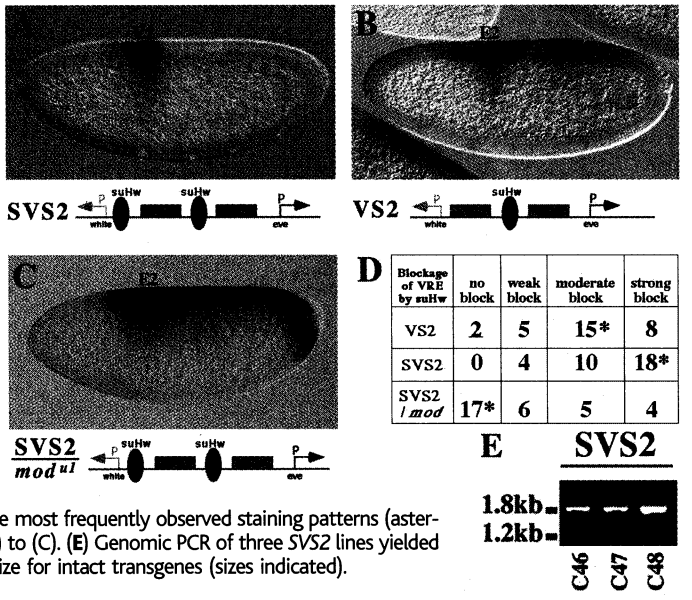

Blockage of VRE by suHw	no block	weak block	moderate block	strong block
VS2	2	5	15*	8
SVS2	0	4	10	18*
SVS2 / *mod*	17*	6	5	4

Fig. 3. Two flanking suHw elements can block VRE more effectively. VRE-mediated dorsal staining is blocked more effectively in *SVS2* embryos (**A**) than in *VS2* embryos (**B**). E2 expression and VRE-mediated repression of E2 are unaffected. (**C**) suHw insulator activity was reduced in embryos hypomorphic for *mod(mdg4)^{u1}*, shown by the intense dorsal stain. (**D**) suHw-mediated blockage of VRE was categorized in three random lines of *VS2*, *SVS2*, and *SVS2/mod(mdg4)^{u1}* embryos. The most frequently observed staining patterns (asterisks) are those shown in (A) to (C). (**E**) Genomic PCR of three *SVS2* lines yielded products of the expected size for intact transgenes (sizes indicated).

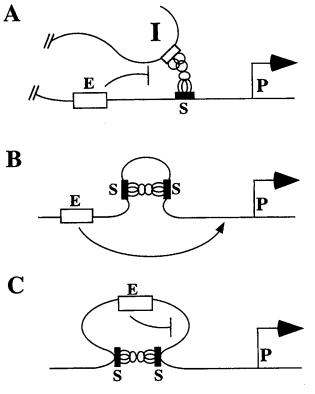

Fig. 4. Insulator-mediated loop formation. (**A**) A suHw insulator (S) may interact with other nuclear sites/insulators (I), separating the enhancer (E) and the promoter (P) into distinct domains and blocking their interaction. (**B**) Interactions between two tandem suHw insulators fail to sequester the enhancer and may even facilitate enhancer-promoter interaction by "looping out" the intervening DNA. (**C**) Enhancer blocking may be strengthened by the preferred interactions between two suHw insulators flanking the enhancer.

tissue-specific enhancers to target promoters by forming alternative chromatin loop domains. It is conceivable that these domains not only block inappropriate enhancers but also facilitate interaction between distant enhancers and the target promoter.

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Loss of Insulator Activity by Paired Su(Hw) Chromatin Insulators

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Chromatin insulators are regulatory elements that block the action of transcriptional enhancers when interposed between enhancer and promoter. The *Drosophila* Suppressor of Hairy wing [Su(Hw)] protein binds the Su(Hw) insulator and prevents enhancer-promoter interaction by a mechanism that is not understood. We show that when two copies of the Su(Hw) insulator element, instead of a single one, are inserted between enhancer and promoter, insulator activity is neutralized and the enhancer-promoter interaction may instead be facilitated. This paradoxical phenomenon could be explained by interactions between protein complexes bound at the insulators.

