Neurofilament Deficiency in Quail Caused by Nonsense Mutation in Neurofilament-L Gene

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Abstract. The existence of a neurofilament-deficient mutant of Japanese quail was recently documented (Yamasaki, H., C. Itakura, and M. Mizutani. 1991. Acta Neuropathol. 82:427-434), but the genetic events leading to the neurofilament deficiency have yet to be determined. Our molecular biological analyses revealed that the expression of neurofilament-L (NF-L) gene was specifically repressed in neurons of this mutant. To search for mutation(s) responsible for the shutdown of this gene expression, we cloned and sequenced the NF-L genes in the wild-type and mutant quails. It is eventually found that the NF-L gene in the mutant includes a nonsense mutation at the deduced amino acid residue 114, indicating that the mutant is incapable of producing even a trace amount of polymerization-competent NF-L protein at any situation. The identification of this nonsense mutation provides us with a solid basis on which molecular mechanisms underlying the alteration in the neuronal cytoskeletal architecture in the mutant should be interpreted.

Neurofilaments (NFs), a member of the intermediate filament (IF) protein family, are major constituents of neuronal cytoskeleton and primarily composed of three different protein subunits referred to as NF-L, NF-M, and NF-H in vertebrates (Shaw, 1991). Although NFs have been actively studied for many years, we can describe the function of NFs only in vague terms. For example, a function of NFs is believed to control axonal calibers as mechanical space filling molecules (Hoffman et al., 1987). However, it is still unclear whether this is the exclusive function of NFs in vivo. Although several lines of evidence have suggested that NFs are not vital for a certain class of neurons, there is no convincing evidence as yet for dispensability of NFs in the nervous system of vertebrates (Palay and Chan-Palay, 1974; Phillips et al., 1983; Lasek et al., 1983, 1985). In this regard, the recent discovery of a NF-deficient mutant of Japanese quail ( Coturnix coturnix japonica), named "quiver" (Quv), strongly arrested our attention because this mutant may shed light on the uncovered function of NFs in vivo. Although several lines of evidence have suggested that NFs are not vital for a certain class of neurons, there is no convincing evidence as yet for dispensability of NFs in the nervous system of vertebrates (Palay and Chan-Palay, 1974; Phillips et al., 1983; Lasek et al., 1983, 1985). In this regard, the recent discovery of a NF-deficient mutant of Japanese quail ( Coturnix coturnix japonica), named "quiver" (Quv), strongly arrested our attention because this mutant may shed light on the uncovered function of NFs in vivo (Mizutani et al., 1992; Yamasaki et al., 1991, 1992).

Previous studies have characterized the lesions in Quv to some extent: Quv shows no big differences in fertility, hatchability, and posthatching mortality (Mizutani et al., 1992); the lesions in Quv are inherited as a single autosomal recessive trait after Mendelian laws (Mizutani et al., 1992); the axons of central and peripheral nervous system of Quv are deficient of 10-nm filaments (i.e., NFs) but with well-developed myelin sheaths, and the size distribution of the axonal calibers significantly shifted to small size classes.

Materials and Methods

Japanese Quails

All quails were kindly provided from Nippon Institute for Biological Science (Tokyo, Japan), where the Quv strain was established. Adult quails from two different strains, TKP and WE, were used as wild-type ones. TKP strain has been used as a wild-type one in previous studies, and the Quv
originated from WE strain (Somes, 1988; Mizutani et al., 1992). Unless otherwise stated, we used quails from TKP strain as wild-type ones, since there were no noticeable differences in the neuronal cytoskeleton between the TKP and WE strains.

**Immunoblotting Analysis of NF Subunit Proteins**

NF-enriched cytoskeleton fractions were prepared from spinal cords of wild-type and Quv quails (Crosneke et al., 1981). Spinal cords or spinal ganglia of wild-type or Quv quails were lysed in loading buffer for SDS-PAGE (1% SDS, 10 mM Tris-HCl, pH 6.8, 1 mM EDTA, 0.1% 2-mercaptoethanol, 5% sucrose) by sonication, and the proteins were analyzed on 12% SDS-PAGE (Laemmli, 1970). For immunoblotting analysis, the separated proteins were electrotransferred onto a nitrocellulose filter and the membrane was incubated at 4°C overnight with mAb against pig NF-L or NF-M in TBS containing 0.5% Tween-20. These mAbs against NF-L and NF-M (NR-4 and NWB18, respectively) were purchased from Boehringer Mannheim GmbH, Biochemica (Mannheim, Federal Republic of Germany). The proteins reacting with the mAbs were colorimetrically detected with a second antibody conjugated with alkaline phosphatase.

