37°C. After the initial incubation, cells were washed twice in staining buffer and incubated for 20 min at 4°C in the presence of biotinylated RA3.3A1. Cells were washed twice in staining buffer and then incubated a second time at 4°C in the presence of phycoerythrin-avidin and either FITC-labeled anti-Ig (187.1, rat monoclonal antibody to mouse κ light chain) or antibody to class I (M1/42.398, rat monoclonal antibody to mouse H2K). Cells were washed twice with staining buffer and analyzed by two-color flow cytometry.

15. Reciprocal loss of CD45 from the cell surface after treatment of B cells with anti-Ig was not detected. Cells express ten times more CD45 molecules on their surface than mIgM (*12, 13*). Thus, if each mIgM interacts with one CD45 molecule, loss of every mIg molecule from the surface of the cell would result in, at most, a 10% decrease in the number of CD45 molecules on the cell. Such a decrease would not be detected in this assay.
16. L. B. Justement, unpublished results. Consistent with the comodulation data, class I H2K molecules were not observed to cocap with CD45.
17. M. R. Gold *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3436 (1991).
18. Splenic B cells were incubated in phosphate-free medium for 1 hour at 37°C, washed once, and permeabilized with α-lysophosphatidylcholine (7) [A. R. Mire, R. G. Wickremasinghe, R. Michalevecz, A. V. Hoffbrund, *Biochim. Biophys. Acta.* **847**, 159 (1985)]. [γ^{32}P]ATP (150 μCi) was added to 6×10^7 cells and AlF_4^- (10 μM $AlCl_2$ and 30 mM NaF) was then added to stimulate phosphorylation of the mIgM-associated protein complex. For each reaction, 2×10^7 cells were lysed in buffer containing 1% digitonin as well as protease and PTPase inhibitors; ^{32}P-labeled phosphoproteins (pp37, −34, −33, and −32) were coimmunoprecipitated with mIg by means of monoclonal antibody (MAb) 187.1 (*14*), as described (7), with the exception that in the last two washings, PTPase inhibitors were omitted. Other membrane proteins or CD45 were isolated from the K46-17μmλ B cell lymphoma. These cells were washed twice in PBS and lysed in buffer containing protease inhibitors (7), 1% NP-40, 0.15 M NaCl, and 0.01 M sodium phosphate, pH 7.2, for 1 hour on ice. The lysates were centrifuged at 12,000*g* for 20 min at 4°C. For each reaction, the supernatant from 5×10^7 cells was incubated with the MAb I3/2.5 (rat anti-CD45) (10 μg/ml) or with either of the control MAbs (187.1 or M1/42.398) (10 μg/ml) at 4°C for 1 hour. Protein-G beads (Pharmacia) were added, and the samples were mixed for an additional hour at 4°C. For the reaction in lane 7, only Protein-G beads were added to the K46-17μmλ lysate. Beads were washed six times in sodium phosphate buffer containing NP-40 (0.2%). To initiate the dephosphorylation reaction, beads bound with ^{32}P-labeled subunits were mixed with beads bound to either CD45, mIgM, or H2K in a final volume of 50 μl. The reaction buffer [N. K. Tonks, C. D. Diltz, E. H. Fischer, *J. Biol. Chem.* **263**, 6722 (1988)] contained 25 mM imidazole-HCl, pH 7.2, 0.15 M NaCl, β-mercaptoethanol (0.1%), and NP-40 (0.6%), which causes dissociation of the α, β, and γ subunits from mIgM (7). Mixtures were incubated at 37°C for varied periods of time and the reaction was stopped by addition of 2× SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer followed by boiling for 5 min. Phosphoproteins were analyzed by SDS-PAGE (10% gel) followed by autoradiography.
19. Splenic B cells (2×10^7), were phosphate-depleted (7) and incubated with saturating concentrations of antibodies (187.1, or I3/2.5, or both) for 10 min on ice. After the addition of primary antibodies, cells were washed three times in ice-cold phosphate-free medium and the secondary cross-linking mAb, RG7 (mouse antibody to rat κ light chain), was added. Cells were incubated for an additional 10 min on ice and washed once. The cells were then permeabilized and mixed with [γ^{32}P]ATP and AlF_4^- (*7, 18*). The cells were incubated for 10 min at 37°C and lysed in buffer containing 1% digitonin. ^{32}P-labeled subunits were immunoprecipitated with Protein-G beads after the addition of 187.1 (10 μg/ml) (*18*). The mIgM-associated phosphoprotein complex was visualized by autoradiography after separation by SDS-PAGE (10% gel) under reducing conditions.
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Tissue-Specific Splicing in Vivo of the β-Tropomyosin Gene: Dependence on an RNA Secondary Structure

