A U1/U4/U5 snRNP Complex Induced by a 2′-*O*-Methyl-Oligoribonucleotide Complementary to U5 snRNA

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Nuclear messenger RNA splicing involves multiple interactions between the five spliceosomal small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4, U5, and U6 and numerous spliceosomal proteins. Here it is shown that binding of a 2′-*O*-methyl-oligoribonucleotide complementary to U5 small nuclear RNA (snRNA) nucleotides 68 to 88 (BU5Ae) disrupts the initial U4/U5/U6 tri–snRNP complex, enhances the U2/U6 interaction, and induces a U1/U4/U5 snRNP complex. The U1/U4/U5 snRNP complex interacts specifically with an RNA oligonucleotide containing the 5′ splice site sequence and may therefore represent a transitional stage in the displacement of U1 from the 5′ splice site by U5 snRNP.

Precursor mRNA (pre-mRNA) is spliced to form mature mRNA by a dynamic complex, the spliceosome, composed of five snRNP particles (the U1, U2, U4, U5, and U6 snRNPs) and of many proteins (*1*). Spliceosome assembly occurs by ordered addition of snRNPs to the pre-mRNA, before the first chemical step of splicing. Initially, U1 snRNP binds the 5′ splice site (5′ SS) of the pre-mRNA, U2 snRNP associates with the branch site, and a preassembled U4/U5/U6 tri-snRNP complex associates with the polypyrimidine tract. Because depletion of U5 blocks association of the U4/U6 snRNP with pre-mRNA (*2*), U5 was thought to associate first with the 3′ SS region. Indeed, one U5 snRNP protein, IBP, binds to the polypyrimidine tract very early in spliceosome assembly (*3*). However, another U5 snRNP protein, p220, can be cross-linked to the 5′ SS, the branch site, the polypyrimidine tract, and the 3′ SS, which suggests that U5 snRNP may juxtapose the 5′ SS, 3′ SS, and the branch site (*4*). The 5′ SS is recognized initially by U1 snRNP, but U1 is replaced by U5 and U6 before the first catalytic step (*5*). U5 snRNA then interacts with the 5′ exon and, after the first catalytic step, with the first nucleotide of the 3′ exon as well. Thus, U5 snRNA itself is implicated in recognition of both exons (*6*).

An RNA oligonucleotide corresponding to a minimal 5′ SS induces formation of a U2/U4/U5/U6 complex (*7*), which presumably represents an intermediate in the spliceosome assembly pathway. We therefore tested whether RNA oligonucleotides directed against other snRNA sequences could induce formation of other potentially informative reaction intermediates. The U5 snRNP is highly resistant to nuclease treatment, and only two short regions of U5 RNA are accessible to chemical modification (*8*). One of these regions is the invariant loop (positions 36 to 46), which interacts directly, albeit weakly, with both exons (*4*, *6*). We therefore focused on the other region (positions 68 to 88), which is not essential for cell viability in yeast (*9*) and is partially accessible to cleavage by ribonuclease (RNase) H and a complementary oligonucleotide in mammals (*3*, *8*).

We tested whether a biotinylated 2′-O-methyl RNA oligonucleotide complementary to U5 nucleotides 68 to 88 (BU5Ae; Fig. 1A) would affect the splicing of an adenovirus (Ad) major late splicing substrate (*10*). Addition of BU5Ae had no effect on splicing activity at concentrations as high as 32 pmol/μl, although a control oligonucleotide complementary to U1 positions 1 to 8 ($U1_{1-8}$) inhibited splicing at concentrations as low as 2 pmol/μl (Fig. 1A). Failure of BU5Ae to inhibit splicing could indicate that the oligonucleotide associates with an inactive subpopulation of U5 snRNPs; however, this cannot be the case because the full array of splicing intermediates can be recovered from the reaction by affinity selection with BU5Ae (Fig. 1B). Pre-mRNA and free intron lariat were as efficiently selected by BU5Ae as they were by antibodies directed against the known U5 snRNP-specific protein p220.

