second leg pairing applies to the trot, the canter, and the transverse gallop. A perfect bound with no phase between either the front or hind legs could be represented by either pairing.
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13. Human measurements were made by digitizing 16-mm film of runners on the semicircular section of an outdoor cinder track. The camera (Bolex H16, operating at 50 frames per second) was mounted on a tripod at the center of the semicircle (18.5-m radius) and panned to track the runner. Ground markers spaced at 1.0-m intervals provided a scale reference and permitted alignment of each frame with respect to the next. Running speed was about 3.8 m sec^{-1}. The data for the cat were obtained by digitizing 16-mm film provided by Wetzel and colleagues [M. C. Wetzel, A. E. Atwater, D. G. Stuart, in *Neural Control of Locomotion*, R. N. Herman, S. Grillner, P. S. Stein, D. G. Stuart, Eds. (Plenum, New York, 1976), pp. 99–136]. Treadmill markers spaced at 0.25-m intervals provided scale and alignment as before. Running speeds for the cat were about 2.2 m sec^{-1} for trotting and 3.1 m sec^{-1} for galloping. Small circular markers attached to the cat's skin made digitizing easier. When digitizing the data for both the human and the cat, I estimated the point of support provided by each foot visually. A straight line from this point to the hip (or shoulder for the cat's front legs) was used to find the leg length and the leg angle. The center of mass of the cat was taken as the point midway between the shoulder and the hip. The pitch angle of the body was the angle between the line connecting shoulder to hip and the horizontal, offset so that $\phi(0) = 0$.
14. Animals do not run with a pattern of motion that is precisely repeatable from one stride to the next, even for a single gait. This variability has been reported in studies of interlimb coordination in the cat [D. G. Stuart, T. P. Withey, M. C. Wetzel, G. E. Goslow, Jr., in *Control of Posture and Locomotion*, R. B. Stein, K. G. Pearson, R. S. Smith, J. B. Redford, Eds. (Plenum, New York, 1973), pp. 537–560; S. Miller, J. van der Burg, F. G. A. van der Meché, *Brain Res.* **91**, 217 (1975); A. W. English, *J. Neurophysiol.* **42**, 229 (1979)]. In principle, variability need not influence symmetry. A legged system can switch from one symmetric pattern of motion on one stride to a different symmetric pattern on the next stride. A legged system can maintain symmetry despite variations because the symmetry equations describe a class of body and leg motions rather than a particular motion. On the other hand, variations may have asymmetric components. Such asymmetric components are expected when a system accelerates as described in (*3*), but asymmetric steady-state variation is also possible. The reported variability has not been analyzed to reveal the relative contributions of symmetric and asymmetric components.
15. I thank M. Wetzel for providing film of the cat, J. Hodgins and J. Koechling for helping film the human runners, and J. Koechling for writing the computer programs used to digitize and process data. Supported by a grant from the System Development Foundation and by contract MDA903-81-C-0130 from the Engineering Applications Office of the Defense Advanced Research Projects Agency.

16 May 1985; accepted 20 November 1985

Bacterial Gene Inserted in an Engineered RNA Virus: Efficient Expression in Monocotyledonous Plant Cells

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Brome mosaic virus (BMV) is a plant virus whose genome consists of three RNA components. A previously described viral complementary DNA expression system has been used to express both wild-type and altered genomic RNA's in barley protoplasts. Variants of BMV RNA3 were constructed in which the coat gene had been removed or replaced with sequences encoding chloramphenicol acetyltransferase (CAT). CAT sequences were also inserted near the 5′ end of the intact coat gene. When inoculated on protoplasts together with transcripts of BMV RNA's 1 and 2, all of these RNA3 derivatives were replicated and produced subgenomic RNA's analogous to the normal subgenomic coat protein messenger RNA. RNA3 derivatives in which the CAT coding sequences were oriented with the same polarity as viral genes produced significant CAT activity in protoplasts. CAT expression was improved by inserting the CAT gene in frame with the upstream coat protein initiation codon, and exceeded expression in plant cells transformed with Ti plasmid-based vectors.

