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Molecular Cloning of Heterotrimeric G-Protein α-Subunits in Chicken Pineal Gland

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Abstract. The avian pinealocytes have an intrinsic circadian clock function that controls rhythmic synthesis of melatonin, and an environmental light signal can reset the phase of the clock. In addition to the photoendocrine function, the melatonin synthesis of the pinealocytes is regulated by neural signals from sympathetic nerves. Thus the avian pinealocytes show diagnostic characters which seem to represent an evolutionary transition from photosensory cells of lower vertebrates to the neuroendocrinal cells of mammals. To understand the evolutionary background of the regulatory mechanism for the melatonin synthesis in this organ, we screened the chicken pineal cDNA library to find α-subunits of heterotrimeric G-proteins involved in the photic and neural regulations. In addition to the transducin-like α-subunit ($G_t\alpha$) supposed to mediate the photic pathway, we isolated cDNA clones encoding $G_{i2}\alpha$, $G_{i3}\alpha$, and $G_{o1}\alpha$ and its splicing variant $G_{o2}\alpha$. The deduced amino acid sequence of each Gα had a potential site for pertussis toxin-catalyzed ADP-ribosylation. As it is known that adrenergic receptor-mediated inhibition of melatonin synthesis is blocked by pertussis toxin, the G-proteins identified in the present study are likely to contribute to this neuroendocrine function of the chicken pineal cells.

Key words: G-protein — cDNA cloning — Pineal gland — Circadian clock — Photoendocrine — Melatonin — Pinopsin — cAMP — Chicken

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Introduction

The mammalian pineal gland is an endocrine organ that secretes melatonin according to noradrenergic signal from the sympathetic nerves (Kappers 1965; Reiter 1981). In contrast, the pineal gland of the lower vertebrates such as the fish and amphibian is photosensitive, and their glands have properties of sensory tissues generating electrical signals in response to light. Also, the pinealocytes of the lower vertebrates show morphological similarities to those of retinal photoreceptors in having an outer segment with a lamellar structure.

Interestingly, in the case of birds, the pineal cell has a circadian clock function regulating rhythmic production of melatonin (Deguchi 1979a), and the outer segment of the photoreceptive cell is replaced by a bulbous cilium or a whorl-like structure (Oksche et al. 1972; Omura 1977). The photoreceptive molecule present in the rudimentary photoreceptors of the chicken pineal gland has been shown to be a member of rhodopsin family, and it was named pinopsin after pineal opsin (Okano et al. 1994). In this case, the captured light signal plays a role as a synchronizer of the endogenous clock. According to the intrinsic clock signal, the intracellular cAMP concentration displays diurnal change, which probably controls the rhythmic production of melatonin (Deguchi 1979b).

In addition to the photoendocrine function, the melatonin synthesis in the avian pineal gland is regulated by the sympathetic nervous system just like mammals. For example, chicken pineal gland is innervated by noradrenaline (NA)- and vasoactive intestinal peptide (VIP)-containing fibers (Takahashi et al. 1989). Thus the avian pineal gland seems to represent a transition state of evo-

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GGGCCCCGCTGCAAGCCCTCGTCGTTCTCATCTACAGGCACTGGAGCCCCCAGGAGCCG -103
GGAGCGGTCCTCGCTGCCCAGGACAGCGCCGACGAGCATTAGGGCCGCGGCCAGCAGCAC  -43
                                        ★
TACGCAACCCCGAGCCAGAGCGCGGAGCGAGGGGCCGCCGCCATGGGCTGCACGGTCAGC   18
                                          M  G  C  T  V  S      6

GCCGAGGATAAGGCGGCCGCCGAGCGCTCCCGCATGATCGACCGCAACCTGCGGGAGGAC   78
A  E  D  K  A  A  A  E  R  S  R  M  I  D  R  N  L  R  E  D    26

GGCGAGAAGGCGGCGCGGGAGGTGAAGCTGCTGCTGCTGGGTGCTGGGGAGTCCGGGAAG  138
G  E  K  A  A  R  E  V  K  L  L  L  L  G  A  G  E  S  G  K    46

AGCACCATCGTCAAACAGATGAAGATCATCCACGAAGATGGCTACTCGGAGGAGGAGTGC  198
S  T  I  V  K  Q  M  K  I  I  H  E  D  G  Y  S  E  E  E  C    66

