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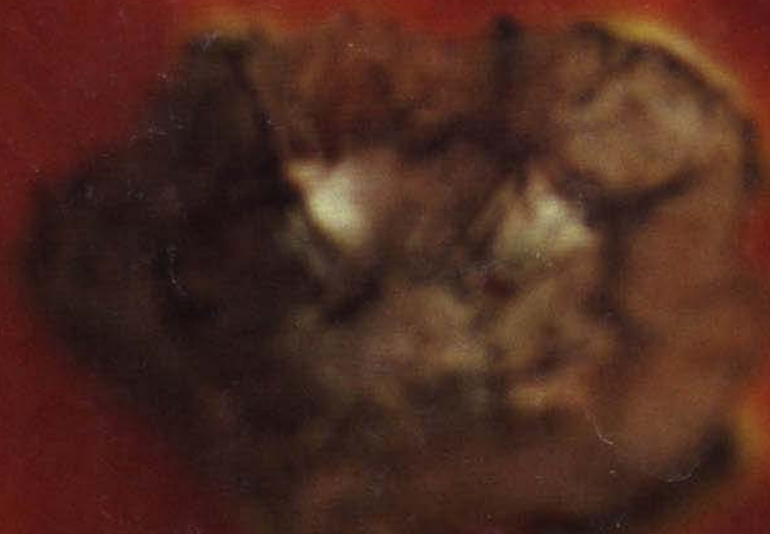
GENETICALLY ENGINEERED INSECTICIDES



FASEB ISSUE

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Expression and effects of the juvenile hormone esterase in a baculovirus vector

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THE endocrine changes that initiate the metamorphosis of insect caterpillars into pupae and, ultimately, into adult moths occur over a very short time period. Thus, it is not surprising that titres of the hormones that control this process seem to be regulated by changes in the rates of both biosynthesis and degradation. A reduction in the titre of juvenile hormone (JH) early in the last larval instar has been shown to initiate metamorphosis and lead to a cessation of feeding behaviour[1]. This reduction in JH is associated with a dramatic increase in the levels of a very active, specific enzyme that hydrolyses the chemically stable, conjugated methyl ester to the biologically inactive JH acid[2]. If this juvenile hormone esterase (JHE) is inhibited, the *in vivo* JH titre remains high enough to keep the larva in the feeding stage, resulting in giant insects[3]. Thus, if sufficient quantities of JHE were expressed *in vivo* at an early stage of development, the reduction in JH titre should cause the affected insect to stop feeding. Because of its low abundance, the purification of sufficient JHE from the blood of caterpillars was very difficult until the development of a highly efficient affinity purification system[4]. This system produced enough JHE for the development of antibodies and for partial amino-acid sequencing of JHE from several species including the major insect pest, *Heliothis virescens*[5,6]. Three similar clones thought to encode JHE were obtained from a complementary DNA library made from fat bodies of *H. virescens*[7]. Here, we describe the expression of JHE in an *in vitro* baculovirus system which will provide enough enzyme for detailed biochemical and physiological studies. The results suggest that *in vivo* expression of JHE may help in the improvement of genetically engineered viral insecticides which work by reducing insect feeding.

To confirm that the clones obtained previously actually encode JHE, it was important to demonstrate that the resulting proteins had catalytic activity when expressed *in vitro*. A baculovirus expression system which uses the moth *Autographa californica* nuclear polyhedrosis virus (AcNPV)[8] was attractive for several reasons. The system is noted for its high levels of expression and for its efficient post-translational modification of many proteins[9–12]. In the case of JHE, however, it offers additional advantages. AcNPV has a broad host range and infects a variety of noctuiid larvae including the genera *Spodoptera*, *Trichoplusia* and, importantly, *Heliothis*. Thus, this system provided the opportunity to study the expression and post-translational modification of an insect enzyme in insect cells derived from species in the same family as *Heliothis*, as well as the possibility of testing the effects of the engineered virus in major insect pests *in vivo*.

The JHE gene was isolated from a Bluescript plasmid[7] and inserted in the transfer vector pAcRP23 (refs 13, 14) to produce pAcRP23.JHE (Fig. 1). This plasmid was co-precipitated with AcNPV DNA and used to co-transfect cells of *Spodoptera frugiperda* (IPLB Sf 21)[15]. In all of the JHE transfections, the hydrolysis of JH was found to be both time-dependent and protein-dependent, whereas no hydrolysis of JH was observed with identical control transfections using the same transfer vector containing the *lacZ* gene. The recombinant virus was purified by five sequential plaque assays with screening for polyhedrin minus plaques using standard procedures[8]. At each stage, the presence of JHE in the plaque was confirmed by a JHE assay on enzyme eluted from agar above each plaque. The highly specific and sensitive JHE assay[16] indicates that the JHE gene may provide a good reporter system for the study of baculovirus promoters. We used the standard esterase assay, except that 150 μl of trichlorethylene was used instead of 250 μl isoctane in the 1.5 ml polypropylene tubes.

In both monolayer and spinner flask culture of uninfected cells of *S. frugiperda*, JHE activity was not detectable by the standard assay. When the virus containing the JHE gene (AcRP23.JHE) was added (at 10 plaque-forming units per cell), the JHE activity in the medium began to increase rapidly at 10 h after infection, reaching a maximum at 48 h. The catalytic activity in the medium was, on average, about 100 nmol min^{-1} ml^{-1} which is about twice the JHE activity seen at peak levels in blood obtained from *H. virescens*. This catalytic activity corresponds to about 75 mg of JHE per litre of medium. In serum-free medium, a single protein band was seen on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) which co-migrated with the affinity-purified native protein. As expected from the sequence of the 19 amino-acid leader, deduced from the complementary DNA (cDNA) sequence of JHE, the protein seemed to be rapidly exported from *S. frugiperda* cells with 94–98% of the total catalytic activity appearing in the media when monitored at 24, 36, 48 and 72 h after infection.

