trogen base containing adenine, histidine, tryptophan, and 2% glucose). After approximately 24 hours, filters were transferred to selective plates containing 2% galactose as carbon source and grown for another 20 to 24 hours. X-gal assays were performed directly on these filters (*19*). Positive (blue) colonies were picked from the filter, streaked to isolate single colonies, and reassayed essentially as described above. Blue colonies were plated on selective plates containing uracil and 5-fluoroorotic acid (1 mg/ml) to select against the reporter plasmid. *ura*$^-$ strains were mated to W303-1b (*MATα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*) harboring either pLGΔ178 or pLG-ARS1+. Five clones expressed β-galactosidase with pLG-ARS1+ but not with pLGΔ178. The library plasmids in these strains were rescued into *Escherichia coli* and, upon reintroduction into fresh reporter-containing yeast, retained their original characteristics. The indicated yeast ARSs were first subcloned into pUC plasmids, excised with Eco RI and Hind III, blunt-ended, and cloned into the unique Xho I site of pLGΔ178. The cloning of ARS1 (derived from pARS1.4.1), ARS2, ARS307 (previously called C2G1), and the 2-μm origin as well as the 138–base pair (bp) Dra I–Alu I *HMR E* fragment into pUC plasmids have been previously described (*16*, *20*). All of these inserts are between 100 and 300 bp except for ARS2, which is 627 bp. Quantitative assays for β-galactosidase were performed, and data were normalized to the cell number as described in (*21*). In all experiments, amounts of β-galactosidase were normalized to the amount produced with the wild-type ARS1 reporter.

23. Mutations (indicated as an X) in the individual elements of ARS1 corresponding to linker substitutions in A (865 to 872), B1 (835 to 842), and B2 (798 to 805) and a double point mutation in B3 (756, 758) (*3*) were cloned into pLGΔ178 in the indicated orientation as described in the legend to Fig. 2. To construct the reporter containing elements A and B1 alone (corresponding essentially to a B2, B3 double mutation), an Eco RI–Pst I fragment from pARS1-WTB(*3*) was blunt-ended and cloned into the blunt-ended Xho I site of pLGΔ178. Quantitative β-galactosidase assays were performed as described above (*22*).
24. Domain A from either wild-type or mutated (865 to 872 linker substitution) ARS1 (*3*) was excised as an Eco RI–Bgl II fragment and cloned into pLGΔ178 as described in the legend to Fig. 1. Arrows indicate the orientation of the ACS. The arrow pointing right represents the ACS in the same orientation as it is in the intact ARS1. In (B), the wild-type domain A was reconstructed by the cloning of the double-stranded oligonucleotide:

```
5′GATCTAAACATAAAATCTGTAG 3′
    |||||||||||||||||||
3′ ATTTGTATTTTAGACATCTTAA 5′
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between the Eco RI and Bgl II sites of pARS-WTB (*3*). This procedure removes sequences 5′ to the T-rich strand of the ACS that are not necessary for ARS function (*3*). Point mutations were constructed in the same manner, except that the double-stranded oligonucleotide contained the indicated point mutations. In each case, the wild-type or mutant ARSs were subcloned into pLGΔ178 and quantitative β-galactosidase assays were performed as described in (*22*). Levels of ARS activity and ORC binding were taken from (*6*).
25. Dbf4 deletions were generated by polymerase chain reaction with the use of oligonucleotides with Bam HI sites for subcloning PCR products into the relevant vectors. The constructs contained the following portions of Dbf4: Full-length (FL), amino acids 1 to 695; C2, amino acids 4 to 416; 152, amino acids 1 to 320; 154, amino acids 1 to 160; 16, amino acids 81 to 416; and 18, amino acids 241 to 416. Because the two interaction assays are quite different, only qualitative X-gal assays are shown. Construct 16 reproducibly activates significant, albeit reduced, levels of β-galactosidase activity in this assay, suggesting a reduction but not elimination of ARS interaction capability. All constructs were tested for their ability to complement a *dbf4-2* mutant at 37°C. In no case was there a significant difference between the lexA and GAD fusions (*12*).