The *Drosophila* gypsy retrotransposon contains a chromatin insulator that consists of cluster of 12 binding sites for the Su(Hw) zinc-finger protein (*1–6*). In the presence of Su(Hw) protein binding, the insulator blocks the activity of an enhancer separated from the promoter by an Su(Hw) binding region. However, this insulator action fails in certain genetic rearrangements that introduce more than one *gypsy* retrotransposon in the region of the *yellow* gene (*7*). The loss of insulator activity might result from intrachromosomal pairing between the two *gypsy* retrotransposons, causing chromatin to fold and allowing the enhancer to contact the promoter. Alternatively, interaction between the pro-

teins bound to two Su(Hw) insulator elements might neutralize insulator action. Here, we analyze insulator activity as affected by insulator element copy number and location.

The *yellow* gene is required for dark pigmentation of *Drosophila* larval and adult cuticle and its derivatives. Two upstream enhancers, En-b and En-w, activate expression in the body cuticle and wing blades, respectively (*8*). When a single Su(Hw) insulator is inserted at position −893 relative to the *yellow* transcription start, between the enhancers and the *yellow* promoter (ESY, Fig. 1), enhancer action is blocked, resulting in yellow instead of dark pigmentation of body and wing cuticle. This block is relieved and pigmentation is restored when the construct is tested in a *su(Hw)*$^-$ background, confirming that the Su(Hw) protein is responsible (*9*). In the ESFSY construct (Fig. 1), a fragment bearing two Su(Hw) insulators, separated by a 1.5-kilobase (kb) spacer fragment, was inserted at position −893. The spacer is derived from the second exon of the *yellow* gene and has no enhancer or insulator activity of its own. In seven ESFSY transgenic lines, *yellow* expression was higher than that in the control ESY lines, and in three lines it was at wild-type levels. When three of the less pigmented ESFSY were tested in a *su(Hw)*$^-$ background, wild-type pigmentation was restored. Thus, the second Su(Hw) insulator partially or completely neutralizes the effect of the first one. Similar results were obtained when the distance between the two Su(Hw) insulators was reduced to 200 base pairs (bp).

The body and wing enhancers in these constructs are responsible for wild-type dark pigmentation because, when they were removed from ESFSY, yielding SFSY (Fig. 1), body and wing pigmentation was yellow in all transgenic lines. Similarly, constructs containing the *yellow* gene alone never result in body or wing pigmentation (*7*). Increasing the distance between upstream enhancers and the *yellow* promoter does not weaken insulator activity because lines containing ESFY, bearing a single Su(Hw) insulator 2.4 kb from the *yellow* transcription start, all had yellow body and wing pigmentation, indicative of the block of wing and body enhancers.

If the loss of insulator activity is due to a steric constraint imposed by a physical interaction between the two insulators, flanking either the enhancers or the target gene with insulators might have the same effect. This was tested with the SFESY construct in which two Su(Hw) insulators frame the wing and body enhancers. Flies from nine SFESY transgenic lines exhibited yellow wing and body pigmentation. When two of these lines were crossed into a *su(Hw)*$^-$ background, wild-type levels of pigmentation were restored, confirming that the proximal Su(Hw) element retained insulator activity.

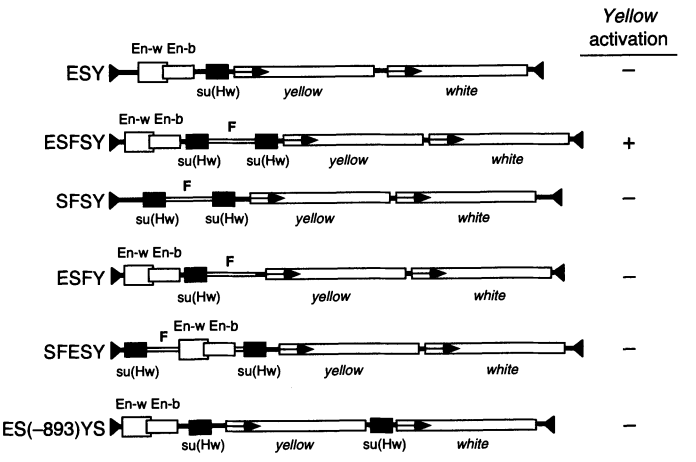

Fig. 1. Transposon constructs used to test insulator action. The maps of the constructs (*20*), not drawn to scale, indicate the *yellow* wing and body enhancers (En-w and En-b, respectively) as partially overlapping white boxes. The Su(Hw) insulator is shown as a black box and the *yellow* and *white* genes as white boxes with an arrow indicating the direction of transcription. F denotes a spacer fragment. The column to the right summarizes the results, with + indicating that the *yellow* gene was activated by its enhancers in the majority of the lines.