APPROPRIATE LINKAGE OF HETEROlogous sequences to a viral genome by recombinant DNA methods can allow those sequences to be amplified as part of an extrachromosomal replicon, transcribed into messenger RNA (mRNA), and translated into protein products. Accordingly, useful gene cloning and expression vectors have been derived from a wide range of bacterial, animal, and plant viruses that replicate by way of DNA intermediates (*1*). Most known viruses infecting eukaryotes, however, have genomes that replicate through only RNA intermediates. We show here that such RNA viruses may also be engineered for experimental purposes, including the efficient expression of foreign coding sequences.

We have developed an in vitro transcription system to express a plant RNA virus, brome mosaic virus (BMV), from cloned complementary DNA (cDNA) (*2, 3*), which allows recombinant DNA techniques to be used for engineering this virus for experimental purposes. Several attributes make BMV attractive for initial studies on designed modification of RNA viruses. Its genome is naturally divided among three segments (designated genomic RNA's), but only two components are necessary for replication in plant protoplasts (*4*), which demonstrates that the replication machinery acts in *trans* and suggests that altered replicating components could be constructed. The virus multiplies to high copy number in plant cells, many details of its biology are known, and its principal hosts are monocotyledonous plants (*5*), in which engineered expression of foreign genes has only recently been reported (*5a*).

Plasmids pB1PM18 (pB1), pB2PM25 (pB2), and pB3PM1 (pB3) contain cDNA copies of BMV RNA's 1, 2, and 3, respectively, adjacent to a modified *Escherichia coli* promoter (*2*). The mixture of capped in vitro transcripts from all three Eco RI–linearized plasmids is infectious to whole barley plants (*3*). To facilitate assays of replication and gene expression, infectivity studies were extended to protoplasts. We found that isolated barley protoplasts, which support BMV infection (*6*), could be efficiently infected with transcripts (Fig. 1B) if the template plasmid DNA was first removed, as by RNA precipitation with 2*M* LiCl (*7*). Typically, as judged by an antibody test (6), 60 percent of the protoplasts became infected when 10^5 viable protoplasts were inoculated (*8*) with a mixture of 1 μg each of pB1, pB2, and pB3 transcripts. Of all single and twofold component combinations, only RNA1 plus RNA2 gave rise to viral RNA replication (Fig. 1B). A previous study had shown that RNA 1 and 2 were sufficient to infect protoplasts (*4*), but these RNA's had not been tested singly. Thus, BMV resembles another tripartite RNA virus, alfalfa mosaic virus (*9*), in requiring two components for RNA replication in isolated cells.

The third BMV component, RNA3, is dicistronic (*10*), encoding genes for both a protein of molecular weight 32,000 (protein 3a) and the virion coat protein. In normal BMV infections, milligram quantities of coat protein are produced per gram of tissue (*5*) from translation of a subgenomic coat mRNA (RNA4) produced in large quantities from RNA3, probably by partial transcription of minus strand (*11*). Replacement of the coat gene with foreign coding sequences might provide high-level expression of the heterologous sequences at the level of both mRNA and gene product. To see if changes could be made in the coat region of RNA3 without preventing either genomic

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RNA replication or subgenomic RNA production, we constructed pB3D, in which most of the coat gene was deleted from pB3 (Fig. 1A). The altered BMV3 transcript from pB3D, when inoculated onto protoplasts with transcripts of pB1 and pB2, was replicated and produced a subgenomic mRNA (Fig. 1B). Densitometry of the autoradiograph of Fig. 1B shows that although accumulation of the altered genomic RNA was reduced threefold from that of the wild type, the molar yield of subgenomic mRNA per genomic RNA was at least 50 percent as high as that of the wild type. The similar ability of wild-type and pB3D genomic RNA3's to direct subgenomic mRNA production suggests that the subgenomic mRNA promoter in BMV3 does not extend farther than 17 bases downstream of the subgenomic start site, where the two sequences diverge (the Sal I site of Fig. 1A). Together with previous in vitro results (*11*), it also suggests that at least a functional core of the subgenomic RNA promoter resides in the 38 bases between the RNA3 Bgl II and Sal I sites (Fig. 1A).