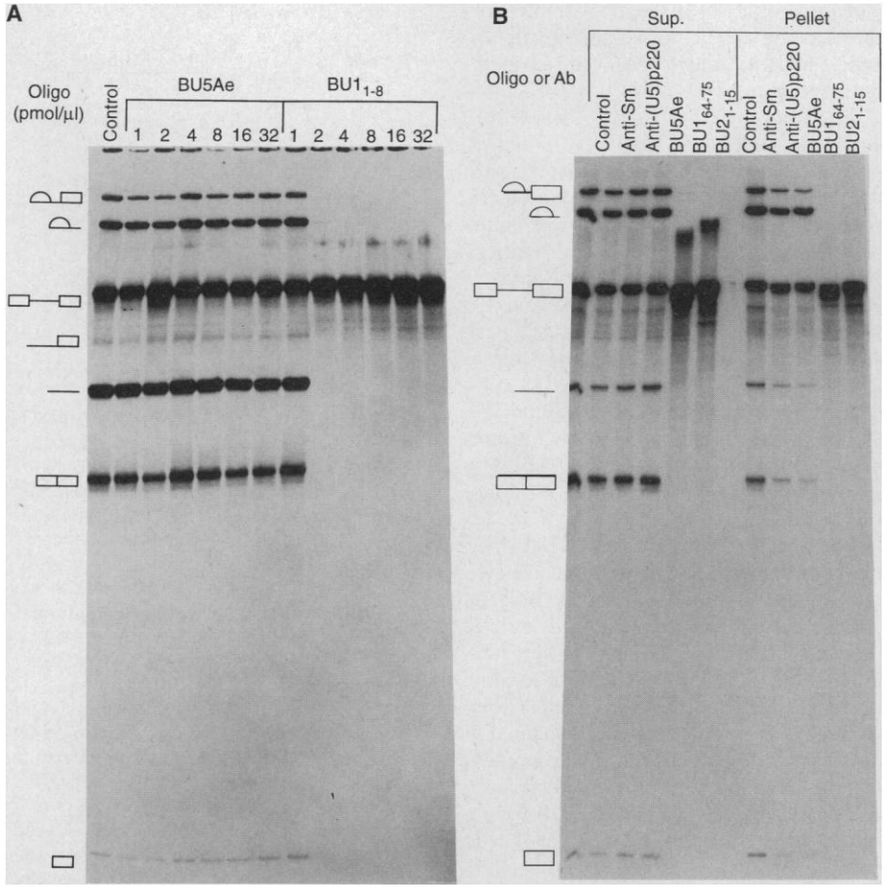

Fig. 1. BU5Ae associates with the active spliceosome but does not inhibit splicing. (**A**) Splicing reactions contained the labeled Ad substrate and oligonucleotide BU5Ae or $BU1_{1-8}$ at the indicated concentration. Incubation was for 1 hour at 30°C. (**B**) Splicing reactions that contained the indicated oligonucleotides (4 pmol/μl) were incubated for 40 min. Left panel, unselected RNA; right panel, selected RNA; anti-Sm and anti-(U5)p220, immunoprecipitation with antibodies to Sm or p220.

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Relatively inefficient selection of lariat 3′ exon molecules and free 5′ exon by BU5Ae and antibody to (U5)p220 may indicate that U5 is partially sequestered during the first catalytic step. All splicing intermediates were recovered by immunoprecipitation with Sm antibodies, which can react with the Sm epitopes on four of the five spliceosome snRNPs (*11*). The ability of BU5Ae to select not only pre-mRNA but also splicing intermediates and products suggests that BU5Ae remains associated with the active spliceosome from formation of complex B (*12*) through the second catalytic step.