**RNA Blotting Analysis of NF Subunit mRNAs**

Total cellular RNAs were prepared from wild-type and Quv quails as described (Chomczynski and Sacchi, 1987). The RNAs were separated on a 1% agarose gel containing 2.2 M formaldehyde, and then transferred onto a nylon membrane. As probes for NF-M and NF-H mRNAs, we used DNA fragments cloned in the chicken NF-M and chicken NF-H genes, respectively, generated by the PCR (for the rat NF-H probe, the residues 1550-2380 of the reported cDNA [Dautigmy et al., 1988]; for the chicken NF-M probe, the residues 868-1494 of the reported cDNA [Zopf et al., 1987]). As a probe for NF-L mRNA, we used quail NF-L cDNA cloned in this study as described below. The probes were labeled with 32P using a random primer labeling kit (Prime-It labeling system: Stratagene, La Plata, MD). Hybridization was performed at 65°C in 6× SSPE (1× SSPE = 150 mM NaCl, 15 mM sodium citrate, 1 mM EDTA) containing 0.1% SDS. The signals were detected by autoradiography with two intensifying screens.

**Cloning and Sequencing of Quail NF-L cDNA and Gene**

First, we tried to clone a NF-L cDNA from a wild-type quail. A brain cDNA library was constructed essentially as described from the instructions of the supplier of a reverse transcriptase (D'Alessio et al., 1988; Gibco BRL, Gaithersburg, MD), which is briefly described below. Total cellular RNA isolated from the whole brain was reverse transcribed with SuperScript RNaseH- (Gibco BRL) using a Not1-adaptor primer (5'-CAAGAC-GGCCGCGACGACT[T31]) into a blunt-ended, double-stranded cDNA, and then ligated with a phosphorylated MluI linker (5'-CGAC-GGCCGAC) into the modified pBluescript SK- vector (Invitrogen Corp., La Jolla, CA). Hybridization was performed at 65°C for 16 h in the presence of 7% SDS as described (Church and Gilbert, 1984). After the hybridization, the membrane was extensively washed at 50°C with 0.2× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, 1 mM EDTA) containing 0.1% SDS. The signals were detected by autoradiography with two intensifying screens.

**Miscellaneous**

Oligonucleotides were synthesized on a Gene Assembler Plus DNA syn-
Analysis of NF Subunit Proteins in Quv

To identify which gene contains mutation(s) responsible for the NF deficiency, we first tried to characterize the changes in the NF subunit production pattern in Quv. Previous studies have already documented that the axons of central and peripheral nervous system of Quv do not contain 10-nm filaments (Yamasaki et al., 1991, 1992). For ascertaining this at the protein level, we prepared NF-enriched cytoskeletal fractions from spinal cords of the wild-type and Quv quails and analyzed them on SDS-PAGE. As shown in Fig. 1 a, Quv did not contain any of the NF triplet proteins in the neuronal cytoskeletal framework while other cytoskeletal components, such as actin, tubulin, and glial fibrillar acidic protein, were present in the same sample (Fig. 1 a).

To further examine whether or not Quv is devoid of NF subunit proteins, we analyzed the proteins in two different neuronal tissues, spinal cords and spinal ganglia, by immunoblotting with anti-NF-L and anti-NF-M mAbs (Fig. 1 b); spinal cords and spinal ganglia are regarded as axon- and perikaryon-enriched sources, respectively. We did not examine the amounts of NF-H since specific detection could not be achieved with anti-pig NF-H mAb commercially available. The results of the immunoblotting analysis of the neuronal tissues in Quv showed remarkable contrasts to those in the wild-type quail (Fig. 1 b): (a) the NF-L band was not visualized in the sample either from spinal cords or from spinal ganglia in Quv; (b) the NF-M band, either the 130- or the 150-kD form, was hardly detected in the sample from spinal cords of Quv; (c) in the sample from spinal ganglia of Quv, only the 130-kD band of NF-M could be detected but was much weaker than that in the wild-type lane. Since Yamasaki et al. (1992) assigned that the 150- and 130-kD forms of NF-M corresponded to phosphorylated and unphosphorylated NF-Ms, respectively, Quv appeared to contain a small amount of only unphosphorylated NF-M mainly in the perikarya of neuronal cells.