26. We thank L. Johnston, J. Blow, J. Harwood, N. Lowndes, and A. Rowley for helpful discussions and critical reading of this manuscript; S. Elledge for the gift of the yeast cDNA library; L. Johnston, N. Lowndes, and B. Stillman for plasmids and yeast strains; B. Amati for advice on quantitative β-galactosidase assays; and I. Goldsmith for oligonucleotide synthesis.

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Convergent Pathways for Steroid Hormone– and Neurotransmitter-Induced Rat Sexual Behavior

S. K. Mani, J. M. C. Allen, J. H. Clark, J. D. Blaustein, B. W. O'Malley*

Estrogen and progesterone modulate gene expression in rodents by activation of intracellular receptors in the hypothalamus, which regulate neuronal networks that control female sexual behavior. However, the neurotransmitter dopamine has been shown to activate certain steroid receptors in a ligand-independent manner. A dopamine receptor stimulant and a D_1 receptor agonist, but not a D_2 receptor agonist, mimicked the effects of progesterone in facilitating sexual behavior in female rats. The facilitatory effect of the neurotransmitter was blocked by progesterone receptor antagonists, a D_1 receptor antagonist, or antisense oligonucleotides to the progesterone receptor. The results suggest that in rodents neurotransmitters may regulate in vivo gene expression and behavior by means of cross-talk with steroid receptors in the brain.

Sexual receptivity in the female rat depends on the ovarian steroid hormones estrogen (E) and progesterone (P) (*1*). The effects of E and P on sexual behavior in rodents appear to require their interaction with specific steroid hormone receptors located in the ventromedial nucleus of the hypothalamus (*2*). The receptors are thought to mediate the actions of these hormones by functioning as ligand-dependent nuclear transcription factors that alter the expression of specific genes or gene networks in the hypothalamus (*3*). The time course of induction and decay of sexual behavior correlates with the amount of inducible progesterone receptor (PR) in the ventromedial nucleus region (*4*). The progesterone antagonist RU 38486, administered before P, inhibits lordosis in E-primed rodents in a dose-dependent manner, which indicates that P occupies its classical nuclear receptor (*5*). Thus, certain actions of steroid hormones in the brain apparently result from activation of intracellular receptors that regulate behavioral functions by modulating gene expression (*6*).

Certain steroid receptors, such as avian PR, human estrogen receptor (ER), and vitamin D_3 receptor, can be activated in a ligand-independent manner by the neurotransmitter dopamine in cultured cells (*7*). Dopamine has no detectable affinity for intracellular steroid receptors and may act by altering phosphorylation of either the receptor itself or a specific transcription cofactor. A single amino acid mutation in the COOH-terminal region of either PR or ER eliminates activation by dopamine but not by the authentic cognate ligand. This ligand-independent activation of certain steroid receptors can also be induced by certain growth factors (*8*). To corroborate that dopamine activation of a steroid receptor has physiological relevance in the central nervous system, we used an established P-dependent behavioral model in female rats. Dopaminergic transmission also has effects on this model because hypothalamic administration of dopamine, or the dopamine receptor stimulant apomorphine, exerts a stimulatory effect on sexual behavior in E-primed female rats (*9*). We examined the effects of inhibition of PR gene expression on dopamine-facilitated sexual behavior of female rats by intracerebroventricular (icv) administration of antisense oligonucleotides to the PR.

Ovariectomized Sprague-Dawley rats previously tested for receptive behavior in the presence of sexually active males were stereotaxically implanted with stainless steel cannulae into the third ventricle to facilitate direct icv injection (*10*). When these animals were primed with E subcutaneously and P was administered by icv injection 48 hours later, they exhibited a high frequency of lordosis [scored and represented by the lordosis quotient (LQ)] in the presence of males (Fig. 1A). Apomorphine, a dopamine receptor stimulant, administered by icv injection 48 hours after E-prim-

S. K. Mani, J. M. C. Allen, J. H. Clark, B. W. O'Malley, Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030, USA.
J. D. Blaustein, Department of Psychology and Neuroscience Behavior Program, University of Massachusetts, Amherst, MA 01003, USA.
*To whom correspondence should be addressed.