We next asked whether BU5Ae interacts with U5 snRNP alone or with larger snRNP/snRNP complexes. When nuclear extract was incubated with BU5Ae under low salt conditions (50 mM NaCl) in the absence of pre-mRNA and then selected on streptavidin agarose beads (*13*), all five spliceosomal snRNAs were retained (Fig. 2A). However, if the NaCl concentration was increased to 250 or 500 mM before selection, interactions involving U2 and U6 were destabilized and an apparently equimolar U1/U4/U5 core complex was retained on the column (*14*). Formation of the U1/U4/U5 complex occurred in the absence of adenosine triphosphate (ATP) or Mg^{2+} but occurred inefficiently on ice (*15*).

To determine whether BU5Ae selects a homogeneous U1/U4/U5 snRNP complex, nuclear extract was preincubated with BU5Ae and then passed over a monomeric avidin column. Bound material was eluted with an excess of free biotin and fractionated according to size by glycerol gradient sedimentation (*8*, *16*). U1, U4, and U5 cosedimented as a homogeneous 25S peak of constant composition (Fig. 2B), which is consistent with a distinct U1/U4/U5 complex rather than separate U1/U5 and U4/U5 complexes. In a parallel control experiment, U1 snRNP was selected with $BU1_{64-75}$ and shown to sediment as a 15S monosnRNP (*15*).

We tested whether the U1/U4/U5 snRNP complex preexisted in nuclear extract or was induced de novo by BU5Ae. Nuclear extract was preincubated with or without BU5Ae, and the extracts were fractionated on a glycerol gradient to separate snRNP/snRNP complexes from small soluble nucleoplasmic factors that remained at the top of the gradient. The 25S gradient fractions, which were enriched for tri-snRNP complexes (*17*), were affinity-selected with BU5Ae at 250 mM NaCl as in Fig. 2A. Affinity selection of the snRNP-containing gradient fractions derived from nuclear extract preincubated without BU5Ae revealed only the preassembled U4/U5/U6 tri-snRNP complex (Fig. 2C). Affinity selection of parallel gradient fractions derived from nuclear extract preincubated with BU5Ae revealed an equimolar U1/U4/U5 complex (Fig. 2C). When the 5′ end of U1 snRNA was removed by digestion with RNase H before preincubation with BU5Ae, only a U4/U5 complex was selected (Fig. 2C).

Several conclusions can be drawn from this experiment. (i) Selection of a U4/U5/U6 complex from extract preincubated without BU5Ae indicates that BU5Ae binds initially to U5 in the U4/U5/U6 tri-snRNP complex. (ii) Selection of a U1/U4/U5 complex from extract preincubated with BU5Ae indicates that binding of BU5Ae to the U4/U5/U6 complex can, in the presence of soluble factors, disrupt the U4/U5/U6 complex and generate a new U1/U4/U5 complex. (iii) Binding of BU5Ae to the U4/U5/U6 complex does not induce or require release of U6. (iv) Because gradient fractions containing the U4/U5/U6 complex also contain U1 and U2 snRNP (*8*, *15*), BU5Ae-induced release of U6 from the U4/U5/U6 complex and formation of the new U1/U4/U5 complex must require an additional factor or factors that can be separated from snRNPs by size fractionation. (v) The 5′ end of U1 snRNA is required for the integrity of the U1/U4/U5 complex but not for release of U6 from the U4/U5/U6 complex.

To investigate whether binding of BU5Ae might push the dynamic network of snRNP/snRNP interactions along the normal reaction coordinate, we used psoralen crosslinking to compare the relative amounts of U4/U6, U2/U4/U6, and U2/U6 interaction in extracts incubated with or without BU5Ae (Fig. 2D). The U2/U6 and U2/U4/U6 interactions increase and the U4/U6 interaction decreases as splicing proceeds (*18*, *19*). These are precisely the changes observed when BU5Ae is incubated with total nuclear extract (Fig. 2D). As expected, addition of a splicing substrate has little further effect (*15*) because only a small fraction of snRNPs participate in the in vitro splicing reaction. Thus, BU5Ae not only affects bulk snRNP/snRNP interactions in the absence of splicing substrates (Fig. 2, A through C) but also induces the U2/U4/U6 interaction characteristic of an active spliceosome (*18*). These data suggest that BU5Ae induces dissociation of U6 from the U4/U5/U6 complex in a form that readily associates with U2 to form the U2/U6 interaction.