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The β-tropomyosin gene in chicken contains two mutually exclusive exons (exons 6A and 6B) which are used by the splicing apparatus in myogenic cells, respectively, before (myoblast stage) and after (myotube stage) differentiation. The myoblast splicing pattern is shown to depend on multiple sequence elements that are located in the upstream intron and in the exon 6B and that exert a negative control over exon 6B splicing. This regulation of splicing is due, at least in part, to a secondary structure of the primary transcript, which limits in vivo the accessibility of exon 6B in myoblasts.

IN MOST HIGHER EUKARYOTES GENES the coding region is interrupted by noncoding sequence segments (intervening sequences or introns) which are excised from the full copy transcript of the gene (pre-mRNA) during the splicing process. Variable use of the coding regions (exons) of the same gene to obtain partially different mRNA's is the widespread process known as alternative splicing.

The β-tropomyosin gene in chicken codes for three related isoforms of tropomyosin and contains a pair of mutually exclusive exons (exons 6A and 6B) that are expressed, repectively, in smooth muscle cells or nonmuscle cells (exon 6A) or in skeletal muscle (exon 6B). Myogenic cells in culture express exon 6A before differentiation and exon 6B after differentiation (myoblast and myotube stage, respectively).

On the basis of computer predictions (*1, 2*) or mutagenesis studies in vivo (*3*) and in vitro (*4*), we have proposed that a secondary structure could be one of the cis-regulating elements that controls alternative splicing of this gene.

The potential influence of secondary structures on alternative splicing has been suggested on the basis of in vitro and in vivo splicing of mRNA precursors containing inverted repeat sequences that flank exons or splice sites (*5, 6*). A threshold value exists in vivo (but not in vitro) for the distance between the two inverted repeats (that is the length of the hairpin loop), which has been related to the existence of a competition between the interaction of the primary transcript with heterogeneous nuclear, spliceosomal ribonucleoproteins (RNP's) or both, and the folding of the newly transcribed region into a secondary structure (*6*). It has been proposed (*6*) that a "window" of about 100 nucleotides (nt) exists behind the transcribing polymerase within which the primary transcript is "naked" and free to fold. However, a naturally occurring example of a secondary structure, which influences alternative splicing in vivo has not yet been reported. Two cases have been described (*7, 8*) in which secondary structures can affect splice site selection in vitro, but these results have not been supported by parallel in vivo splicing studies.

We have earlier shown (*3*) that the sequence near the acceptor site of exon 6B contains at least two, nonoverlapping, cis elements that are part of a negative splicing control preventing the use of exon 6B in myoblasts. Mutation of either of them activates splicing of the exon even in myoblasts or nonmyogenic cells. One of these elements is contained in the long polypyrimidine stretch (about 90 bp) located between the branchpoint (position −105) and the terminal AG of the intron, and the other is contained in the exon itself. Earlier we proposed a model whereby a secondary structure of the primary transcript would be responsible in myoblasts for the skipping of exon 6B, while exon 6A would be spliced as a default choice. Furthermore, a specific factor would be expressed in myotubes, which would disrupt the structure (or pre-

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vent its formation) and allow the exclusive recognition of the specific exon (6B).

We suggested (*3*) that the mutations introduced would disrupt this structure (or an equivalent higher order organization of the primary transcript) and relieve the inhibition thus making the exon accessible. To test this hypothesis we have used one of our previously described mutant minigenes, Mut16 (*3*). In this minigene a 15-nt change is introduced at the very beginning of exon 6B; this change affects the formation of one stem (stem I) (*9*) of the secondary structure (Fig. 1, A and B). This mutation results in the activation of splicing of the exon in myoblasts.