ing, also facilitated lordosis response. The measured response was equivalent to the response of P at the doses administered. A selective D_1 agonist, SKF 38393, also mimicked P in the facilitation of lordosis response in E-primed rats (Fig. 1B). At a 60- to 100-ng dose, virtually all animals exhibited reliable receptive behavior; lower doses of SKF 38393 were partially effective. In contrast, quinpirole, a D_2 agonist, had little or no stimulatory effect on lordosis in E-primed animals at any of the doses administered (Fig. 1C). Facilitation of sexual behavior by the D_1 agonist was suppressed by the D_1 receptor antagonist SCH 23390 (Fig. 1D).

The administration of apomorphine or D_1 agonist directly into the hypothalamic area and blockade by a D_1 antagonist indicate that dopamine is acting at its cell membrane receptor to produce a behavioral response similar to that initiated by P. These responses are in contrast to some earlier reports of the inhibitory effects of dopaminergic agonists on sexual behavior (*11*). The observed facilitatory effects could be due to the local administration of the compounds into the third cerebral ventricle as compared with the systemic or oral administration in the earlier studies. Similar to our results, local micro-infusions of dopamine or apomorphine into the medial preoptic area and arcuate-ventromedial area have been reported to stimulate sexual behavior in female rats (*9*).

The possibility of indirect actions of dopamine agonists on the adrenal gland is unlikely because facilitatory effects of apomorphine on sexual behavior have been demonstrated in adrenalectomized and ovariectomized rats (*12*). Nevertheless, we performed separate studies in dexamethasone-suppressed, E-primed rats that confirmed that icv administration of SKF 38393 induced lordosis independent of the presence of the adrenal glands (*13*). Also, serum P was measured in E-primed rats after icv injections with SKF 38393, and no significant elevations were observed compared with matched controls injected with vehicle (*14*). SKF 38393 also mimicked P's biphasic influence on sexual behavior of female rats (*15*); standard doses were ineffective, and larger doses caused facilitation of lordosis during the period of refractoriness (*16*). The selectivity for a D_1 receptor agonist and not for a D_2 receptor agonist in the potentiation of lordosis is consistent with an inhibitory role of D_2 receptors on lordosis behavior (*17*).

Innervation of neurons containing steroid receptors by apparent dopaminergic neurons in the rodent brain have been reported (*18*). Moreover, E and P increase dopamine transmission at dopaminergic synapses (*19*). Catecholamines increase the number of intracellular receptors for E and P (*20*). Thus, both P and dopamine could induce lordosis through effects on PRs within the brain. To test this hypothesis, we examined P- and dopamine-facilitated sexual behavior in E-primed rats in the presence of PR antagonists RU 38486 and ZK 98299 (Fig. 2). The receptor-specific antiprogestins blocked the facilitation of lordosis by P in the E-primed female rats (*21*). However, icv administration of the PR antagonists 60 min before the injection of the D_1 agonist SKF 38393 also suppressed sexual behavior (Fig. 2), suggesting that PR is involved in the dopamine-facilitated lordosis response. It is likely that the antiprogestin creates an unresponsive form of receptor that cannot be activated either by P or by dopamine (*22*).