In the ES(−893)YS construct, the *yellow* gene is flanked by Su(Hw) insulators, one at position −893 and the other downstream of the *yellow* gene (Fig. 1). In nine ES(−893)YS transgenic lines, *yellow* expression in the body and wings was blocked. When two of these lines were crossed into a *su(Hw)*$^-$ background, wild-type pigmentation of wings and body was restored. Thus, the second insulator, downstream of *yellow*, does not prevent the insulating function of the first.

Next, we tested a different enhancer-promoter combination. The *white* gene is required for eye pigmentation and is regulated by its eye-specific enhancer. Roseman *et al.* (*5*) found that interposing the Su(Hw) insulator between the eye enhancer and *white* promoter completely blocked enhancer activity, whereas bracketing the *mini-white* gene between two Su(Hw) insulators protected *white* expression from position effects. In the EyeSYW construct, the eye enhancer was inserted between the *yellow* wing and body enhancers and was flanked by Flp recognition target (FRT) sites to permit its excision from transgenic flies (*10*). The three enhancers are separated from the *yellow* and *white* genes by a Su(Hw) insulator. Flies of 20 EyeSYW lines displayed eye pigmentation levels like those produced by an enhancerless *white* transposon, that is, ranging from pale yellow to red, depending on the insertion site. In two red-eyed EyeSYW lines, the deletion of the eye enhancer by Flp-dependent excision did not influence eye color, implying that in these two lines the *white* gene was activated by some genomic enhancer element. Thus, one Su(Hw) insulator interposed between eye enhancer and *white* gene blocks enhancer-promoter communication. The body and wing enhancers of the *yellow* gene were also blocked in these lines, indicating that the insulator functioned normally. Similarly, if the insulator was placed in front of the *white* gene, to give EyeYSW, the 14 transgenic lines obtained had eye colors in the range expected in the absence of eye enhancer. Deletion of the eye enhancer in five dark orange–eyed lines did not change eye pigmentation. Thus, the eye enhancer is blocked by one copy of the Su(Hw) element inserted either near or far from the *white* promoter.

The EyeSYSW construct uses the same enhancer configuration described above and contains one Su(Hw) insulator at position −893 relative to the *yellow* transcription start and another inserted between the *yellow* gene and the *mini-white* promoter (Fig. 2). Therefore, just one insulator intervenes between the enhancers and *yellow* but two insulators between the enhancers and *white*. In 19 of 21 transgenic EyeSYSW lines, wing and body pigmentation were yellow, indicating that the *yellow* enhancers were blocked, whereas *white* expression was stronger than in lines bearing the *mini-white* gene without eye enhancer. To demonstrate that the eye enhancer stimulates *white* expression in these lines, we excised it by Flp-induced recombination between FRT sites. In nine ΔEyeSYSW lines tested, the deletion of the eye enhancer strongly diminished eye pigmentation, indicating that the enhancer can activate the *white* gene despite the two intervening insulators. Therefore, also in this case, two insulators between enhancer and promoter neutralize one another. However, interaction between the two insulators does not simply inactivate them, because the upstream insulator can still block the activation of the *yellow* gene.