In a first minigene construction (MutA16, Fig. 1C) the other arm of stem I has been changed. The sequence of the mutation was designed to provide the changes necessary to restore base pairing of the two mutated sequences in Mut16 and MutA16 when combined on the same minigene (Fig. 1D). Mutation A16 has an activating effect on the use of exon 6B when compared to the wild type (Fig. 2, lanes 6, A and B), even if this effect is not as dramatic as in Mut16 (compare sections WT, Mut16, and MutA16). The ratio of splicing of exon 6A to exon 6B is about 1:1, while in wild type it is of the order of 10 to 30:1. When the two mutations are combined on the same minigene (Mut16/A16, clone 39, Fig. 1D), the splicing pattern, which was completely biased toward the inclusion of exon 6B in mutant Mut16, partially reverts, leading to the reexpression of exon 6A (Fig. 2, lanes 6A and 6B of corresponding section). In a variant of this mutant (Mut16/A16, clone 40), which was obtained by chance, 2 nt of the mutated region of Mut16—located in the loop of the hairpin, positions 7825 to 7826 (Fig. 1D)—have reverted to the wild type. The analysis of the transcripts derived from this mutant (Fig. 2, Mut16/A16, clone 40) showed a better reversion of the splicing pattern that was indistinguishable from the wild type. These data show that the mutation introduced in MutA16 partially compensates the activating effect of Mut16 on the utilization of exon 6B (compare sections Mut16 and Mut16/A16 in Fig. 2), and suggest a role for at least two of the nucleotides in one loop region.