To test whether foreign coding sequences could be expressed by linkage to BMV RNA, we constructed pB3 derivatives in which the coding sequences for the bacterial chloramphenicol acetyltransferase (CAT) gene from plasmid pBR325 (*12*) were inserted in place of the normal BMV coat sequence (Fig. 2). Because the CAT gene contains an internal Eco RI site, the Eco RI site originally used for runoff termination of pB3 transcripts (*2, 3*) could not be used in these constructs. Hence, a synthetic oligonucleotide (*2*) was used to fuse a Pst I site, which does not occur in either BMV cDNA or the CAT gene, to the 3′ end of BMV cDNA in pB3, creating pB3P (Fig. 2). Capped transcripts of Pst I–cut pB3P, like those of Eco RI–cut pB3, were replicated when inoculated onto barley protoplasts with transcripts of pB1 and pB2. On the sense strand of pB3P, the 3′ BMV cDNA sequence (underlined) adjoining the inserted Pst I site (bracketed) is (5′) . . . AAGAGAC[CTGCAG] . . . (3′). This sequence predicts that the runoff-terminated BMV3 transcript produced from Pst I–cut pB3P would lack the terminal A residue of mature BMV virion RNA, but this base may be restored by transfer RNA (tRNA) nucleotidyl transferase after recognition of the tRNA-like structure of the BMV 3′ end (*13*), as has been shown for both turnip yellow mosaic virus RNA and BMV RNA (*14*). Such an event may be a normal part of BMV RNA maturation (*15*).

CAT coding sequences were inserted in pB3P downstream of the coat AUG (Fig. 2), either in frame (pB3CA42) or out of frame (pB3CA61, pB3CA71) with the coat AUG. RNA blot analysis (Fig. 3A) of nucleic acid extracts from protoplasts inoculated with transcripts of these plasmids, combined with transcripts of pB1 and pB2, shows that these chimeric BMV RNA's both replicate and give rise to subgenomic mRNA's. Similar RNA replication results were obtained with pB3 derivatives containing the CAT gene in the opposite orientation, and also without deleting coat sequences before CAT gene insertion, indicating some tolerance for the sequence of the insertion and the overall length of the final RNA3 derivative. Densitometry of blots in Fig. 3A, and of similar ones using an RNA3-specific probe, revealed that from 5 to 16 ng of chimeric RNA3 accumulated per 10^5 protoplasts by 20 hours after infection, or 5- to 15-fold less RNA3 than is produced by infection with pB3 transcripts. Insertion of CAT sequences also reduced the molar ratio of subgenomic mRNA to parent genomic RNA, which is about 1.0 for wild type (*6*) to around 0.5. Reduced accumulation of genomic and subgenomic RNA from RNA3 derivatives may reflect decreased synthesis, increased degradation, or both. Increased degradation may result from lack of RNA encapsidation in