These results are in essential agreement with the previous data by Yamasaki et al. (1992), from which they hypothesized that the NF deficiency in Quv results from the inability of NF assembly due to the lack of NF-L. Although this hypothesis was a likely one, the amount of information was not sufficient to confidently determine which gene is to be investigated at the nucleotide sequence level. Hence, we proceeded to examine the NF subunit gene expression at the mRNA level for obtaining more information about the changes in the NF gene expression in Quv.

Analysis of NF Subunit mRNA Levels in Quv

Fig. 2 shows the results of RNA blotting analyses for NF subunit mRNAs in Quv. The most prominent change was observed in the NF-L mRNA level, although the mRNA level of NF-H was also lowered to some extent; under the exposure conditions used in Fig. 2 (5 h at −70°C), we could not see any NF-L mRNA band in the Quv lane whereas a strong NF-L mRNA band appeared in the wild-type lane under the same conditions. However, the autoradiogram obtained after a longer exposure time (24 h at −70°C) showed that the brain RNA from Quv contained a detectable amount of NF-L mRNA; judging from the signal intensity, the amount of the NF-L mRNA in the brain of Quv drastically decreased to <5% of that in the wild-type quails (data not shown).
The Quv NF-M gene is surely capable of directing the synthesis of NF-M protein in neurons, though most of neurons in Quv were not stained with an anti-NF-M antibody (Yamasaki et al., 1992; Fig. 3). Since the previous workers and we have used this strain as a wild-type quail in the experiments (Yamasaki et al., 1991, 1992), other NF subunit gene expression would be also affected if something is wrong with the regulatory machinery. Therefore, as a first choice, we proceeded to focus on the structural characterization of the Quv NF-L gene in order to check whether or not there are any obvious changes leading to the suppression of this gene expression.

**Sequence Analysis of the Wild-Type and Quv NF-L Genes**

Although the nucleotide sequences for several NF-L cDNA and genomic clones have been reported to date, no sequence information was available for avian NF-L genes in GenBank DNA database (release No. 73). Thus, we first isolated and sequenced NF-L cDNA clones from both the wild-type and Quv quails, and then proceeded to genomic cloning for this gene. The nucleotide sequences of the NF-L cDNAs (2.1 kb) isolated from the wild-type and Quv quails are shown with their deduced amino acid sequences in Fig. 4. The wild-type quail NF-L cDNA was found to encode a protein composed of 556 amino acid residues, which is highly homologous to the other vertebrate NF-L proteins (Lewis and Cowan, 1986; Julien et al., 1987; Charnas et al., 1992). Although there are 13 nucleotide differences between the wild-type and Quv sequences, most of them seem to be irrelevant to the NF deficiency since they result in silent or synonymous changes in amino acid residues. However, a significant difference is found at the nucleotide residue 352: a point mutation from C to T generates a stop codon at the position corresponding to the amino acid residue 114 of the NF-L protein.