In the same EyeSYSW lines, *white* ex-

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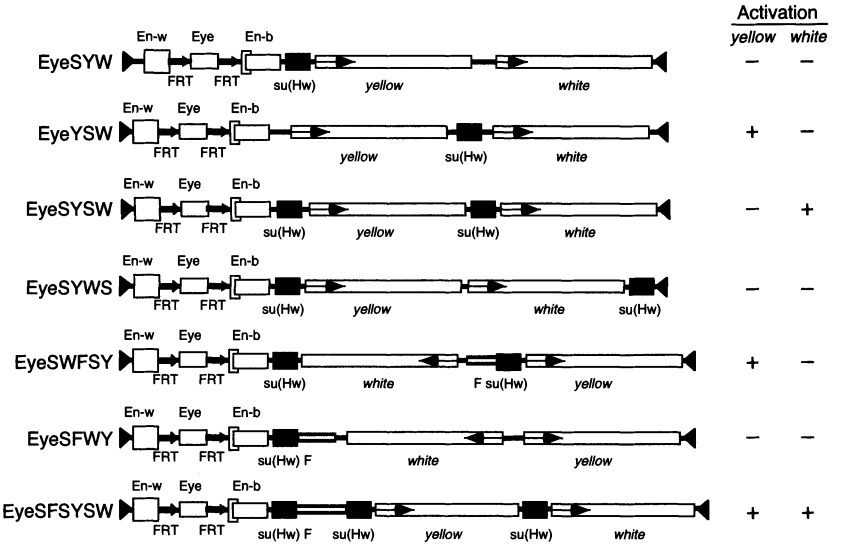

Fig. 2. Transposon constructs to test *white* enhancer action. The white box (Eye) indicates the eye enhancer of the *white* gene, and the thick arrows marked FRT represent the target sites of the Flp recombinase. The other symbols are the same as in Fig. 1. The two columns on the right summarize the results, with + indicating that the *yellow* or *white* genes were activated by their respective enhancers in the majority of the lines.

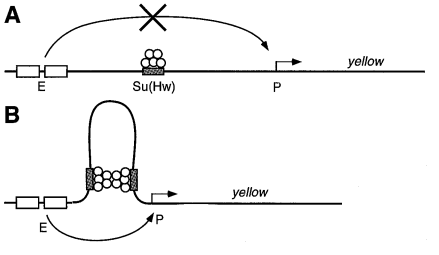

Fig. 3. Model of the double insulator bypass. **(A)** A single insulator blocks enhancer-promoter interaction. **(B)** Two insulators may interact with one another through the protein complexes bound to them, forming a loop and bringing the enhancers closer to the promoter.

pression was studied in a *su(Hw)*$^-$ background. In five lines, the absence of Su(Hw) protein reduced *white* expression, implying that the Su(Hw) protein actually has a positive role, facilitating enhancer-promoter interactions. In four other lines, the absence of Su(Hw) protein had no effect. Thus, the stimulating effect of the two Su(Hw) insulators may depend on genomic context and/or local chromatin structure. To show that the Su(Hw) protein does not by itself activate *white* expression, we crossed five lines bearing the EyeSYSW transposon with deleted eye enhancer (ΔEyeSYSW) into a *su(Hw)*$^-$ background. The absence of Su(Hw) protein did not influence *white* expression.

To determine what configuration of two insulators neutralizes their insulator activity, we constructed EyeSYWS, in which the two Su(Hw) insulators frame the *yellow* and *white* genes. Fourteen EyeSYWS lines displayed weak expression of both *white* and *yellow*, indicating that all three enhancers upstream of the interposed Su(Hw) insulator were blocked. However, when the *mini-white* gene flanked by two Su(Hw) insulators was inserted at position −893 relative to the *yellow* transcription start site (EyeSWFSY, Fig. 2), the *yellow* gene was expressed in the body and wings. In a *su(Hw)*$^-$ background, *yellow* expression decreased in three lines and did not change in one line, showing that the activation of the *yellow* promoter by distant *yellow* enhancers is improved by an interposed insulator pair. Thus, for both *white* and *yellow*, the insertion of two Su(Hw) insulators between the respective enhancers and promoters may facilitate their interaction instead of blocking it. When the Su(Hw) insulator between *white* and *yellow* genes was removed, yielding EyeSFWY (Fig. 2), *yellow* expression in the body and wings was suppressed, showing again that a single insulator blocks the wing and body enhancers. Two copies of Su(Hw) do not simply neutralize one another by an exclusive binary interaction. In the EyeSFSYSW construct, three insulator copies intervene between eye enhancer and *white* gene and two copies are between the *yellow* enhancers and the *yellow* gene. In 12 of 16 lines carrying this transposon, both *yellow* and *white* are activated, producing flies with strongly pigmented eyes and wing and body cuticle.