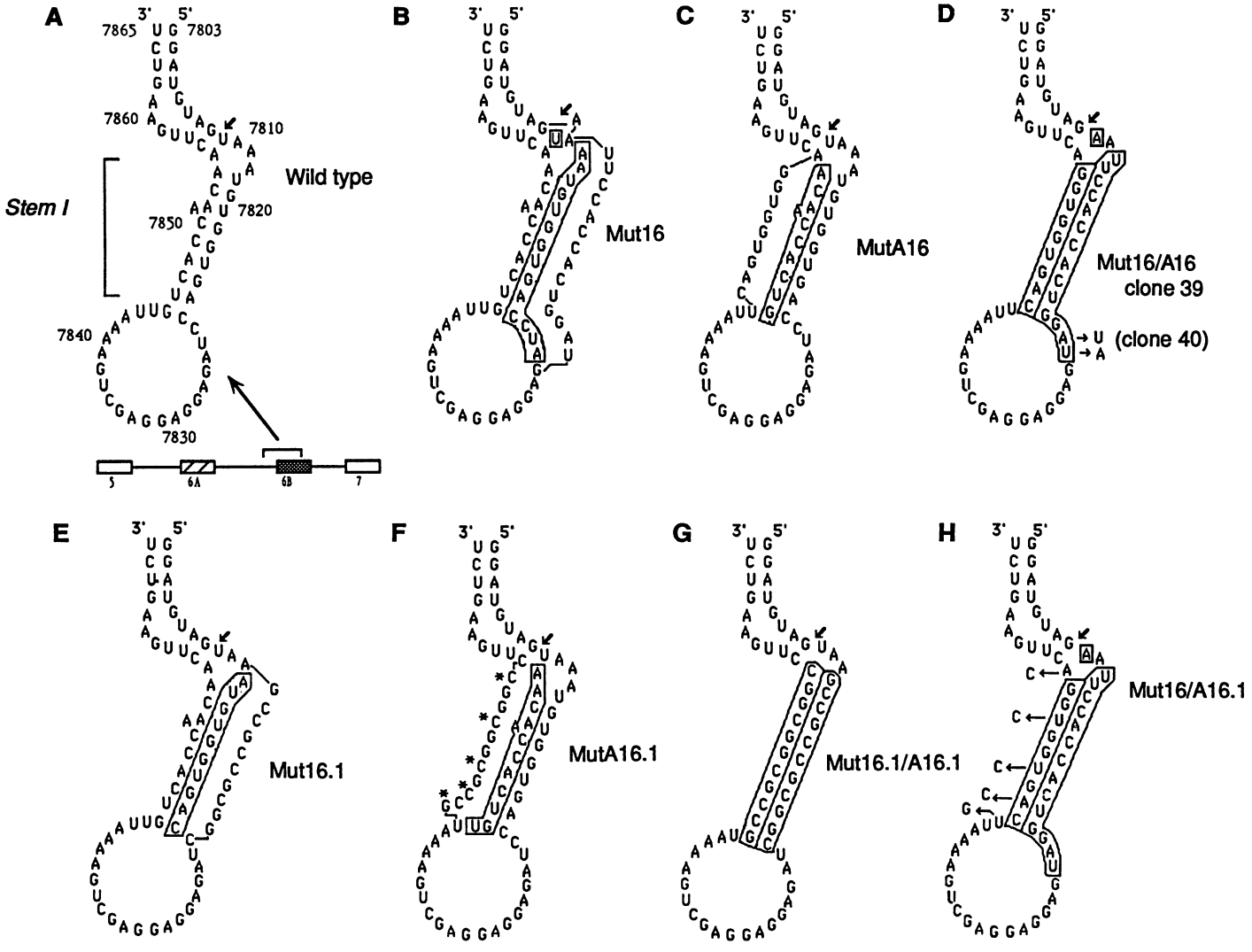

Fig. 1. (**A**) Zuker folding (*16*) of the wild-type primary transcript around exon 6B (table 1 in *17*). The rough position of the folded region is indicated in the bottom schematic diagram of the construction used for transfection (Fig. 2 legend). The acceptor site of exon 6B is shown on the secondary structure by a short arrow. Stem I is indicated. (**B** to **H**) The sequence of the analyzed mutations is reported on the wild-type secondary structure model. In the single mutants (B, C, E, and F) the wild-type sequence that has been changed is boxed, and the sequence of the mutation is indicated nearby. In the double mutants Mut16/A16 (D) and Mut16.1/A16.1 (G) the boxed sequence is the mutated one. In (D) the 2-nt reversion in clone 40 (position 7825 to 26) is indicated. In (F) the differences between the sequences of MutA16 and MutA16.1 are indicated by asterisks. In (H), the differences between the sequences of Mut16/A16 (boxed sequence) and Mut16/A16 (the relative changes are indicated nearby) are shown to evidence the lower stem stability in this last mutant.

The lack of complete activation of exon 6B in mutant MutA16 when compared to Mut16 was unexpected. One possible reason is that MutA 16 is shorter than Mut16 since the mutation affects only the stem of the hairpin, whereas in Mut16 5 nt in loop regions were mutated (2 nt upstream and 3 nt downstream of the stem). A second possibility is that the mutated sequence in MutA16 can induce a different (or partially different) folding of the region, which would be capable (at least in part) of favoring exon 6B skipping.

To choose between these possibilities, we constructed two new mutants in this region. First, a shorter mutation was introduced in the same location as in Mut16 to obtain Mut16.1, in which only the stem of the hairpin has been mutated to a sequence containing four base changes with respect to Mut16 (Fig. 1E). Second, we constructed MutA16.1 (Fig. 1F) which brings an 11-nt mutation in the same location as MutA16, but differs from it by its length (11 nt compared with 9 nt) and by the sequence of the common region (two mutations from T to C and one from A to C).

The five changes introduced in MutA16.1 were designed to lessen the stability of the stem in the double mutant Mut16/A16.1 (Fig. 1H), which should result in an activation of exon 6B as in Mut16. However, when coupled with the mutation Mut16.1, which contains the compensatory changes (Mut16.1/A16.1), the stem of the hairpin is expected to be more stable, and splicing of exon 6B should be inhibited to a greater degree than in wild type. Moreover, a new sequence is expected to fold differently compared to MutA16 and possibly to have a different splicing pattern.

The splicing pattern of mutant Mut16.1 (Fig. 2) is similar to the one of Mut16. Exon 6B choice is the main splicing pathway (even if it is somewhat less activated than in mutant Mut16), and exon 6A is barely detectable. This suggests that the activating effect on splicing of exon 6B observed in Mut16 is mainly due to the mutations introduced in the stem region. Moreover, when the splicing pattern of MutA16.1 is examined, a similar activation of exon 6B is observed as in Mut16.1 (Fig. 2, corresponding section).

The double mutant Mut16/A16.1, in which the stability of the stem is lower than in the double mutant Mut16/A16 or in the wild type, behaves as if the negative control around exon 6B was again relieved and this exon becomes a constitutive one (Fig. 2). In the double mutant Mut16.1/A16.1, whose primary transcript folds in a more stable stem-and-loop structure, exon 6B is less accessible than in the wild type or in mutant Mut16/A16, and exon 6A is spliced exclusively (Fig. 2, section Mut16.1/A16.1).