For further confirmation of the presence of this nonsense mutation and search for any other mutations in the Quv NF-L gene, we next proceeded to isolate and sequence the NF-L genomic clones from the wild-type and Quv quails. In this case, we tried to isolate complete genes including 5'-flanking regions, since we intended to examine the structure of all the regions that may affect the gene expression. Using the quail NF-L cDNA as a probe, we could isolate entire NF-L genes for the wild-type and Quv quails as two BgIII-digested fragments (total 10 kb). The genomic clones for the wild-type NF-L gene were isolated from an individual in TKP strain, since the previous workers and we have used this strain as a wild-type quail in the experiments (Yamasaki et al., 1991, 1992). Actual nucleotide sequences for the 5'-flanking region, four exons, parts of introns, and the 3'-flanking region are shown in Fig. 5. The sequence analysis of the wild-type NF-L gene (7.9 kb) revealed that the exon–intron structure is tightly conserved during vertebrate evolution. On the other hand, it is interesting to note that the nucleotide sequences in the proximal 5'-flanking and untranslated regions of this gene have low homology to those of the mouse and human genes although these regions are well conserved between mouse and human (Lewis and Cowan, 1986; Julien et al., 1987; Beaudet et al., 1992). This may suggest that the promoter activity of quail NF-L gene is under a control of
regulatory machinery different from that in mammals. The comparison of the nucleotide sequences of the genomic clones for the wild-type and Quv NF-L genes demonstrates that poly(A) addition signals (underlined in Fig. 5) and exon–intron boundary sequences are not mutated, suggesting that poly(A) addition and splicing processes are not disturbed in Quv. However, we find many sequence variations particularly in the flanking regions and introns. The sequence variation rates are summarized in Table I. It is interesting to note that the sequence variation rate in the non-coding region is significantly lower than that in other regions. Since quail strains were established on the basis of obvious apparent phenotype (e.g., egg shell color) and maintained as randombred in a closed colony originated from more than 20 individuals, the genetic homogeneity might not be expected for individual quails even in the same strain.

Figure 4. The comparison of the nucleotide sequences of the wild-type and Quv NF-L cDNAs. The nucleotide sequences of NFoL cDNAs isolated from the wild-type and Quv quails are indicated with their deduced amino acid sequences. Amino acid residues are presented above (for the wild-type sequence) and below (for the Quv sequence) the nucleotide sequences. The nucleotide residues in the Quv cDNA identical to those in the wild-type one are shown as hyphens, and the amino acid residues (or stop codon) for Quv NF-L are indicated only when they are different from those for the wild-type NF-L. The nucleotide sequences in the coding and noncoding regions are illustrated in upper and lower cases, respectively. These sequence data are available from EMBL/GenBank/DDBJ under accession number D13222 and D13223.

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Figure 5. The comparison of the nucleotide sequences of the wild-type and Quv NF-L genes. The nucleotide sequence of the NF-L gene from Quv is compared with that either from WE strain (WILD1) or from TKP strain (WILD2). The nucleotide residues are numbered from the transcription start site as +1, which was experimentally determined by a modified primer extension method (Ohara et al., 1990). The nucleotide residues in WILD1 and WILD2 identical to those in Quv are indicated by hyphens, and blanks are introduced when insertions/deletions are present. The exon/intron and 3'-noncoding regions were assigned by comparing the structures of the genomic and cDNA clones. The upper case letters are used for the residues in the mature mRNA, and the lower case letters mean that they are in introns or 3'-noncoding regions. Canonical poly(A) addition signals (AATAAA) are underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number D13222 and D13223.

Therefore, most of the observed sequence variations are likely to have nothing to do with the repression of the NF-L gene expression. In fact, there are two nucleotide differences between the cDNA and genomic clones for the wild-type genes. Actual residue numbers for the wild-type and Quv NF-L genes are 7,933 and 7,913 bp, respectively.

Table I. The Sequence Variation Rate between Wild-Type and Quv NF-L Genes

<table>
<thead>
<tr>
<th>Region</th>
<th>Length*</th>
<th>Variations†</th>
<th>Variation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-Flanking</td>
<td>2,712</td>
<td>59 (3)</td>
<td>2.2</td>
</tr>
<tr>
<td>5'-Noncoding</td>
<td>31</td>
<td>0 (0)</td>
<td>0.0</td>
</tr>
<tr>
<td>Coding</td>
<td>1,671</td>
<td>11 (0)</td>
<td>0.6</td>
</tr>
<tr>
<td>Introns</td>
<td>2,655</td>
<td>71 (9)</td>
<td>2.7</td>
</tr>
<tr>
<td>3'-Noncoding</td>
<td>398</td>
<td>0 (0)</td>
<td>0.0</td>
</tr>
<tr>
<td>3'-Flanking</td>
<td>473</td>
<td>10 (1)</td>
<td>1.9</td>
</tr>
<tr>
<td>Total</td>
<td>7,940</td>
<td>151 (13)</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* The indicated numbers include some gaps due to the presence of deletion/insertion, and thus are different from actual residue numbers for the FN-L genes. Actual residue numbers for the wild-type and Quv NF-L genes are 7,933 and 7,913 bp, respectively.
† The numbers in parentheses indicate those of insertion/deletion (from 1 to 9 bp) included in the variations. All other mutations are single-base replacements.
quencing pattern is shown in Fig. 6. Since the nucleotide sequence of the region around the deduced amino acid residue 114 is shown. The predicted structures of the wild-type and Quv NF-L protein are also illustrated, demonstrating that the Quv NF-L protein misses most of the rod and tail regions of the molecule. The rod region, which is thought to play a key role in filament assembly, is indicated as filled boxes.