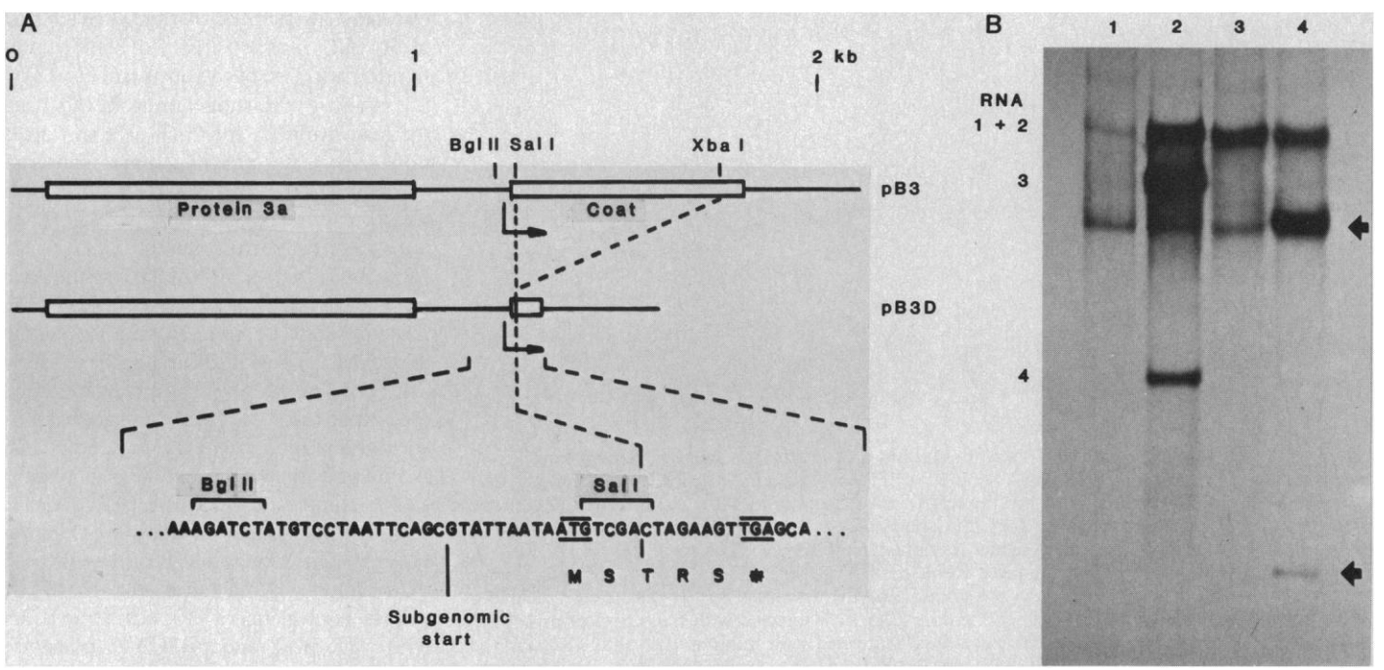

Fig. 1. Construction and replication of a BMV RNA3 derivative with a 0.5-kb deletion in the coat gene. (A) Schematic diagram of BMV cDNA regions in plasmids pB3 and pB3D, showing the 3a and coat coding regions as open boxes. pB3D was constructed by cleaving pB3 DNA with Sal I and Xba I, repairing the ends with DNA polymerase I large fragment, and recircularizing the larger DNA fragment with T4 DNA ligase (*21*). The sequence spanning the Sal I–Xba I junction in pB3D was confirmed by dideoxy sequencing (*22*) after subcloning in M13mp18. (B) Fluorograph of gel-electrophoresed ^{3}H-labeled RNA's synthesized in protoplasts inoculated with BMV cDNA transcripts and cultured 20 hours in the presence of ^{3}H-labeled uridine (40 μCi/ml) (*6*), but without actinomycin D or cephaloridine. Total nucleic acids were extracted and subjected to electrophoresis and fluorography (*6*). Protoplasts were mock-inoculated (lane 1) or inoculated with in vitro transcripts from the following plasmids: pB1+pB2+pB3 (lane 2), pB1+pB2 (lane 3), or pB1+pB2+pB3D (lane 4). In addition to viral RNA's, two host ribosomal RNA's can be seen (compare lane 1 with lanes 2 to 4). The position of wild-type BMV RNA's 1, 2, 3, and 4 are numbered in the margin, and the RNA products from pB3D are indicated with arrows. RNA1 and RNA2 were not resolved on this gel, and the deleted version of RNA3 from pB3D co-migrated with the smaller labeled host RNA.