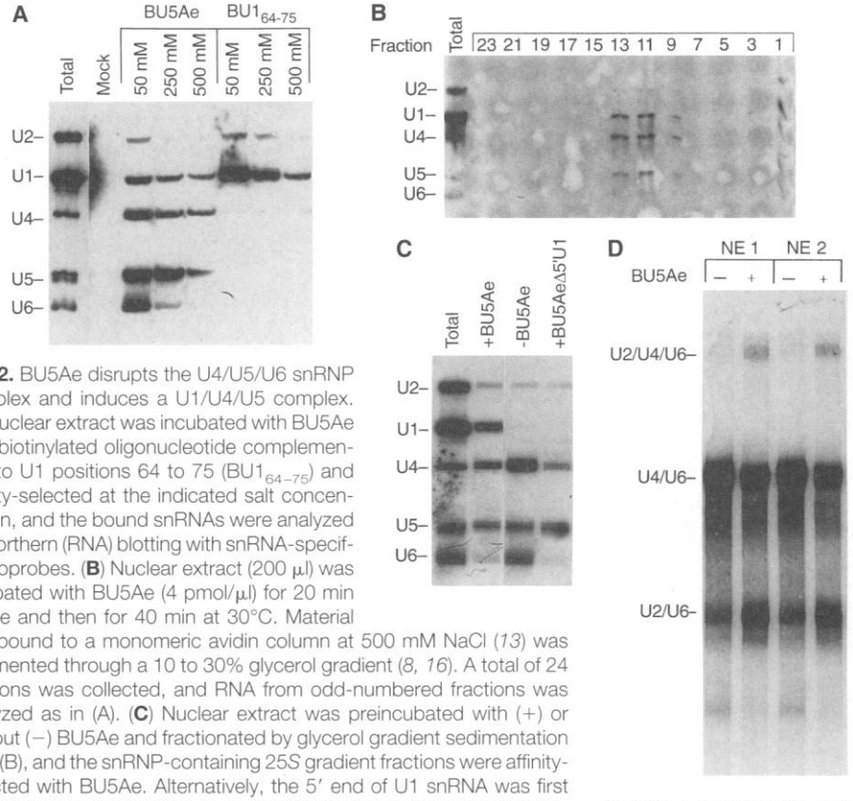

Fig. 2. BU5Ae disrupts the U4/U5/U6 snRNP complex and induces a U1/U4/U5 complex. (**A**) Nuclear extract was incubated with BU5Ae or a biotinylated oligonucleotide complementary to U1 positions 64 to 75 ($BU1_{64-75}$) and affinity-selected at the indicated salt concentration, and the bound snRNAs were analyzed by Northern (RNA) blotting with snRNA-specific riboprobes. (**B**) Nuclear extract (200 μl) was incubated with BU5Ae (4 pmol/μl) for 20 min on ice and then for 40 min at 30°C. Material that bound to a monomeric avidin column at 500 mM NaCl (*13*) was sedimented through a 10 to 30% glycerol gradient (*8*, *16*). A total of 24 fractions was collected, and RNA from odd-numbered fractions was analyzed as in (A). (**C**) Nuclear extract was preincubated with (+) or without (−) BU5Ae and fractionated by glycerol gradient sedimentation as in (B), and the snRNP-containing 25S gradient fractions were affinity-selected with BU5Ae. Alternatively, the 5′ end of U1 snRNA was first digested by treatment of extract with RNase H and oligonucleotide $U1_{1-14}$, and treated extract was then incubated with BU5Ae and sedimented as above (+BU5AeΔ5′U1). Northern analysis was done as in (A). (**D**) BU5Ae induces a U2/U4/U6 snRNP complex and enhances the U2/U6 snRNA interaction. Splicing reactions (*13*) containing either of two different nuclear extracts were incubated with (+) or without (−) BU5Ae for 10 min at 30°C. After addition of psoralen (40 μg/ml), the reactions were cross-linked for 30 min on ice with fresh psoralen (40 μg/ml) added every 10 min. RNA was resolved by denaturing 5% PAGE, blotted, and probed for U6 snRNA or U2 (*15*).