Figure 6. The presence of nonsense mutation in the Quv NF-L gene at the deduced amino acid residue 114. An actual sequencing gel pattern of the region around the deduced amino acid residue 114 is shown. The predicted structures of the wild-type and Quv NF-L protein are also illustrated, demonstrating that the Quv NF-L protein misses most of the rod and tail regions of the molecule. The rod region, which is thought to play a key role in filament assembly, is indicated as filled boxes.

Discussion

We here describe detailed characterization of the NF deficiency in Quv on a molecular basis. The most striking contrast between the wild-type and Quv quails was found in the expression of NF-L gene at both the protein and mRNA levels. From these results, we supposed that the mutation leading to the NF deficiency was introduced in the NF-L gene, and thus characterized NF-L cDNA and genomic clones from both the wild-type and Quv quails. It was eventually found that the Quv NF-L gene carried a nonsense mutation at the amino acid residue 114. The observation that NF-L protein was not detectable in spite of the presence of the mRNA is consistently explained by this nonsense mutation. However, another question would arise as to why the NF-L mRNA level in Quv was only 5% of that in the wild-type quail. One plausible explanation is to assume that the nonsense mutation found in the Quv NF-L gene is a cause not only for the absence of intact NF-L protein but also for the decline in the NF-L mRNA level, since occurrence of a nonsense mutation has been reported to posttranscriptionally lower the mRNA level in many cases (Brawerman, 1989; Daar and Maquat, 1988; Gotoda et al., 1991; Kadowaki et al., 1990; Hentze, 1991; Urlaub et al., 1989).

The earlier breeding experiment suggests that the NF deficiency in Quv is generated by a single mutational event, and thus it is most likely that this nonsense mutation is an exclusive cause of the NF deficiency. However, we could not completely exclude other possibilities for the decline in the Quv NF-L mRNA level. At the beginning of this study, we suspected that the NF deficiency is due to the decline in the Quv NF-L mRNA level rather than to the premature translation termination since the change in the mRNA level was so conspicuous. This is the reason why we extensively analyzed the Quv NF-L gene structure including a long 5'-flanking region. However, the highly polymorphic nature of the quail NF-L gene made it impossible to conclude the absence of mutations lowering the promoter activity from only the sequence data.

Although the drastic decrease in the Quv NF-L mRNA strongly arrested our attention at the initial stage of this study, it is now unclear how important the lowered NF-L mRNA level is in the mechanism leading to the total loss of the NF-L proteins. In contrast to this, the meaning of the nonsense mutation is clear: the Quv NF-L gene directs the synthesis of a truncated NF-L protein lacking 80% of the carboxy-terminal part of the intact molecule, regardless of amounts of its mRNA. Since the deleted portion was corresponding to the region indispensable for filament formation (Gill et al., 1990), the resultant Quv NF-L protein completely loses the competence for NF formation. We therefore concluded that the genetic defect of Quv is the lack of the functional NF-L subunit protein caused by the nonsense mutation. In other words, all the symptoms in Quv are thought to originate from complete inability of the synthesis of the functional NF-L proteins.