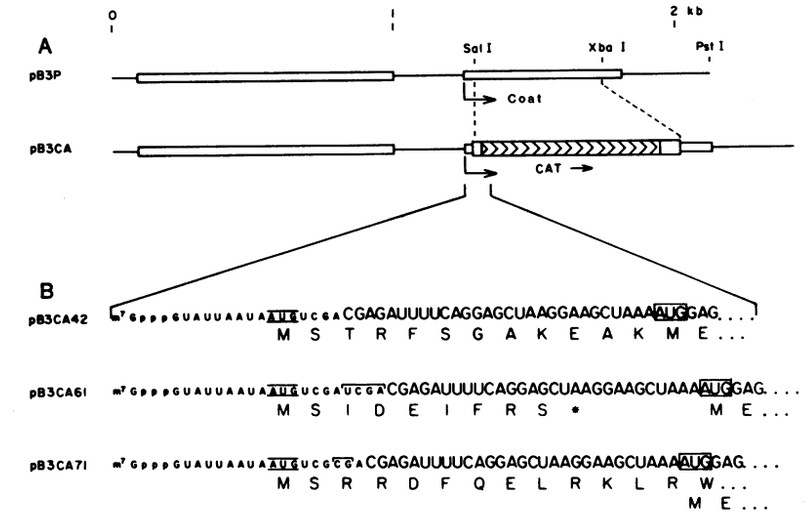

Fig. 2. (A) Schematic diagram of inserted CAT sequences in pB3P derivatives pB3CA42, 61 and 71. The pB3CA42 was constructed by ligating the 773-bp Taq I fragment of pBR325, containing the entire CAT coding sequence (cross-hatched), between the Sal I and Xba I sites of pB3P. All restriction fragment ends were filled out with DNA polymerase I large fragment to generate blunt ends before ligation. Plasmid pB3CA42 was further modified by cutting with either Sal I (for pB3CA61), or Acc I (for pB3CA71), repairing the 5′-overhangs with DNA polymerase I large fragment, and recircularizing with T4 DNA ligase (*21*). Acc I recognizes the same sequence as Sal I but leaves a two-, rather than four-, base overhang. The sequence across the BMV-CAT sequence junction in all three constructs was verified by dideoxy sequencing as in Fig. 1A. (B) 5′-Terminal sequences of subgenomic mRNA's and their translation products generated from RNA3-CAT chimeras, with the site of subgenomic mRNA initiation being assumed to be the same as for wild-type BMV RNA3. Sequences derived from the CAT gene are shown with larger letters than those from subgenomic BMV RNA4, the BMV coat AUG is denoted by lines above and below, and the CAT AUG is boxed. Brackets denote bases duplicated to construct pB3CA61 and pB3CA71 from pB3CA42. Translation from the first (BMV coat) AUG would continue in phase with (pB3CA42), terminate before (pB3CA61), or continue well past (pB3CA71) the CAT AUG.