We also administered antisense oligonucleotides to PR mRNA to reduce the amount of PR in the ventromedial nucleus. Antisense oligonucleotides to PR have been shown to inhibit P-facilitated lordosis (*23*). Ovariectomized rats with indwelling cannulae were primed with E (subcutaneously) and concurrently given an icv injection of antisense oligonucleotides to PR. Oligonucleotides were readministered 24 hours later, and a maximal effective dose of P (2 μg) or apomorphine (100 ng) was administered (by icv injection) 48 hours after the initial E-priming. The animals were examined for sexual behavior in the presence of males and the LQ determined (Fig. 3A). Neither E nor apomorphine alone had an effect on the sexual behavior of female rats; after E treatment, both P and apomorphine induced equivalent intensities of lordosis. Antisense oligonucleotides

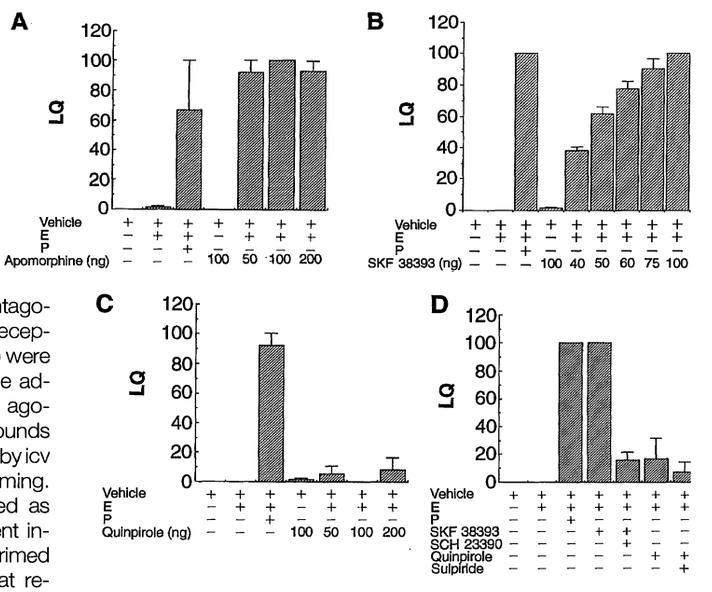

Fig. 1. The effect of apomorphine and dopamine receptor agonists and antagonists on sexual behavior of female rats. Ovariectomized rats were primed with estradiol benzoate (E) in sesame oil (10 μg). Various doses of (**A**) apomorphine, (**B**) SKF 38393 or, (**C**) quinpirole were administered. (**D**) The D_1 dopamine receptor antagonist SCH 23390 (100 ng) or D_2 receptor antagonist sulpiride (100 ng) were administered 30 min before the administration of their respective agonists (100 ng). All the compounds other than E were administered by icv injection 48 hours after E-priming. Sexual behavior was measured as described (*28*). Each experiment included control groups of E-primed and non–E-primed animals that received vehicle only. The agonists and antagonists were dissolved in saline and administered by icv injection. The dose of P used (2 μg in sesame oil) gave maximal response. Statistical analyses were done as described (*29*). Kruskal-Wallis one-way ANOVA on ranks method of analysis indicated a statistically significant effect ($P < 0.001$) of all treatments compared with E-treated controls ($n = 6$ in each group). Dunn's method for comparison indicated a statistically significant difference ($P < 0.05$) in lordosis response between apomorphine- or SKF 38393 (75 ng or more)–treated animals versus E-treated animals. No significant effect on sexual behavior was observed in animals that were administered quinpirole when compared with controls ($P > 0.05$). The Mann-Whitney rank sum test indicated an effect of SCH 23390 on SKF 38393–facilitated lordosis ($P = 0.002$), whereas no significant difference in lordosis response was observed between quinpirole ± sulpiride–treated animals ($P > 0.1$).

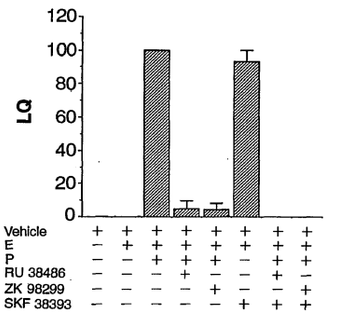

Fig. 2. Effect of P antagonists on P- or SKF 38393–facilitated sexual behavior. Ovariectomized rats were primed with E (Fig. 1). Forty-eight hours later, the animals were given icv injections of RU 38486 (2 μg) or ZK 98299 (2 μg) in sesame oil. Then 60 min later the animals were administered icv injections of P (2 μg in sesame oil) or SKF 38393 (100 ng in saline), and sexual behavior was quantitated as described (*28*). Control animals received vehicle only or vehicle and E only. Statistical analysis was done as described (*29*). Statistically significant differences ($P < 0.05$) were observed in P- or SKF 38393–facilitated lordosis responses in the animals that received the antagonists compared with those that did not ($n = 6$ for all groups).