CGGCAATACAAAGCCGTGGTCTACAGCAACACCATCCAGTCCATCATGGCCATCATCAAG  258
R  Q  Y  K  A  V  V  Y  S  N  T  I  Q  S  I  M  A  I  I  K    86

GCTATGGGGAACCTGCAGATCGACTTTGGGGACTCCTCCAGAGCGGATGATGCCCGCCAG  318
A  M  G  N  L  Q  I  D  F  G  D  S  S  R  A  D  D  A  R  Q   106

CTCTTTGCGCTCGCCTGCACCGCAGAGGAGCAGGGCATCATGCCTGAAGACCTCGCCAAC  378
L  F  A  L  A  C  T  A  E  E  Q  G  I  M  P  E  D  L  A  N   126

GTCATCCGGAGGCTGTGGGCTGACCACGGGGTCCAGGCCTGCTTCAATCGCTCCCGTGAA  438
V  I  R  R  L  W  A  D  H  G  V  Q  A  C  F  N  R  S  R  E   146

TACCAACTGAATGACTCTGCTGCCTACTACCTGAACGACCTGGAGAGGATAGCGCGGGCC  498
Y  Q  L  N  D  S  A  A  Y  Y  L  N  D  L  E  R  I  A  R  A   166

GACTACATCCCCACCCAGCAGGACGTGCTGCGCACCCGGGTGAAGACCACCGGCATCGTG  558
D  Y  I  P  T  Q  Q  D  V  L  R  T  R  V  K  T  T  G  I  V   186

GAGACCCACTTCACCTTCAAGGACCTGCACTTCAAGATGTTCGACGTGGGCGGCCAGCGC  618
E  T  H  F  T  F  K  D  L  H  F  K  M  F  D  V  G  G  Q  R   206

TCAGAGCGGAAGAAGTGGATCCACTGCTTCGAGGGGGTGACAGCCATCATCTTCTGCGTG  678
S  E  R  K  K  W  I  H  C  F  E  G  V  T  A  I  I  F  C  V   226

GCCCTGAGTGCCTATGACCTGGTGCTGGCAGAAGATGAGGAGATGAACCGGATGCACGAG  738
A  L  S  A  Y  D  L  V  L  A  E  D  E  E  M  N  R  M  H  E   246

AGCATGAAGCTATTCGACAGCATCTGCAACAACAAGTGGTTCACGGACACGTCCATCATC  798
S  M  K  L  F  D  S  I  C  N  N  K  W  F  T  D  T  S  I  I   266

CTCTTCCTCAACAAGAAGGACCTCTTTGAGGAGAAGATCGTGCACAGCCCCCTGACCATC  858
L  F  L  N  K  K  D  L  F  E  E  K  I  V  H  S  P  L  T  I   286

TGCTTCCCGGAGTACACAGGTGCCAACAAGTACGACGAGGCCGCCGGCTACATCCAGAGC  918
C  F  P  E  Y  T  G  A  N  K  Y  D  E  A  A  G  Y  I  Q  S   306

AAGTTTGAGGACCTGAACAAGCGGAAGGACACCAAGGAGATCTACACGCACTTCACCTGT  978
K  F  E  D  L  N  K  R  K  D  T  K  E  I  Y  T  H  F  T  C   326

GCCACCGACACCAAGAACGTGCAGTTTGTCTTCGATGCCGTCACCGACGTCATCATCAAA 1038
A  T  D  T  K  N  V  Q  F  V  F  D  A  V  T  D  V  I  I  K   346

AACAACCTGAAGGACTGCGGGCTCTTCTGAGGGCGGCAAGGACGCACCGATGAAGGG    1095
N  N  L  K  D  C  G  L  F  stop                               355
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Fig. 1. The nucleotide and deduced amino acid sequences of the clone C2 encoding chicken pineal $G_{i2}\alpha$. Nucleotides are *numbered* beginning with the first methionine codon in the longest open reading frame. A potential site for ADP-ribosylation by pertussis toxin is *boxed*. In-frame stop codon in the 5′ flanking region is marked by a *star*.