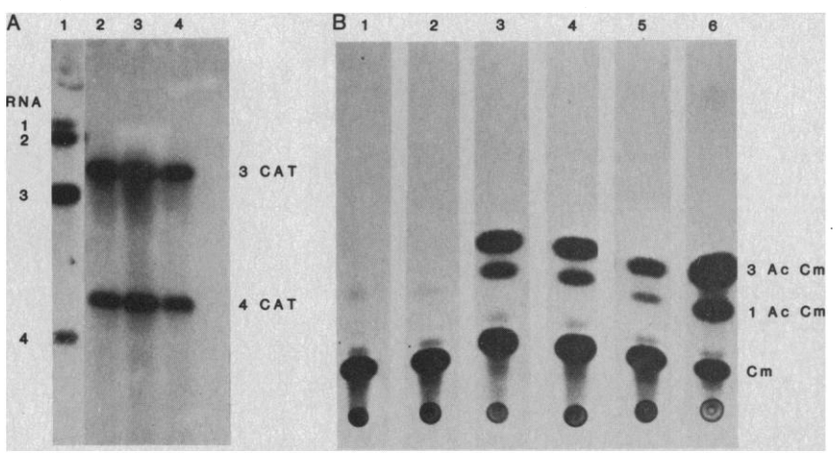

Fig. 3. (A) BMV and BMV-CAT RNA's synthesized in protoplasts inoculated with transcripts of pB1 and pB2, plus pB3 (lane 1), pB3CA42 (lane 2), pB3CA61 (lane 3), or pB3CA71 (lane 4). After electrophoresis on a 1 percent agarose gel, the RNA was electrophoretically transferred to a Zeta-probe membrane (Bio-Rad) and hybridized (*21*) with (lane 1) nick-translated pB1, pB2, and pB3 or (lanes 2 to 4) with ^{32}P-labeled RNA from CAT sequences cloned in pSP64 (*23*). The positions of BMV RNA 1, 2, 3, and 4, as well as BMV-CAT chimeric RNA3 (3 CAT) and RNA4 (4 CAT), are noted in the margins. (B) Assay of protoplast extracts for CAT enzyme activity. Lane 1, unreacted ^{14}C-labeled chloramphenicol (Cm); lane 2, extract representing 1000 cells infected with transcripts of pB1, pB2, and pB3. Lanes 3 to 5, extract representing 200 cells infected with transcripts of pB1, pB2, and the indicated pB3P derivative: lane 3, pB3CA42; lane 4, pB3CA61; and lane 5, pB3CA71. Lane 6, extract from 1000 mock-inoculated cells plus 5×10^{-5} unit (*17*) of bacterial CAT (Pharmacia P-L Biochemicals). Protoplasts were harvested 20 hours after inoculation, resuspended in 0.25*M* tris-Cl, *p*H 7.5, 0.5 m*M* leupeptin, and lysed by one freeze-thaw cycle before cell debris was removed by centrifugation. Portions of the supernatant were assayed for CAT activity in 50 μl of the same buffer plus 0.5 m*M* acetyl coenzyme A and 0.125 μCi of ^{14}C-labeled chloramphenicol (47 mCi/mmol, New England Nuclear) for 45 minutes at 37°C. Chloramphenicol and its acetylated derivatives (1AcCm, 3AcCm) were extracted with ethyl acetate and resolved by thin-layer chromatography (*17, 24*). After autoradiography (24 hours), spots were cut out and quantified by liquid scintillation counting. Percentage acetylation of chloramphenicol (2.67 nmol) by extracts of 200 cells (600 ng of protein per 200-cell portion) were: pB3CA42, 21.5 percent; pB3CA61, 14.0 percent; and pB3CA71, 3.3 percent.

the absence of coat protein expression or from a lack of adaptation of CAT coding sequences for stability in plant cytoplasm, for which the viral RNA is presumably selected.

Extracts of protoplasts inoculated with pB1, pB2, and pB3CA42 transcripts exhibited considerable CAT activity (Fig. 3B). Omission of either pB1 or pB2 transcripts from the inoculum prevented protoplasts from synthesizing CAT, which indicates that the chimeric RNA3 must be replicated to be detectably expressed. CAT expression was reduced by 50 percent when the Sal I–Xba I fragment spanning the BMV coat gene (Fig. 2A) was reinserted downstream of the CAT gene in pB3CA42, and CAT expression was eliminated when the orientation of CAT sequences was inverted. Constructs in which CAT codons were out of frame with the coat AUG (pB3CA61 and pB3CA71) (Fig. 2B) also expressed CAT activity (Fig. 3B), but at levels dependent on the reading frame used. When ribosomes initiating at the first AUG of the subgenomic mRNA reached a termination codon before passing the CAT gene initiation codon (as with pB3CA61) (Fig. 2B), CAT activity was still 65 percent of that derived from pB3CA42 (Fig. 3B). When the initial reading frame extended past the start of the CAT gene, as in pB3CA71, CAT activity was only 15 percent of that derived from pB3CA42. Such sensitivity of CAT expression to the frame of the upstream coat AUG is consistent with subgenomic RNA's being the major mRNA for CAT protein translation (*16*). CAT activity comparisons may overestimate the level of translational initiation of internal AUG's, since a CAT protein translated from the second AUG of the pB3CA61 and pB3CA71 subgenomic mRNA's would have a natural NH_2-terminus and might be more enzymatically active than the CAT fusion protein initiated at the first AUG in the pB3CA42 mRNA. However, these results suggest that internal initiation and reinitiation of translation do occur at biologically significant levels in plant as well as animal and yeast cells (*16*), and such pathways may occur in the natural expression of plant cellular and virus genes.

In assays such as those of Fig. 3B, the extract equivalent of 40 cells transfected with pB1, pB2, and pB3CA42 transcripts generated a strong CAT activity signal, and extract representing a single cell had activity easily detectable by overnight autoradiography. Such protoplasts produced 1.8×10^{-2} units (*17*) of CAT activity per milligram of total extracted cellular protein, which is 7- and 23-fold higher, respectively, than CAT expression reported by Herrera-Estrella *et al.* (*18*) for the light-induced ribulose 1,5-

bisphosphate carboxylase small-subunit promoter and the nopaline synthase promoter in Ti-plasmid transformants.