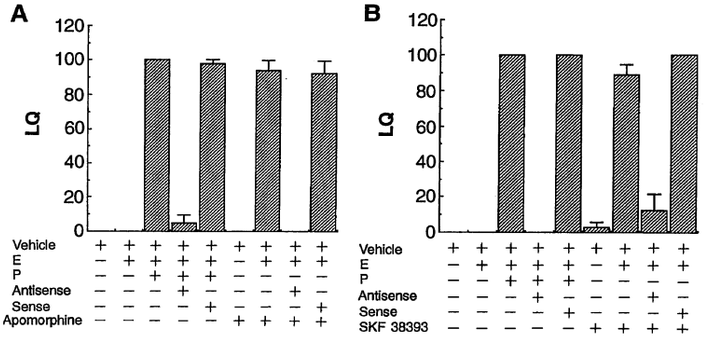

Fig. 3. Effects of (**A**) apomorphine and (**B**) selective D_1 dopamine receptor agonist SKF 38393 in the presence of oligonucleotides to PR mRNA on sexual behavior of E-primed female rats. Rats were treated with E as described (Fig. 1) and concurrently administered 4 nmol of antisense or sense oligonucleotides to the PR mRNA in saline. Twenty-four hours later the oligonucleotide treatment was repeated. Apomorphine or SKF 38393 in saline was administered by icv injection at a dose of 100 ng 48 hours after E-priming. The sexual behavior of female rats was observed and scored as described (*28*). The antisense and sense oligonucleotides used in these experiments were to the PR mRNA sequence 5′-TGTTGTCCCCGCTCATGAGC-3′. Another set of oligonucleotides to the sequence in the PR mRNA 5′-CCCGCTCATGAGCCGGCCAG-3′ gave similar results. All oligonucleotides were directed against sequences around the translational initiation region of the rat PR mRNA. Statistical analysis was done as described (*29*). Statistically significant ($P < 0.05$) differences in P-, apomorphine-, or SKF 38393–facilitated lordosis were evident in animals that received antisense oligonucleotides compared with those that did not. Sense oligonucleotides had no significant effect ($P > 0.05$) on P-, apomorphine-, or SKF 38393–facilitated lordosis ($n = 6$ in every group).

to PR suppressed lordosis initiated either by P or apomorphine (Fig. 3A). In contrast, control animals that received sense oligonucleotides to PR responded to either P or apomorphine. In an identical experimental paradigm, icv administration of the selective D_1 agonist SKF 38393 also induced lordosis that was blocked by antisense, but not sense, oligonucleotides to PR (Fig. 3B). These results were confirmed with two separate sets of oligonucleotides to PR mRNA and their matched sense oligonucleotide controls.

To demonstrate that icv administration of antisense oligonucleotides to PR mRNA reduced the amount of PR in the rat hypothalamus, we measured the amount of cellular PR in the mediobasal hypothalamus of E-primed animals that received antisense or sense oligonucleotides (4 nmol) to PR mRNA 48 hours after E-priming (*24*). Although E-priming induced a 2.6-fold increase in PR concentration compared with that in untreated animals, administration of PR antisense oligonucleotides reduced the E-induced PR concentration by 52.2%. The control PR sense oligonucleotides had no significant effect (*25*). Lordosis was abolished when the amount of PR was decreased by only 52%, similar to the decreases in the amount of PR that occur after administration of P during the state of hyposensitivity to P (*1*, *15*, *24*, *26*). It appears that a critical threshold concentration of PR is essential for P or dopamine to initiate lordosis.

Our results provide evidence for the requirement of PR for physiological actions of both P and dopamine in the central nervous system and suggest the presence of a convergent pathway through which steroids and neurotransmitters can control sexual behavior. Dopamine regulation of lordosis, which also requires activation of intracellular PR, suggests that dopamine induces a ligand-independent activation of PR. This dual mechanism for activating steroid hormone receptors may represent an important physiological communication mechanism by which neurotransmitters can affect steroid receptor-dependent gene transcription through events initiated at their cell membrane receptors.

Regulation of gene expression by means of cross-talk between membrane receptors for neurotransmitters and intracellular steroid receptors could be a general aspect of integration of neural information in the neuroendocrine system. The operation of such a mechanism could provide a means by which neuronal responses to environmental, behavioral, or emotional events could selectively alter steroid effects on behavior and physiology.