In light of accumulating evidence that U5 may displace U1 from the 5′ SS (*4*–*6*), we asked whether BU5Ae could select a 5′ SS. Nuclear extract was preincubated with BU5Ae and then mixed on ice with end-labeled RNA oligonucleotides corresponding to the 5′ SS, the branch point (BP), or the polypyrimidine tract and the 3′ SS (Py-3′ SS) (Fig. 3). Selected snRNP complexes were captured on streptavidin agarose, and the labeled oligonucleotides were visualized by denaturing polyacrylamide gel electrophoresis (PAGE) and autoradiography. BU5Ae was able to select the labeled 5′ SS (Fig. 3); however, selection did not require the 5′ end of U1 snRNA, which could be sequestered by a 2′-O-methyl RNA oligonucleotide complementary to U1 snRNA positions 1 to 10 ($U1_{1-10}$) or removed by digestion with RNase H. This suggests that either the U1/U4/U5 complex induced by BU5Ae, or a larger complex also containing the U2 or U6 snRNPs, which can form in the presence of a 5′ SS (*7*), recognizes the 5′ SS through an interaction with U5. In fact, BU5Ae can select a U4/U5 complex essentially free of U1 snRNP when the 5′ end of U1 is digested by RNase H [Fig. 2C and (*15*)]. Selection of the 5′ SS by BU5Ae was strongly dependent on the presence of the Py-3′ SS (Fig. 3), decreasing eightfold when the polypyrimidine tract was omitted (*20*) or sequestered by preincubation with a complementary 2′-O-methyl-oligonucleotide (α-Py). This may reflect an interaction between the 5′ SS and Py-3′ SS through a protein bridge (*21*), or a direct interaction of U5 snRNP with the Py-3′ SS (*5*), or selection by BU5Ae of a larger U2- or U6-containing complex, which can interact with the Py-3′ SS in other ways (*7*).

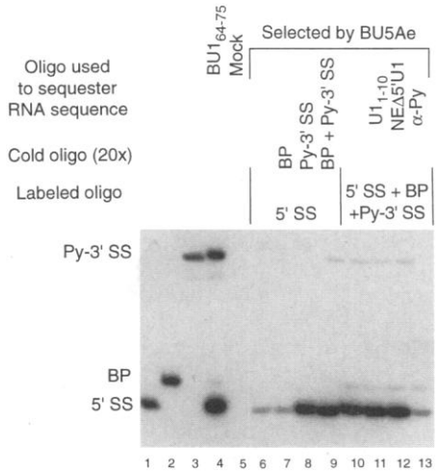

Fig. 3. Binding of the U1/U4/U5 complex to the 5′ SS does not require the 5′ end of U1 snRNA. Nuclear extract was incubated with BU5Ae together with the indicated combinations of labeled or unlabeled RNA oligonucleotides containing the sequence of the 5′ SS, the branch point (BP), or the polypyrimidine tract and the 3′ SS (Py-3′ SS). Oligonucleotides bound to U1/U4/U5 were the affinity-selected with BU5Ae, and labeled oligonucleotides were visualized by PAGE followed by autoradiography (lanes 6 through 13). As controls the three labeled oligonucleotides were subjected directly to PAGE (lanes 1 through 3), or mixed with extract and then affinity-selected with $BU1_{64-75}$ (lane 4) or mock-selected (lane 5) before PAGE. Formation of a commitment complex (*23, 24*) presumably accounts for selection of the Py-3′ 5S along with the 5′SS (lane 4). Where indicated, the 5′ end of U1 snRNA was sequestered by preincubation of nuclear extract with a 2′-*O*-methyl RNA oligonucleotide complementary to U1 nucleotides 1 to 10 (lane 11, $U1_{1-10}$) or digested by RNase H treatment as in Fig. 2C (lane 12, NEΔ5′U1). Alternatively, the polypyrimidine tract in Py-3′ SS was sequestered with the complementary 2′-*O*-methyl oligonucleotide α-Py (lane 13).

