinity of a certain promoter (Fig. 1G) is less probable, as promoters need to be a limited distance from a transcription initiation site. The mode of cryptic promoter activation in the reporter gene without rearrangement (Fig. 1H) is also unlikely, because it needs a rare trans-acting factor that is activated only in limited populations — that is, in less than about 0.5% of neuroepithelial cells, neurons, and astrocytes — and also causes expression of multiple transcripts from the single reporter gene. Since the Southern blot analysis showed that the cloned P19 cell contained a full-length reporter gene, we can also exclude the possibility that the *lacZ* gene became integrated in the vicinity of neural cell-specific gene promoters of the cloned P19 cell before the induction treatment. The 2 transcripts with unknown sequences (exons 1c and 1d in Fig. 5) in the 4th mode (Fig. 1E) indicate genomic DNA rearrangements, and the other 23 clones seem to be examples of the 5th mode, indicating DNA rearrangement by one of the integrations (Fig. 1F). Finally, these many different 5'-end sequences of the reporter gene transcripts expressed in neural cells suggest that the transcripts may be a result of DNA rearrangement through re-integration of the reporter gene into the host genomic DNA, and that the recombination signals for site-specific V(D)J rearrangement of immunoglobulin genes do not work in the neural differentiation of P19 cells. This conclusion is consistent with the finding that only a small portion of total neural cells were β -galactosidase-positive, because the 5' RACE analysis revealed that many transcripts did not include the translation start codon of the reporter gene. This suggested that far more transcripts not detected by the 5' RACE were expressed in the neural cells.

The mechanisms of DNA re-integration specific to neural differentiation are yet to be elucidated. DNA integration, on the other hand, is known to include generation and rejoining processes of double-strand

breaks (DSBs). Non-homologous end joining and homologous recombinational repair are known to be involved in DSB repair [28]. Recent investigations in the immune system demonstrated that DNA repairs, especially base excision repair and homologous recombination, play a key role in the pathway of class-switch recombination and gene conversion [29]. Non-homologous end-joining repair is involved in the repair of DSBs generated by RAG-1 and RAG-2 [7]. These observations indicate that DNA repair plays an important role in DNA rearrangements, such as class-switch recombination, gene conversion, and V(D)J recombination.

The findings of the present study suggest that DNA rearrangement activity as a result of integration may increase during neurogenesis, and that the V(D)J recombination signal in the immune system does not work during neural differentiation. Increased activity to repair DSBs during chromatin remodeling in neurogenesis may lead to DNA rearrangement of the unstable integrated reporter gene. Further investigation of the molecular mechanisms underlying the generation and repair processes of DSBs in neurogenesis will elucidate the entity of neuronal differentiation.

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