We can now describe the sequence of events leading to the NF deficiency in Quv. Quv carries the defective NF-L gene in a homozygous state, which results in the total absence of a polymerization-competent, mature NF-L protein. The loss of the normal NF-L protein makes it impossible to form NFs in perikarya even in the presence of NF-M and NF-H subunits, as shown by the previous results (Yamasaki et al., 1992), which is in accord with the data in vitro (Geisler and Weber, 1981; Liem and Hutchison, 1982; Scott et al., 1985; Hishanaga and Hirokawa, 1989; Balin and Lee, 1991). Since most of the NF-M subunit in perikarya of Quv were hardly transported into the axons, the filament formation might be prerequisite to the axonal transport of NF subunit proteins. In this regard, NF-L overproducing transgenic mice make a great contrast to Quv: in the transgenic mice, NF-L proteins seem to form filaments primarily by themselves and travel...
into axons (Monteiro et al., 1990). These two extreme cases in vivo clearly indicate that the NF-L subunit plays a key role in assembly of the NF subunits, which has been suggested by the data of in vitro studies (Geisler and Weber, 1981; Liem and Hutchison, 1982; Scott et al., 1985; Hisanaga and Hirokawa, 1989; Balin and Lee, 1991). Moreover, the lack of the functional NF-L subunit could affect the stabilities of other NF subunits, since susceptibility of the NF subunits to proteolysis in perikarya is probably dependent on their physical (assembled or unassembled) and phosphorylation states as suggested from aluminum intoxication (Shea et al., 1992). Thus, the NF-M and NF-H subunit proteins remaining in perikarya of Quv might be more readily degraded by proteolytic enzymes than those of the wild-type quail, which would explain the low levels of these protein subunits in most neurons of Quv without altering these mRNA levels drastically. In this way, the deficiency of the functional NF-L protein in Quv can consistently account not only for disappearance of NFs but also for great reduction in amounts of NF-M and NF-H subunits in Quv. To our best knowledge, Quv is the first example of a vertebrate mutant with convincing genetic evidence for NF deficiency, although an entire invertebrate phylum, the arthropods, has been reported to exist without NFs apparently (Lasek et al., 1983, 1985).

Strictly regulated expression pattern of different types of IFs in different tissues and at different developmental stages has been considered as an indication of uncovered biological importance of IF cytoskeleton. Several experimental approaches have been taken to try to decipher the biological roles of IFs in general. However, most of the results in cultured cells so far obtained have suggested that IFs are dispensable for many cellular events in which IFs were supposed to be involved (Schultheiss et al., 1991). In this respect, Quv provides us with the first definite in situ evidence of dispensability of a class of IFs (i.e., NFs) for maintaining vertebrate life. Quv appears to compensate the total loss of NFs, to some extent, with upregulation of tubulin synthesis (Yamasaki et al., 1991), suggesting that tubulin could function as a mechanical integrator in place of NFs, as previously discussed for the arthropod nervous system (Lasek et al., 1983).

Although the existence of Quv evidently indicates that NFs are not essential for survival of quails, it does not necessarily mean that NFs are useless in the nervous system. In fact, tight conservation of the NF subunit gene structure during vertebrate evolution has raised the possibility that NFs are very important for the nervous system in vertebrates. Since the lack of NFs resulted in disappearance of axons with large diameters (>3 μm) without disturbing the basic function of neurons (Yamasaki et al., 1991; Sakaguchi, T., M. Okada, T. Kitamura, and K. Kawasaki, manuscript submitted for publication), the biological significance of NFs might be directly connected to the importance of axons in a large size class (i.e., with high conduction velocity) in the nervous system as a whole; the size distribution of axonal calibers may have a critical meaning in organismal behaviors. If so, the biological importance of NFs could not be appreciated by observing individual neurons; we might pay more attention to the defect(s) resulting from disturbed organization of the whole nervous system rather than to the functions of individual neurons in Quv. Although Quv seems to be tolerable against such an organismal perturbation in the nervous system under the breeding conditions used, it is still unclear whether or not the dispensability of NFs can be generalized for other animals or under different breeding conditions even in the case of quails.

In this study, we focused on the identification of the genetic defects leading to the NF deficiency in Quv. During the course of this study, we noticed that some changes in expression of neuronal cytoskeletal genes other than the NF-L gene occurred in Quv probably because of secondary effects of the NF-L deficiency. For example, the density of microtubules in axons increases in Quv as reported by Yamasaki et al. (1991). If the nonsense mutation in NF-L gene is an exclusive genetic defect in Quv, how does this information travel to tubulin gene? This is an important and interesting issue to be examined, and Quv would provide us with a unique and useful model system for study of the interactive regulation of neuronal cytoskeletal gene expression in vertebrates.

The authors wish to thank Dr. Mizutani for kindly providing us Japanese quails; Drs. Yamada and Okada for helping in some experiments; and Drs. Yamasaki and Iikura for continuous stimulation and helpful discussions.

Received for publication 6 October 1992 and in revised form 30 December 1992.

References