Our data show that mutation in either one of the two strands of the stem can alone relieve the negative control around exon 6B and modify the splicing pattern toward the almost exclusive use of this exon in myoblasts. That this effect is indeed mediated by the disruption of the stem is shown by the restoration of the negative regulation in the double mutants in which the stem is reconstituted (Mut 16/A16 and Mut16.1/A16.1), but not in the mutant with two mutated regions that cannot undergo base-pairing (Mut16/A16.1). A stronger inhibition of exon 6B splicing is observed in Mut16.1/A16.1, the stem is expected to be more stable than in the wild-type structure. The data also suggest that the differences in the splicing patterns between MutA16 and MutA16.1 may be due to the existence in MutA16 of an alternative folding of the region that can inhibit splicing of the exon although to a lesser extent compared to the wild type (*10*).

We analyzed the splicing pattern of minigenes bearing a reconstituted stem I (Mut16/A16, clone 40, and Mut16.1/A16.1) in myotubes (*11*) in order to ascertain that the negative control could still be overcome in differentiated cells as in the wild type. In transcripts derived from both minigenes we observed a significative change in the ratio of splicing of exon 6A to exon 6B to levels close to those of the unmodified minigene. Finally, most of the

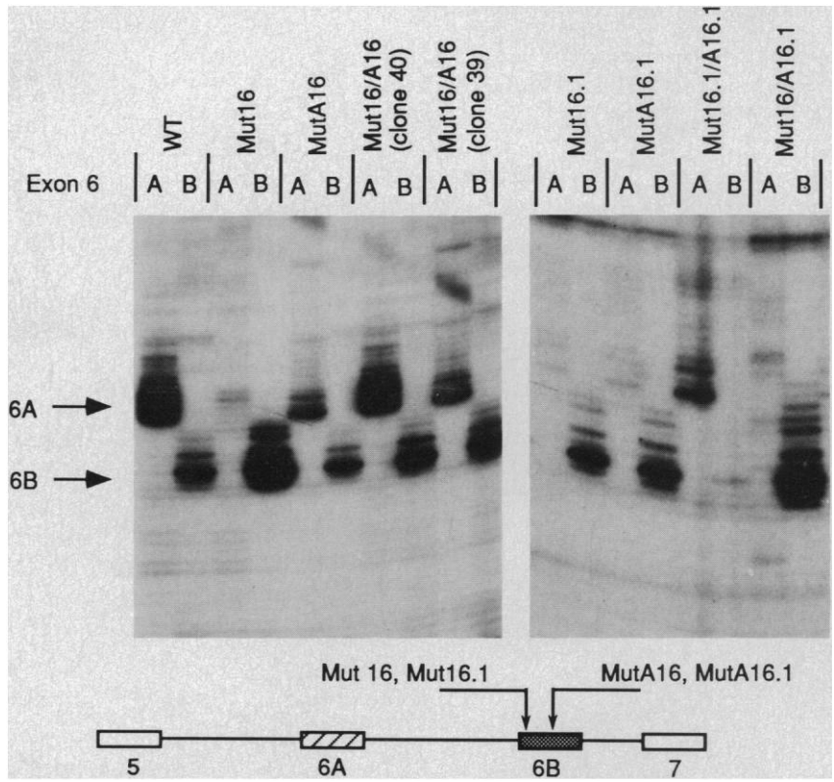

Fig. 2. Controlled primer extension (*2, 3, 18*) of transcripts derived from myoblasts transfected with the various minigene constructions (transient expression) (Fig. 1 for sequences). All the mutations were introduced by site-directed mutagenesis (*19*) directly on the single-stranded form of the wild-type minigene previously described (*2*) and extensively sequenced to ensure authenticity. All minigenes contained exons 5 to 7 of the β-tropomyosin gene (*1*) inserted between SV40 sequences reponsible for transcription initiation and termination. The transfections were obtained by the calcium-phosphate precipitation (*20*) with 10 μg of plasmid per 10^6 cells and the transcripts were extracted after 24 to 48 hours (each minigene was transfected at least twice to overcome variability in transfection efficiency). The splicing pattern of each minigene was analyzed by controlled primer extension (*2, 3, 18*). In this method, total mRNA's are first cleaved by oligonucleotide-directed treatment with ribonuclease H and then subjected to reverse transcription from a common primer directed against SV40 sequences. By the use of oligonucleotides pairing with the sequences of exons 6A or 6B (lanes labeled, repectively, 6A and 6B for each section in the figure), two specific run-off products of reverse transcriptase can be detected which indicate the use of one or the other exon. A band at nucleotide 101 (shown by an arrow and labeled 6B in the figure) indicates a run-off product due to the use of exon 6B while the multiple bands (probably due to nuclease nibbling) around 108 (shown by an arrow and labeled 6A) show that exon 6A is used in the splicing reaction. Our results have been repeated by polymerase chain reaction analysis of the transcripts. The sections indicate the origin of mRNA's. The position of the mutations is shown in the bottom.

nucleotides located in the loop region (with the exception of nucleotides 7825 and 7826) could be modified without affecting the splicing pattern both in myoblasts and in myotubes (*11*).