These results demonstrate that an RNA virus can be both engineered and used as a convenient and efficient gene expression vector at the level of single cells. Direct infectivity of poliovirus cDNA (*19*) and the likelihood that in vitro transcription will allow expression of cloned cDNA to other RNA viruses (*3*) suggest that other plant, animal, and bacterial RNA viruses should be amenable to similar manipulation. The stability of nonselected sequences in RNA genomes has been questioned (*20*) because of high estimated mutation rates. Such effects were not apparent in this study, but future experiments to test RNA virus expression of heterologous genes in whole plants over many replication cycles will allow further assessment of this question.

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25. We thank L. S. Loesch-Fries for valuable advice on protoplast methodology. Supported by a grant from Agrigenetics Research Associates.

28 May 1985; accepted 15 November 1985

Bilateral Syringeal Interaction in Vocal Production of an Oscine Bird Sound

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The vocal organ, or syrinx, of oscine birds has two parts, each of which has generally been presumed to operate independently of the other. A significant counter-example is now demonstrated in the production of a common vocalization by the black-capped chickadee (*Parus atricapillus*), in which the two acoustic sources interact in a nonlinear fashion. This bird produces a sound with multiple frequency components that are heterodyne products resulting from cross-modulation between two signals, thus providing evidence that avian phonation can involve cooperative coupling between the two syringeal sources.

THE AVIAN SYRINX IS UNIQUE among the sound-generating organs of vertebrates. Most notably, many birds, including members of the suborder oscines or the "true songbirds," possess a two-part syrinx. Situated at the junction of the two primary bronchi, each half of this organ has an independent set of muscles, membranes, and nerves thought to be involved in phonation (*1–3*). While this morphological peculiarity has been known since the 1800's, it was only recently suggested that the two sides of an oscine syrinx might be functionally independent, thus enabling a bird to sing an "internal duet." Initial support for this hypothesis came from acoustic analyses of birdsongs in which elements presumed to arise from two independent sources were isolated spectrographically or through the use of frequency filters (*2, 4*). Further evidence arose from the discovery that transection of the hypoglossal motor nerve to either side of the organ leads to a predictable loss of certain acoustic elements in a complex song; the deleted elements are presumed to have been generated by the disabled side of the syrinx (*3*). On the basis of this evidence, it is now generally accepted that songbirds are capable of using their two voices simultaneously and independently (*5*), and oscine phonation thus has become an important model system for studying the lateralization of motor control (*3*).

While it is likely that the two sides of an oscine syrinx can be functionally decoupled in the production of some sounds, it may be premature to assume that the two halves never interact cooperatively during phonation. Given their close proximity, and the number of structural and aerodynamic connections between them, the acoustic sources could also be coupled. We here report the production of a complex signal by the black-capped chickadee (*Parus atricapillus*) through a previously unreported mechanism of interaction between the two syringeal sides.

The "dee" syllable, the terminal note in the "chick-a-dee" call from which this species derives its name, is composed of 12 to 20 temporally overlapping frequency components (Fig. 1). These components, evenly spaced at approximately 400-Hz intervals, were originally considered to be a source-generated harmonic series (*2*), but two facts contradict this hypothesis. (i) Significant energy is only rarely present at the predicted fundamental frequency and at what would be the second and third harmonics. Since the avian vocal tract is presumed to have little effect as an acoustic filter (*2*), it is unclear how to account for these missing frequencies. (ii) In 32 percent of a sample of "dee" syllables examined, the frequencies of the spectral components were clearly not integral multiples of the spacing between them, yielding an ambiguous value for a possible fundamental frequency (*6*). The lowest frequency component that consistently displays significant energy invariably occurs at what would be the fourth harmonic, or approximately 1600 Hz (Fig. 1). The next highest frequency component (about 2000 Hz) is also invariably of strong amplitude (*7*). If these two frequency components are labeled as peaks 1 and 2, there is generally an increase in the energy of the successive peaks up to peaks 5 to 8 (about 3000 to 4000 Hz), followed by a decrease. Most of the spectral energy occurs below 7000 Hz.

If the "dee" syllable is not a harmonic

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