In summary then, when two or more Su(Hw) insulators are introduced between enhancer and promoter, their enhancer-blocking effect is neutralized in most cases and enhancer-promoter communication is often improved. Entirely similar results, using different promoter and enhancer combinations have been obtained by Cai *et al.* (*11*). The implication is that two insulators interact, probably through the protein complexes bound to them. This interaction by itself does not neutralize the blocking action, because when the insulators frame the enhancers or the target gene, the block still occurs. A possible explanation is that the "looping out" of the sequences separating enhancer and promoter displaces the insulators out of the way and, by bringing the enhancer and promoter closer, may even stimulate expression (Fig. 3). This may explain why the stimulating effect increases with the distance between enhancers and promoter.

These effects may have a bearing on the mechanism of insulator action. A possible way to envision how the insulator interferes with the access of the enhancer to the promoter is by associating with the nearest Su(Hw)-related complexes in the nucleus (*12, 13*). The effect of this association would be to tether loops containing members of an enhancer-promoter pair, thereby interfering with the interaction of the enhancer on one loop with the promoter on another loop. When two Su(Hw) elements are placed between enhancer and promoter, the loop would form preferentially between the two neighboring Su(Hw) elements, thereby shortening the distance between enhancer and promoter rather than inhibiting their interaction. This type of mechanism may also help to explain the role of boundary elements in the *Drosophila* bithorax complex (*14*). In the *Abd-B* regulatory region, boundary elements like *Fab-7* and *Fab-8* flank the *iab* enhancer regions, insulating them from the silencing or activating effects of adjacent regulatory regions (*15–17*). However, as insulators, the boundary elements would also block activation of the *Abd-B* promoter by more distant *iab* enhancers, thus defeating the purpose of these enhancers. Although other explanations are possible, our results with insulator pairs may account for this discrepancy. Interaction between boundary elements flanking each enhancer may effectively protect the *iab* enhancers from outside repressing effects and facilitate, instead of blocking, enhancer-promoter communication. It is possible, in fact, that one role of certain kinds of insulator is to promote the interaction between distant enhancers and promoters.

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20. The transposon constructs were based on the CaSpeR series and derivatives. The entire *yellow* gene was contained in an 8-kb fragment with the partially overlapping body and wing enhancers located, respectively, at positions −1266 to −1963 and −1808 to −2873 from the transcription start site. The *white* Eye enhancer fragment contained eye and testis enhancers (*18*). The Su(Hw) insulator was a 430-bp fragment containing 12 Su(Hw) binding sites, amplified by polymerase chain reaction from the *gypsy* retrotransposon. Details of the constructions are available upon request. The constructs were injected in $y^{-}ac^{-}w^{1118}$ embryos, and the transgenic flies were identified by their eye color. The transformed lines were tested by Southern blot hybridization for transposon integrity, copy number, and presence of the enhancers and Su(Hw) insulators. Only lines with single-copy inserts were used. Lines in a *su(Hw)*$^{-}$ mutant background were obtained by consecutively crossing transgenic males with *C(1)RM,yf; D/ T(2;3)Xa, C(1)RM,yf; su(Hw)*^{v}*/T(2;3)Xa, C(1)RM,yf; su(Hw)*^{2}*sbd/T(2;3)Xa* females as described (*19*).
21. Supported by grants from the Human Frontiers Science Program and from INTAS to P.G. and V.P. P.G. was an International Research Scholar of the Howard Hughes Medical Institute and received an award from the Volkswagen Stiftung Foundation, and A.G. was supported by a stipend from the Center for Medical Studies, University of Oslo.

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Crystal Structure of an Initiation Factor Bound to the 30S Ribosomal Subunit

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Initiation of translation at the correct position on messenger RNA is essential for accurate protein synthesis. In prokaryotes, this process requires three initiation factors: IF1, IF2, and IF3. Here we report the crystal structure of a complex of IF1 and the 30*S* ribosomal subunit. Binding of IF1 occludes the ribosomal A site and flips out the functionally important bases A1492 and A1493 from helix 44 of 16*S* RNA, burying them in pockets in IF1. The binding of IF1 causes long-range changes in the conformation of H44 and leads to movement of the domains of 30*S* with respect to each other. The structure explains how localized changes at the ribosomal A site lead to global alterations in the conformation of the 30*S* subunit.