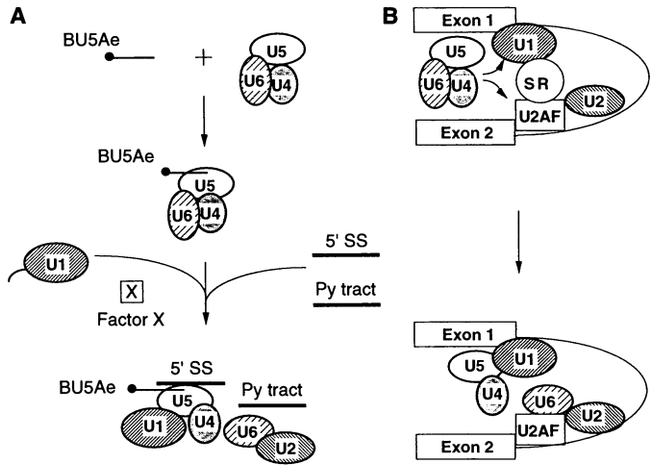

Fig. 4. The U1/U4/U5 complex may represent a transitional stage in displacement of U1 from the 5′ SS by U5. (**A**) A model for BU5Ae action in vitro. BU5Ae binds initially to U5 snRNA in the U5/U4/U6 complex. In the presence of soluble factor X, the U4/U5/U6 complex is transformed into a U1/U4/U5 complex, U6 snRNP is released in a form that interacts with U2 snRNP, and U5 binds to the 5′ SS independently of the 5′ end of U1 snRNP. The polypyrimidine tract enhances binding of the 5′ SS, perhaps by protein-protein (*21*) or protein-snRNP interactions (see text). (**B**) A possible role for the U1/U4/U5 complex in the normal splicing reaction. Initially, U1, U2, and the U4/U5/U6 snRNPs interact with the 5′ SS, branch site, and 3′ SS respectively (upper panel). In the substep presumably mimicked by BU5Ae binding, U6 snRNP interacts with U2 snRNP as U5 displaces U1 from the 5′ exon (lower panel).

Our data are most consistent with the model shown in Fig. 4. BU5Ae associates with a subpopulation of U5 in the preassembled U4/U5/U6 complex. This induces a conformational change in U5, which triggers a cascade of events (Fig. 4A) resembling substeps in the normal splicing reaction (Fig. 4B). The U4/U5/U6 complex is converted into a transitional U1/U4/U5 complex, which allows U5 to displace U1 from the 5′ exon (*5*, *6*), and U6 is released in a form that preferentially associates with U2 snRNP (*19*). Conversion of U4/U5/U6 into U1/U4/U5 requires an additional soluble factor (designated X) that can be separated from the snRNPs by velocity sedimentation. The 5′ end of U1 snRNA is required for the integrity of the U1/U4/U5 complex but not for release of U6 from the U4/U5/U6 complex or for binding of the U4/U5 complex to the 5′ SS. Stabilization of the BU5Ae-induced U1/U4/U5 complex by the 5′ end of U1 snRNA suggests that U1 and U5 may associate before U5 displaces U1, an interpretation supported by our recent cross-linking data (*15*). Indeed, the U1/U5 interaction may bring U5 to the 5′ SS, ultimately enabling the invariant U5 loop to pair with the last nucleotides of the 5′ exon (*4*, *6*, *18*).