lution from photosensory tissue of the lower vertebrates to the neuroendocrinal organ of mammals. It should be noted, however, that the same adrenergic input gives opposite effects on the avian and mammalian pineal glands, i.e., inhibition and stimulation of melatonin synthesis, respectively (Klein and Weller 1973; Deguchi 1979b). This is explained by a switch of the subtypes of adrenergic receptors from α_2 in the chicken (Pratt and Takahashi 1987) to β-subtype of mammals (Deguchi and Axelrod 1972; Klein and Weller 1973). In general, the α_2 and β receptors are known to couple with heterotrimeric G-proteins, G_i and G_s, respectively, leading to the inhibition and activation of adenylyl cyclase. Functionally similar VIP receptors seem to be expressed in the avian and mammalian pineal glands, and they couple with G_s to elevate cAMP level and consequently stimulate melatonin synthesis in both cases (Yuwiler 1983; Kaku et al. 1985; Takahashi et al. 1989). Thus the information about the receptors and G-proteins expressed in the pineal gland may provide a clue to the answer to the question how the pineal function has altered during phylogeny.

This study was undertaken to reveal molecular identities of chicken pineal G-protein α-subunits (Gα) by cDNA cloning. To date, immunohistochemical studies on various animals have suggested the presence of transducin-like pineal G-protein (van Veen et al. 1986; Foster

Table 1. Amino acid identities among G-protein α-subunits in the G_i subfamily[a]

			$G_t\alpha$			$G_o\alpha$				$G_i\alpha$			
			Rat $G_{gust}\alpha$	Human $G_{t2}\alpha$	Human $G_{t1}\alpha$	Chicken $G_{o2}\alpha$	Human $G_{o2}\alpha$	Chicken $G_{o1}\alpha$	Human $G_{o1}\alpha$	Chicken $G_{i3}\alpha$	Human $G_{i2}\alpha$	Chicken $G_{i2}\alpha$	Human $G_{i1}\alpha$
Human	Genome	$G_{i1}\alpha$	65.8	69.5	65.7	71.4	70.8	71.4	71.4	**97.7**	**86.2**	**86.7**	**100**
Chicken	Pineal	$G_{i2}\alpha$	66.4	70.1	67.4	70.3	69.8	70.0	69.8	**87.3**	**94.9**	**100**	
Human	Leukocyte	$G_{i2}\alpha$	66.7	70.1	66.9	70.1	69.5	69.5	69.5	**86.4**	**100**		
Chicken	Pineal	$G_{i3}\alpha$	66.1	69.8	66.6	72.0	72.0	71.7	72.5	**100**			
Human	Brain	$G_{o1}\alpha$	62.3	61.5	63.0	**93.8**	**94.4**	**98.3**	**100**				
Chicken	Pineal	$G_{o1}\alpha$	62.3	61.8	63.0	**94.6**	**92.9**	**100**					
Human	Genome	$G_{o2}\alpha$	61.2	59.8	61.0	**96.9**	**100**						
Chicken	Pineal	$G_{o2}\alpha$	61.2	59.8	60.7	**100**							
Human	Retinal rod	$G_{t1}\alpha$	**80.0**	**82.9**	**100**								
Human	Retinal cone	$G_{t2}\alpha$	**79.7**	**100**									
Rat	Taste cell	$G_{gust}\alpha$	**100**										

[a]Values greater than 75% are in *boldface characters*. G-proteins sequenced in this study are *boxed*