We compared the sequences of the chicken β-tropomyosin gene with the corresponding *Xenopus laevis* and rat genes as far as stem I is concerned. In this region, the three genes have similar structures and the mutually exclusive exons 6A and 6B have identical splicing expression patterns (*1, 11, 12*). A single nonconservative change is present in stem I both for the rat and the *X. laevis* genes at position 7820, which causes an internal G-A mismatch instead of a A·U base pair (*13*), while most of the changes are located in the large loop or lead to G·T versus G·C base pair substitution. A similar conservation is not present in the cognate chicken α-tropomyosin gene (three nonconservative base changes can be observed in the stem I), in spite of the high conservation of the protein sequence in this region. This was expected because of the different expression pattern of exon 6B in the α tropomyosin gene (*14*).

The mechanism of this interference of splicing by secondary structures is still unclear. The integrity of stem I is necessary but not sufficient for the establishment of the negative control around exon 6B. Moreover, further data are needed to unambiguously define the overall structure of the region. We have described other nonoverlapping mutations (located in the upstream intron) capable of independently exerting the same activating influence as the stem mutations described above (*3, 4*). We have also obtained evidence that additional negative cis elements are present upstream of the branchpoint of exon 6B but not in the intron between exons 6B and 7 (*15*).

We favor the hypothesis that intron and exon cis elements are part of a complex regulatory system in which secondary structures and possibly trans acting factors act together competing with the binding of splicing factors to the branchpoint upstream of exon 6B in myoblasts. Finally, data in myotubes, when both exons 6A and 6B can be recognized by the splicing apparatus, a competition between the two exons is responsible for the preferential choice of the skeletal muscle specific exon (*15*).

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Subunit Communication in the Anthranilate Synthase Complex from *Salmonella typhimurium*

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The anthranilate synthase–phosphoribosyl transferase complex of the tryptophan biosynthetic pathway in *Salmonella typhimurium* is an allosteric, heterotetrameric ($TrpE_2$-$TrpD_2$) enzyme whose multiple activities are negatively feedback-regulated by L-tryptophan. A hybrid complex containing one catalytically active, feedback-insensitive and one catalytically inactive, feedback-sensitive mutant TrpE subunit was assembled in vitro and used to investigate communication between regulatory and catalytic sites located on different subunits. The properties of the hybrid complex demonstrate that the binding of a single inhibitor molecule to one TrpE subunit is sufficient for the propagation of a conformational change that affects the active site of the companion subunit.

REGULATION OF CARBON FLOW IN metabolic pathways is often achieved by the ligand-dependent modulation of the activity of strategically located allosteric enzymes. Allosteric enzymes are generally oligomeric in structure and have multiple, topologically distinct binding sites for substrates and regulatory effectors. Ligand binding is accompanied by conformational changes in the enzyme that alter its affinity for the binding of additional ligand molecules and are usually manifested by cooperativity in the reaction kinetics (*1, 2*). A pivotal question in the elucidation of the allosteric mechanism is whether the binding of a single effector molecule to one of the regulatory sites of an enzyme is sufficient to elicit the conformational effects that modulate the functioning of all of its catalytic sites. We report results of experiments that test the possibility of such a concerted transition in the feedback-regulated anthranilate synthase–phosphoribosyl transferase (AS-PRT) complex of the tryptophan (Trp) biosynthetic pathway from *S. typhimurium*.

The AS-PRT complex is a multifunctional, heterotetrameric enzyme composed of the two TrpE and two TrpD polypeptides, each with a molecular weight of ~57 kD (*3, 4*). The complex catalyzes the first two steps of Trp pathway: (i) the conversion of chorismate (Chr), the branchpoint intermediate in aromatic amino acid biosynthe-

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