Although BU5Ae affects snRNP/snRNP interactions, it appears to remain stably associated with the spliceosome throughout the splicing reaction and does not inhibit splicing. Such paradoxical behavior is not unprecedented. The 2′-O-methyl RNA oligonucleotide U4b associates with U4 in the active spliceosome (*22*), and the monoclonal antibody H1B2 recognizes a spliceosomal protein but does not affect splicing (*23*). BU5Ae may push the network of snRNP/snRNP interactions along the normal reaction pathway without affecting equilibria or subreactions that are rate-limiting for splicing activity overall. Indeed, binding of BU5Ae to U5 snRNP may mimic or functionally replace a normal spliceosomal interaction, perhaps one involving a factor that binds to the same accessible region of U5 snRNA as BU5Ae itself.

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13. For affinity selection, 200 mg of streptavidin agarose beads (Pierce) were washed once with WB-50 buffer [20 mM tris (pH 7.6), 0.01% NP-40, 1.5% NaN_3, 50 mM NaCl, and 2 mM $MgCl_2$]. Beads were preblocked with glycogen (0.1 mg/ml), bovine serum albumin (1 mg/ml), and tRNA (0.1 mg/ml) in WB-50 buffer for 20 min at 4°C, then washed four times with 1 ml of WB-50 and once with WB-250 (WB-50 brought to 250 mM NaCl). After resuspension in an equal volume of WB-250, 50-mg aliquots of beads were gently rocked for 1 hour at 4°C with 25 μl of a standard splicing reaction containing 60% v/v HeLa nuclear extract but no substrate or, in later experiments, with either 250 μl of each glycerol gradient fraction. Beads were washed four times with 0.5 ml WB-250 buffer, and bound RNA was purified by digestion with proteinase K, phenol extraction, and ethanol precipitation. Affinity selection on a monomeric avidin column was performed according to the manufacturer (Pierce) with WB-500 as the wash buffer. The three RNA oligonucleotides used in Fig. 4 were 5′-CAGGUAAGUAdT-3′ (5′ SS), 5′-CAUACUUAUUCCdU-3′ (BP), and 5′-CCCUUUUUUCCACAGCUdC-3′ (Py-3′ SS). The sequence of α-Py is 5′-GGAAAAAAGGG-3′.
14. BU5Ae interacts efficiently with the accessible subpopulation of U5 snRNP, although only 7 ± 3% of total U5 snRNP is retained on the streptavidin column after extensive washing to reduce background binding (Fig. 2C). In a typical experiment, 25 to 50% of total U5 snRNP in nuclear extract can be cleaved by RNase H and the corresponding DNA oligonucleotide spanning the same positions as BU5Ae (*8*). Pretreatment of nuclear extract with BU5Ae completely blocks RNase H cleavage (*15*), implying that BU5Ae interacts quantitatively with the entire cleavable subpopulation of U5 snRNP. Trace amounts of U2 selected by BU5Ae (Fig. 2A, left) presumably reflect the known U1/U2 association [D. L. Black, B. Chabot, J. A. Steitz, *Cell* **42**, 737 (1985)] because trace amounts of U2 snRNP (<0.8%) were also retained by a biotinylated RNA oligonucleotide complementary to U1 positions 64 to 75 ($BU1_{64-75}$), which selects U1 almost exclusively (Fig. 2A, right).
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20. Both labeled and unlabeled Py-3′ SS oligo support 5′ SS selection, although labeled oligo is present at 20-fold lower concentration than unlabeled oligo.
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24. We thank J. A. Steitz for many useful suggestions, oligonucleotides, and Y12 antibody; members of her laboratory for suggestions; A. D. Bailey and T. A. Pavelitz of our laboratory for discussions and critical reading of the manuscript; and M. Moore for the gift of p220 antibody. G.A. was supported by a Human Frontiers Science Program Organization Long-Term Fellowship. This work was funded in part by NIH award GM31073 to A.M.W.