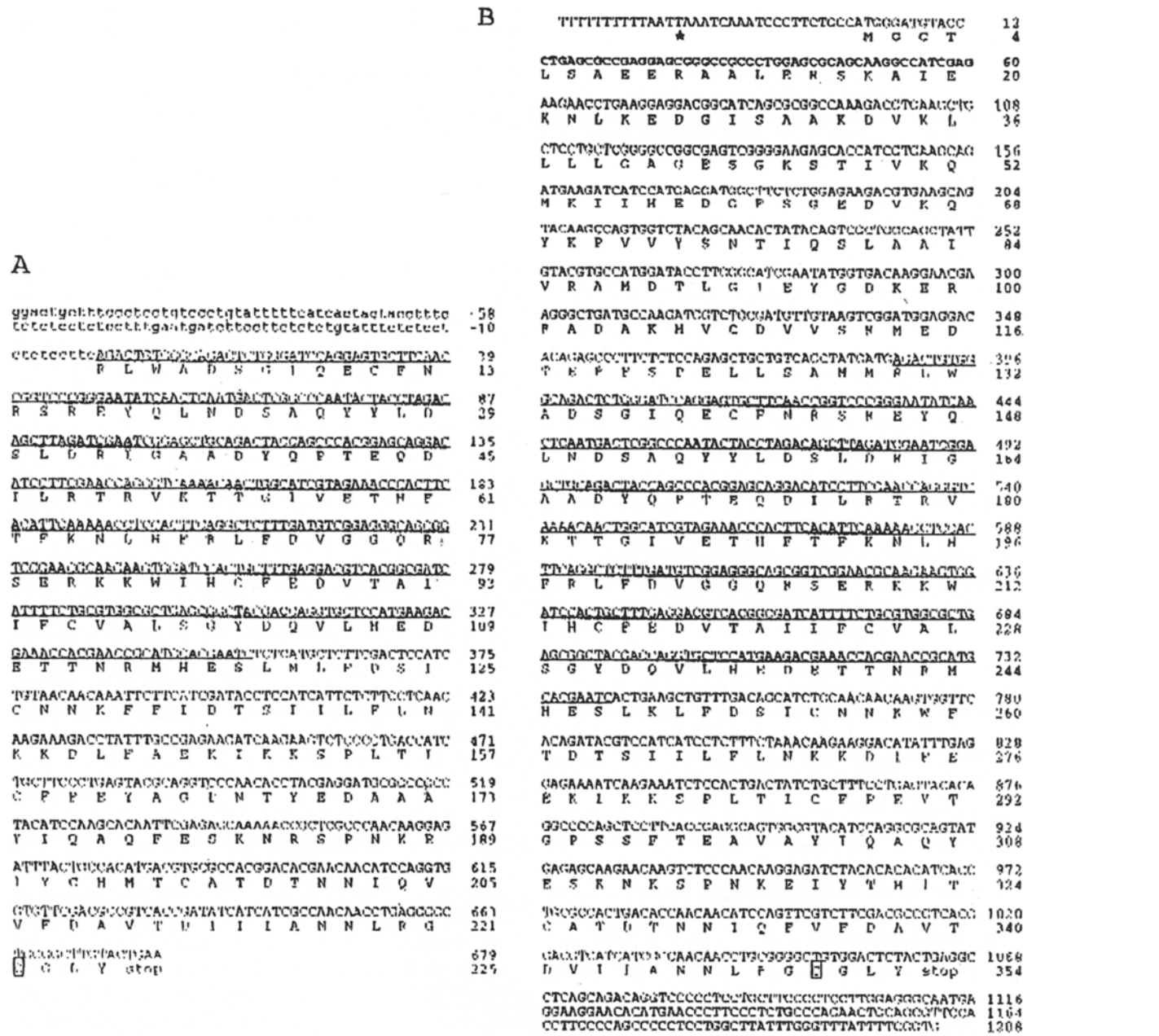
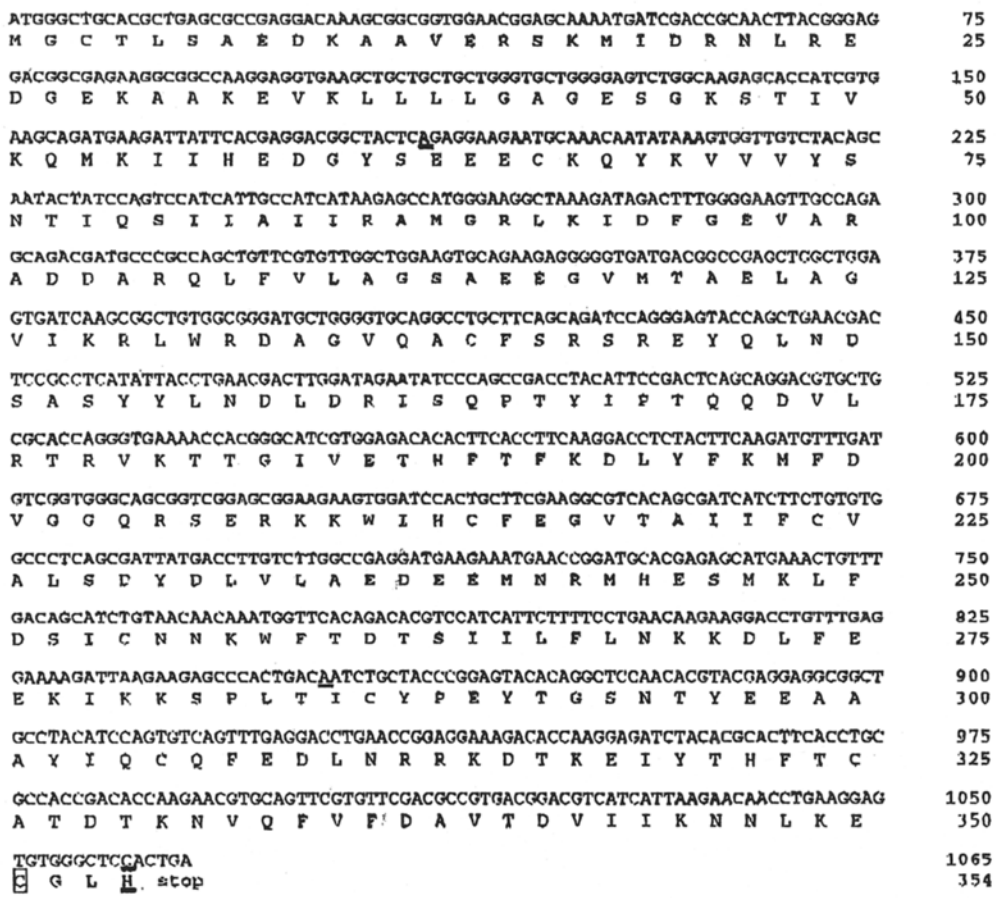