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25. Tissue samples were homogenized in 10 mM tris-HCl (pH 7.4) containing 1.5 mM Na_2EDTA, 10% glycerol, and 12 mM monothioglycerol and centrifuged at 48,000*g* for 30 min; hormone binding assays were done by incubating the samples with 0.4 nM [^{3}H]R5020 in the presence and absence of 100 nM unlabeled P for 4 hours. Bound and free [^{3}H]R5020 were separated by gel filtration on Sephadex LH-20 and expressed as femtomoles of [^{3}H]R5020 specifically bound per milligram of protein. Protein was determined by the method of Bradford (*27*). The following receptor concentrations were obtained: vehicle = 2.75 ± 0.28; E = 7.18 ± 0.34; E + sense oligonucleotides to PR = 6.63 ± 0.27 and E + antisense oligonucleotides to PR = 3.43 ± 0.55. Each value is the mean ± SEM of four to six independent determinations. One-way analysis of variance (ANOVA) indicated a statistically significant difference ($P < 0.001$) in the mean values among the treatment groups. A pairwise multiple comparision by the Student-Newman-Keuls method indicated a significant effect of antisense oligonucleotides on estradiol pretreatment ($P < 0.05$), whereas sense oligonucleotides had no significant effect on PR concentration ($P > 0.05$).
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28. Sexual behavior was measured in terms of number of lordosis responses of each female rat in the pres-

ence of a male. The number of mounts attempted by a male was also scored and recorded. The results of all the experiments were converted to lordosis quotient (LQ), defined as a percentage of full lordosis response (perineum elevated, all four legs extended from the initial crouch position and the head at an angle of 45° from the floor) divided by the number of mounts. Each group consisted of six animals, and each female was tested for 10 min in the presence of a male. The animals were tested for lordosis response at 30 min and at 3 hours after injection of compounds. The effects of the compounds were not transient, because identical results were obtained at 30 min and at 3 hours after infusion.

29. Statistical analysis was done by either of two methods as appropriate: Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's method for comparision of all groups versus control group, or one-way ANOVA followed by Dunnett's method for comparision of all groups versus control group. Sigma Stat (Jandel Corporation) was used.

30. We thank K. Mayo for the sequence of the rat PR mRNA, J. Turcotte for assistance with the binding assays, the staff of the Center for Comparative Medicine at Baylor College of Medicine, and D. Scarff for help in the preparation of the figures. Supported in part by N.S. 19327 (NIH) and Research Scientist Development Award grant MH-00885 to J.D.B. and a NIH (National Institute of Child Health and Human Development) grant to B.W.O.

27 April 1994; accepted 8 July 1994

TECHNICAL COMMENTS

Accounting for Endothermy in Fishes

A basic premise of the recent article by B. A. Block *et al.* (*1*) is that a molecular phylogeny of scombroid fishes provides novel insight into the evolution of endothermy in fishes. Specifically, nucleotide sequence data from a portion of the mitochondrial *cytochrome b* gene were used to infer multiple origins of endothermy—in billfishes, butterfly mackerel and tunas—and to hypothesize selective, causative forces. Block *et al.* note that two recent hypotheses based on morphological data (*2, 3*) differ in the placement of the butterfly mackerel and billfishes, "taxa that are key to the study of the evolution of endothermy within the suborder" and state that "[i]n an attempt to resolve these relationships, we have produced a hypothesis of scombroid phylogeny based on molecular data" (*1*, p. 212).

We contend that the molecular phylogeny infers nothing new about the number of origins of endothermy in scombroid fishes. Despite their differences, both of the hypotheses based on morphology and that based on *cytochrome b* require three origins of endothermy (Fig. 1). The strategies for elevating body temperature in the three endothermic groups are strikingly different (*4*), and we perceive no arrangement of taxa in which a hypothesis of one (or two) origins of endothermy would be more parsimonious than one requiring three. For example, a single-origin hypothesis of endothermy in the form of a brain heater derived from the superior rectus muscle in the ancestor of billfishes and scombrids (Fig. 1A) not only requires the loss of the condition in "other scombrids" and "bonitos," but the conversion of the lateral rectus eye muscle in the butterfly mackerel to a brain heater, and finally the evolution of a "whole body" form of endothermy in the tunas produced by countercurrent mechanisms (as opposed to thermogenic organ) in the brain, muscle, and viscera. Block *et al.* (*1*) note that despite their derivation from different muscles, the thermogenic organs of billfishes and butterfly mackerel could be homologous, citing evidence of structural and biochemical similarity (*5*). But even in the absence of morphological and physiological data that suggest the three types of endothermy are nonhomologous, a hypothesis of independent origins requires the fewest evolutionary steps (three as opposed to a minimum of four, Fig. 1).