29 November 1995; accepted 8 February 1996

Genetically Engineered Resistance to Dengue-2 Virus Transmission in Mosquitoes

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The control of arthropod-borne virus diseases such as dengue may ultimately require the genetic manipulation of mosquito vectors to disrupt virus transmission to human populations. To reduce the ability of mosquitoes to transmit dengue viruses, a recombinant Sindbis virus was used to transduce female *Aedes aegypti* with a 567-base antisense RNA targeted to the premembrane coding region of dengue type 2 (DEN-2) virus. The transduced mosquitoes were unable to support replication of DEN-2 virus in their salivary glands and therefore were not able to transmit the virus.

DEN viruses (serotypes 1 to 4; Flaviviridae) contain a positive sense RNA genome (11 kb) that encodes three structural and seven nonstructural proteins (*1*). The viral genome is the only viral mRNA found in infected cells and is translated as a single polyprotein (350 kD), which is cleaved by both host cell and virus-encoded proteases to generate individual proteins (*2*). The natural cycle involves only mosquitoes, which develop a lifelong, persistent, noncytocidal infection, and humans, who may manifest either DEN fever (DF), an acute, debilitating illness, or DEN hemorrhagic fever (DHF), a severe disease that usually results from sequential infections by different viral serotypes (*3*, *4*). Female *Ae. aegypti* acquire DEN viruses by biting and taking a bloodmeal from an infected human. The virus enters the midgut, replicates, and disseminates to other mosquito organs, including the salivary glands. After further replication of the virus in salivary glands, the virus in mosquito saliva can be transmitted to humans during the ingestion of the next bloodmeal (*5*).

DEN viruses are a major health concern to urban populations throughout tropical regions of the world (*3*, *4*, *6*). Conventional measures to limit the proliferation of the disease have failed in part because of the lack of effective vaccines and the collapse of health care and mosquito control infrastuctures in many developing countries (*4*, *6*). Novel control strategies are now being considered, including the genetic alteration of mosquito vectors, to control the rapid proliferation of arthropod-borne pathogens.

A potentially powerful control strategy is to prevent replication of DEN viruses in the midgut or salivary glands of the mosquito by expressing part of the virus's own genetic material in those organs and establishing intracellular immunity to the virus (*7*). Engineering pathogen refractoriness into arthropod vectors has been difficult because of a lack of efficient DNA expression systems (*8*). Viral transducing systems derived from infectious complementary DNA (cDNA) clones of the mosquito-borne RNA virus Sindbis (SIN; Togaviridae) have recently been developed that achieve efficient, long-term gene expression in mosquitoes and can be used to rapidly assess promising gene-based approaches for pathogen control (*9*). The TE/3′2J double subgenomic SIN (dsSIN) transducing viruses contain a second subgenomic promoter, between the end of the structural protein coding region and the viral 3′ noncoding region. These dsSIN viruses transcribe one genomic and two subgenomic mRNAs in infected cells, and heterologous proteins may be translated from the second subgenomic mRNA (*9*). The dsSIN viruses replicate in the cytoplasm of infected mosquito cells, which obviates potential problems such as mRNA splicing, mRNA transport, and poorly characterized DNA promoters in mosquitoes. We have demonstrated that dsSIN viruses can efficiently express heterologous proteins and antisense RNAs in the head and salivary gland tissues of infected *Ae. triseriatus* mosquitoes more than 20 days after infection (*10*). Previous studies also have demonstrated that dsSIN viruses can transduce cultured mosquito cells with RNAs complementary to specific RNA sequences of unrelated arthropod-borne viruses, such as La Crosse (Bunyaviridae) and DEN-2 viruses, and can establish intracellular immunity to those viruses (*11*–*13*).

A dsSIN virus, designated D2prMa, has been generated that expresses a 567-base antisense RNA targeted to the premembrane

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