Fig. 2. The nucleotide and deduced amino acid sequences of the clones Ar11 (**A**) and Ar8 (**B**) encoding two types of chicken pineal $G_o\alpha$. **A** Ar11 contains a partial cDNA of chicken pineal $G_{o1}\alpha$ fused with another piece of unknown cDNA. Nucleotides are *numbered* beginning with the first nucleotide corresponding to the $G_{o1}\alpha$ cDNA. Nucleotides possibly arising from unrelated cDNA are shown in *lowercase*. **B** Ar8 contains the entire coding region of chick pineal $G_{o2}\alpha$ cDNA. Nucleotides are *numbered* beginning with the first methionine codon in the longest open reading frame. Potential sites for ADP-ribosylation by pertussis toxin are *boxed*. *Underlined* are identical sequences between Ar11 and Ar8. In-frame stop codon in the 5′ flanking region is marked by a *star*.

A



B

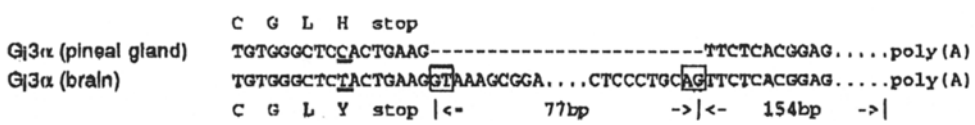


Fig. 3. The nucleotide and deduced amino acid sequences of the coding region of the clone KAN1 encoding chicken pineal $G_{i3}\alpha$. **A** Nucleotides are *numbered* beginning with the first methionine codon in the longest open reading frame in KAN1. Three nucleotides different from those of chicken brain cDNA sequence are *underlined,* among which two alternations at positions 183 and 849 are synonymous. A potential site for ADP-ribosylation by pertussis toxin is *boxed.* **B** The nucleotide sequence of the 3′ flanking region of KAN1 is compared with that of chicken brain $G_{i3}\alpha$ cDNA (Kilbourne and Galper 1994), which has additional 77 nucleotides. Consensus sequences for the 3′ and 5′ splice sites are *boxed.*

et al. 1987; Yoshikawa et al. 1994), but the biochemical information is still limited. Pertussis toxin-catalyzed ADP-ribosylation reaction of chicken pineal cell membranes has revealed 40–41-kDa Gα which appears to represent $G_i\alpha$ coupling with α_2-adrenergic receptor (Takahashi et al. 1989). In the present study, we show the primary structures of chicken pineal Gα's, all of which have a potential ribosylation site by pertussis toxin, and they may be involved in the neural regulation of melatonin synthesis.