Hypothetically, it would be equally parsimonious to propose (i) a single origin of endothermy (in any of the three forms) with subsequent modifications and (ii) independent origins only if billfishes, tunas, and butterfly mackerel form a monophyletic group. Likewise, a sister-group relationship between any two of the endothermic taxa would render a double-origin hypothesis as likely as one requiring three events. None of those clades has been proposed, and thus the hypothesis of independent origins goes unchallenged. The cladograms based on morphology are derived mostly from characters independent of endothermy, and exclusion of the one endothermy character in each hypothesis does not affect the topology. In summary, the morphological data clearly suggest three origins of endothermy.

Block *et al.* (*1*) state that the molecular data are important because they provide information about the affinities of billfishes and butterfly mackerel. However, none of the six nodes that determine the placement of butterfly mackerel in their phylogeny [figure 2 in (*1*)] was supported in greater than 50% of 300 replications of the bootstrap calculation (*1*, p. 212). The molecular data also provide little evidence for the clade comprising endothermic tunas, the monophyly of which has not been questioned.

In a comparative review of recent studies

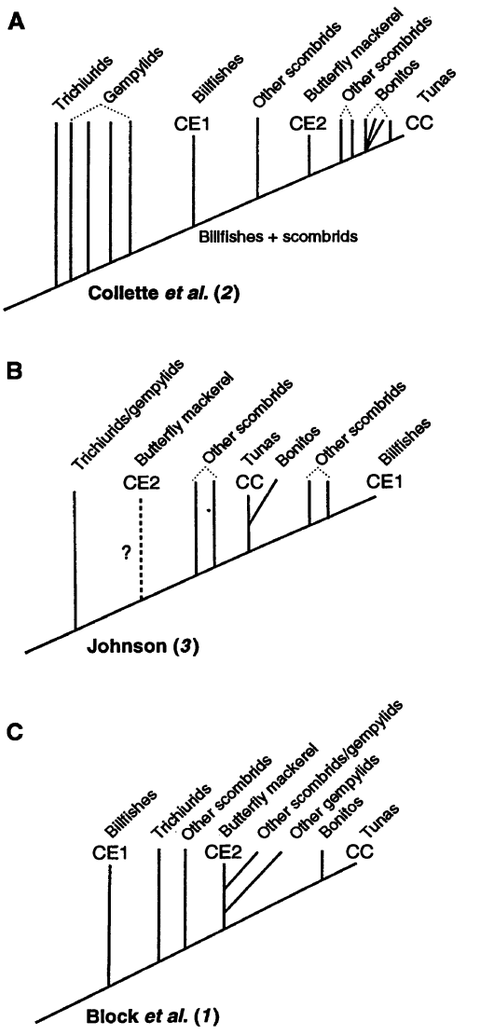

Fig. 1. Simplified versions of three recently proposed phylogenies of scombroid fishes. (**A**) and (**B**) are based on morphology (*2* and *3*, respectively) and (**C**) on sequences of a portion of the mitochondrial *cytochrome b* gene (*1*). CE1, cranial endothermy by a thermogenic organ derived from superior rectus eye muscle fibers; CE2, cranial endothermy by a thermogenic organ derived from lateral rectus eye muscle fibers; CC, whole body endothermy by counter-current heat exchangers in the brain, muscle, and viscera.

based on morphological or on molecular data, Patterson *et al.* (*6*, p. 179) found that "[c]ongruence between molecular phylogenies is as elusive as it is in morphology and as it is between molecules and morphology." In the absence of additional hypotheses of scombroid phylogeny based on molecular data, it is premature to suggest the *cytochrome b* data have "resolved" scombroid relationships. One potential problem is that addition of taxa in molecular phylogenies often leads to decreased support for a previously preferred cladogram (*6*). The studies based on morphology included examination of all scombroid genera, whereas that based on molecular data (*1*) was limited to repre-