The synthesis of functional polypeptides requires initiation of translation to occur at the correct mRNA codon. In prokaryotes, selection of the start codon involves formation of a 30*S* initiation complex containing the small (30*S*) ribosomal subunit, three protein initiation factors (IF1, IF2, and IF3), and initiator tRNA (formyl-methionine-$tRNA_f^{Met}$) base-paired to the mRNA start codon in the ribosomal P site (*1*–*3*). IF3 acts to ensure that the 30*S* subunit dissociates from the large (50*S*) ribosomal subunit (*4*). It also cooperates with IF2 to prevent incorrect P-site interactions by ensuring that only initiator tRNA is present in the P site (*5*–*7*) and that it interacts only with the start codon (*8*). The 50*S* subunit binds the 30*S* initiation complex after IF3 has been displaced, triggering hydrolysis of the guanosine 5′-triphosphate (GTP) bound to IF2. Subsequently, IF2 is released, allowing initiator tRNA to form the first peptide bond with the first elongator aminoacyl tRNA (aa-tRNA), which is delivered to the A site in complex with the elongation factor EF-Tu.

The role of IF1 is the least well defined of the three initiation factors (*2*). It has been implicated in subunit dissociation preceding initiation (*4*, *9*), stimulating 30*S* complex formation (*10*, *11*), release of IF2 from the 70*S* (*12*, *13*), and blocking of the A site to tRNA binding (*3*; *14*). The gene encoding IF1 is essential in *Escherichia coli* (*15*), suggesting that one or more of its functions are crucial in vivo. Here we present a 3.2 Å resolution crystal structure of the complex of IF1 with the 30*S* ribosomal subunit from *Thermus thermophilus*. The structure allows us to discuss the functions of IF1 at a molecular level and also provides an atomic resolution view of factor-induced conformational changes occurring within the small ribosomal subunit.

The large solvent channels found in the 30*S* subunit crystal form made it possible to soak IF1 directly into crystals prepared as described previously (*16*, *17*). Diffraction data extending to 3.2 Å were collected from these crystals (Table 1), and a single round of refinement against the native 30*S* coordinates resulted in a model with R/R_{free} of (0.239/0.278). The electron density for IF1 was visible in σ_A-weighted $2mF_o - DF_c$ maps (Fig. 1A). The nuclear magnetic resonance (NMR) structure of *E. coli* IF1 (*18*) was unambiguously placed in the density and rebuilt with the sequence of the *T. thermophilus* protein (Fig. 1). The Cα root-mean-square deviation between our final refined structure and the NMR structure is 1.41 Å. The major changes in the 30*S* structure occurred in helix 44 (H44), although small shifts in the relative positions of the RNA domains were also observed. The statistics of a final round of refinement including IF1 are shown in Table 1.

IF1 is a member of the S1 family of OB fold proteins (*19*, *20*), consisting of a barrel of five β strands with the loop between strands 3 and 4 capping one end of the barrel (*18*). It binds to the 30*S* subunit in a cleft formed between H44, the 530 loop, and protein S12 (Fig. 2, A and B). The face of IF1 that interacts with the ribosome is rich in basic residues, whereas most of the acidic residues are on the solvent-exposed surface. This highly asymmetric charge distribution is probably important in stabilizing binding to the 30*S* subunit. Conserved residues in IF1 make tight electrostatic and hydrogen bonding interactions with the phosphate backbone of the 530 loop. A loop from IF1 inserts into the minor groove of H44, forms contacts with the backbone of several nucleotides, and flips out bases A1492 and A1493 (Fig. 2A). A1493 is buried in a pocket on the surface of IF1, whereas A1492 sits in a cavity formed at the interface between IF1 and S12 (Fig. 2D). In both cases conserved arginine residues (Arg^{46} and Arg^{41}, respectively) are in a position to stack against the bases and stabilize the interaction. In contrast to the antibiotic paromomycin, which flips out A1492 and A1493 so that they are stacked together (*21*), IF1 causes them to be splayed apart.

The structure agrees well with most biochemical and mutagenesis data. The sequestering of bases A1492 and A1493 into protein pockets explains why IF1 completely protects them from chemical modification (*14*). The abolition of IF1 binding in A1492G or A1493G mutants is consistent with the bulkier guanine base being unable to fit in either binding pocket (*22*). The increase in reactivity of A1408 (*14*) is explained by the disruption of the base pair it makes with A1493 in the native structure (*16*, *23*). Finally, muta-

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