Materials and Methods

All molecular biology manipulations were carried out according to standard methods (Sambrook et al. 1989). The chick pineal cDNA library was constructed by using purified poly(A)$^+$ RNA with the aid of commercial kits (cDNA Synthesis System Plus and cDNA Rapid Cloning Module λgt11, Amersham; Gigapack II Gold packaging kit, Stratagene). The cDNA inserts in recombinant phages isolated from the library were excised and subcloned into pBluescript II KS+ (Stratagene) or pUC119 plasmid vector (TaKaRa) for sequencing by the dideoxynucleotide chain termination method (Sequenase Ver.2.0,

United States Biochem) with [α-^{32}P]dCTP, or by the cycle sequencing method with fluorescence-labeled sequencing terminators (model 373S-18, Perkin Elmer).

Results and Discussion

A chicken pineal cDNA library was screened with probes of full-length and partial cDNA for chicken $G_t\alpha$ (to be published elsewhere). Then we isolated a clone termed C2 (2.2 kbp) which contained a long open reading frame for Gα in full length of 355 amino acid residues (Fig. 1). The sequence was most closely related to $G_{i2}\alpha$; 94.9% identical to human $G_{i2}\alpha$ (Beals et al. 1987), 86.7% to human $G_{i1}\alpha$ (Bray et al. 1987), and 87.3% to chicken (brain) $G_{i3}\alpha$ (Kilbourne and Galper 1994), while it showed much lower identities to the other subtypes of Gα (Table 1).

The second clone isolated (termed Ar11, 3.4 kbp) seemed to have two different pieces of cDNAs, one of which (675 bp) encoded a C-terminal two-thirds of $G_o\alpha$ (225 amino acids, see Fig. 2A); the other was unrelated. This is probably due to an unexpected ligation during the construction of the library. By using Ar11 as a screening probe, we isolated a clone Ar8 (1.4 kbp) encoding a full-length sequence of $G_o\alpha$ composed of 354 amino acid residues (Fig. 2B). Interestingly, the Ar11 and Ar8 shared a common nucleotide sequence followed by diverged regions (Fig. 2), suggesting that these two clones were splicing variants, $G_{o1}\alpha$ and $G_{o2}\alpha$, transcribed from a single gene, as reported in human brain and heart (Hsu et al. 1990; Strathman et al. 1990; Tsukamoto et al. 1991). Because the two proteins coded by Ar11 and Ar8 were highly similar (96.6% and 95.2%) to mouse brain $G_{o1}\alpha$ and $G_{o2}\alpha$, respectively, we concluded that Ar11 and Ar8 were the two transcriptional variants of a chicken $G_o\alpha$ gene.

The third clone isolated (termed KAN1, 3.1 kbp) was identical in amino acid sequence to chicken brain $G_{i3}\alpha$ (Kilbourne and Galper 1994) except for one amino acid at the C-terminus (Fig. 3A). In addition, this clone lacked the 77-bp sequence found in the 3′ flanking region of the chicken brain $G_{i3}\alpha$ cDNA (Fig. 3B). This may reflect that the two were splicing variants derived from the same gene.

Based on the amino acid identities among Gα's, phylogenetic trees were constructed by the neighbor-joining method (Fig. 4). The branching patterns of the chicken Gα's in respective subtypes are highly reliable. As clearly shown by Fig. 4, the tree topology in each subtype of Gα's agreed well with the phylogeny. Thus it seems likely that a gene duplication has not occurred after the divergence among Gα subtypes $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, and $G_o\alpha$.

Expression of all the genes described above was detected in the chick pineal gland by Northern blot analysis

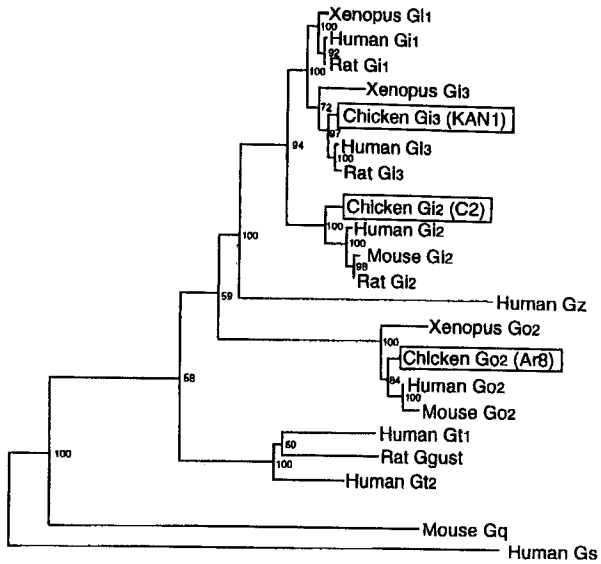


Fig. 4. A phylogenetic tree of G-protein α-subunits constructed on the basis of amino acid identities. The tree was constructed by the neighbor-joining method (Saitou and Nei 1987) using PHYLIP software (Felsenstein 1989). The values at the nodes are the bootstrap probabilities (%) estimated by 1,000 times replications, and the *lengths of the horizontal lines* are evolutionary distances. The amino acid sequences were obtained from GenBank. The tree does not include $G_{o1}\alpha$, which is a splicing variant of $G_{o2}\alpha$.

(not shown). The four kinds of Gα's identified in the present study will be involved in the pertussis toxin-sensitive signal-transducing pathways, because each Gα has a potential site for the ADP-ribosylation catalyzed by the toxin (Figs. 1–3). The α_2-adrenergic receptor-mediated inhibition of melatonin synthesis in the chicken pineal cells is sensitive to pertussis toxin treatment, and the calculated molecular weights of the four Gα's cloned (40,057–40,517; disregard for a possible post- or co-translational modification) are consistent with the observed mass value (40 kDa) of the major ADP-ribosylated protein in the gland (Pratt and Takahashi 1988). In particular, $G_{i2}\alpha$ and $G_{i3}\alpha$ are the most probable candidates for the adrenergic regulation of melatonin production in the chicken pineal cells, because the pathway is mediated by a reduction of intracellular cAMP level (Zatz and Mullen 1988a).

In the case of mammals, melatonin production of the pineal gland is primarily regulated by a circadian pacemaker localized in the suprachiasmatic nucleus through the superior cervical ganglia. This transmission is mediated by activation of pineal β-adrenergic receptors (Deguchi and Axelrod 1972; Klein and Weller 1973). In contrast, the chicken pineal gland has an endogenous circadian oscillator contributing to the rhythmic production of melatonin, whereas the noradrenergic input discussed above only transiently suppresses the melatonin synthesis. As compared with such a neural regulation, the light signal has relatively strong effects on the melatonin production in the chicken: A light pulse given at

nighttime acutely inhibits the melatonin synthesis, and more importantly causes a phase shift of the pineal oscillator (Zatz and Mullen 1988b). As the former effect was sensitive to pertussis toxin, a transducin-like G-protein has been postulated to mediate the pathway. In fact, we confirmed that the α-subunit of chicken pineal transducin had the ADP-ribosylation site (to be published elsewhere). But it is still possible that one or some of the Gα's cloned in this study might be responsible for the light-dependent endocrine function. Pinopsin, recently identified as a chicken pineal photoreceptive molecule, was a member of the rhodopsin family (Okano et al. 1994) capable of interacting with G-proteins other than transducin (Cerione et al. 1986).

In the other tissues, $G_o\alpha$ is known to regulate calcium channels dependent on hormone and neurotransmitter (Hesheler et al. 1987; Neer and Clapham 1988; Kleuss et al. 1991). In the chicken pineal cells, the influx of extracellular calcium does not appear to influence the endogenous circadian oscillator, but the reduction of calcium influx suppresses the melatonin synthesis (Zatz and Mullen 1988c; Zatz 1989). The two splice variants of $G_o\alpha$ identified in this study might be involved in the calcium-dependent regulation of melatonin synthesis, though there is no evidence for the hormonal regulation of the calcium influx in the chicken pineal cells.

It is intriguing to see which of the pineal Gα's interacts with which of the receptors in vivo. This question would be answered by the in vitro study using expressed proteins and by investigating the intracellular localization of the receptors and G-proteins. Further characterization of the signal-transduction pathways in the chicken pineal cells and the comparison with those in the lower and higher vertebrates would provide important clues to understand how the pineal function has altered during phylogeny.

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