The JHE activity in the medium surrounding infected *S. frugiperda* cells was compared, in several ways, with the activity in haemolymph collected from larvae of third or fourth instar *Trichoplusia ni* infected with the engineered virus (see below) and with the natural enzyme in *H. virescens* blood in the second day of the last larval instar. In each case, chromatographic analysis indicated that JH acid was the only metabolite produced from tritiated JH, and in each case hydrolysis was directly dependent upon incubation time and protein concentration over a wide range. The enzymes proved stable to repeated freeze thaw cycles, incubation at 5 or 30°C for several days, as well as to a variety of reagents. The natural and expressed enzymes were very resistant to eserine, a potent general esterase inhibitor, and to DFP (*O,O*-diisopropylphosphofluoridate), a choline esterase inhibitor, but were inhibited by phenylmethyl sulphonyl

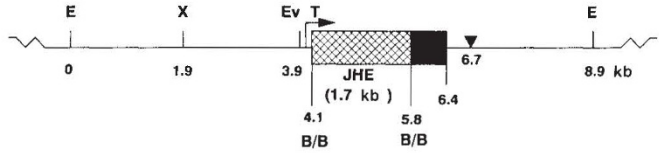

FIG. 1 Structure of the AcRP23.JHE virus. E, *Eco*RI sites; X, *Xho*I site; Ev, *Eco*RV site; B/B, *Bam*HI/*Bgl*II ligation; T, transcription start site. The hatched area shows the coding region of JHE and the dark area the remaining polyhedron sequence.
METHODS. To prepare the transfer vector, the 3 kilobase (kb) JHE gene at the *Eco*RI cloning site of a Bluescript SK$^-$ plasmid (plasmid 3hv16; ref. 7) was linearized by partial digestion with *Eco*RI. The ends were repaired with the Klenow fragment of *Escherichia coli* DNA polymerase and a *Bgl*II linker was placed at the 5′ end of the JHE gene. After ligation and transformation the plasmid was linearized, again by partial digestion at a *Cla*I site just downstream from the natural translation stop codon, and a second *Bgl*II linker was inserted at the 3′ end of the gene (pJHE16B). This process resulted in the removal of the large non-coding 3′ region of the JHE but did not remove the 1.7 kb coding sequence. The resulting plasmid was digested with *Bgl*II and the 1.7 kb JHE fragment isolated by agarose gel electrophoresis. The above procedure allowed the JHE gene to be inserted into a *Bam*HI cloning site of the pAcRP23 transfer vector without disrupting an internal *Bam*HI site in the JHE gene. After transfection, this pAcRP23.JHE transfer vector places the JHE under the control of the polyhedrin promoter of the AcNPV[13,14]. Thus, neither AcRP23.JHE nor the control virus expressing the HIV coat protein produced the polyhedron protein. Use of the polyhedron promoter reduces oral infectivity of the resulting virus.

fluoride ($I_{50} = 10^{-4}$ M) and strongly inhibited by a trifluoromethyl ketone, designed to be a selective JHE inhibitor [3-(1-decyl)thio-1,1,1-trifluoropropan-2-one; $I_{50} = 10^{-9}$ M] (ref. 4). In each case, the catalytic activity could be precipitated by a polyclonal antibody raised to the affinity-purified JHE of *H. virescens*[7].

The natural haemolymph enzyme of *H. virescens* and the enzyme expressed by cells of *S. frugiperda* infected with AcRP23.JHE were both purified by affinity chromatography as described previously[4]. However, analysis by SDS-PAGE with western and glycan[17] blotting indicated that the proteins were immunologically similar but had slightly different relative molecular masses as a result of extensive glycosylation of JHE in the *S. frugiperda* cells (Fig. 2). After the natural and expressed proteins were digested with endonuclease F to remove sugars, they had identical relative molecular masses. A major advantage of the baculovirus system is that it carries out many post-translational modifications, and insect JHEs from several species are known to have varying levels of glycosylation[5,6]. However, the extensive glycosylation carried out by Sf 21 cells in this study indicates that an investigation of post-translational modification in different host cells for engineered AcNPVs may be needed.

When crude supernatant from AcRP23.JHE-infected cells of *S. frugiperda*, or JHE purified by open-column and high-performance liquid chromatography anion exchange chromatography, was injected into larvae of *Manduca sexta*, the caterpillars turned black at the next molt. This effect could be reversed by the application of epofenonane (a juvenoid lacking the methyl ester of JH; Fig. 3*a*)[18]. Blackening is a common bioassay for chemical anti-juvenile hormones[19,20] and has been used to characterize the biological activity of natural enzymes after affinity purification[21]. This indicates that the expressed JHE does have anti-juvenile hormone activity.

When the polyhedrin minus AcRP23.JHE was fed to first instar larvae of *T. ni*, the feeding and growth of many of the infected larvae were profoundly reduced compared with either untreated control larvae or larvae treated with a control virus (Fig. 3*b*). The blood JHE levels in all of the stunted larvae assayed approached the levels normally seen at ecdysis, but were only about 10% of the levels seen in the normal last larval instar (Fig. 4*a*). On the basis of these data, and the observation that the level of JHE activity in the haemolymph rises in control animals between head capsule slippage and ecdysis[22], we suggest that a reduction in JH titre caused by high JHE may lead to this reduction in feeding (Fig. 4*b*). When the infected larvae were treated with a juvenoid, feeding increased and growth was partially restored. These levels of juvenoid had no significant effect on the rate of growth in the early instars of control larvae or on larvae infected with similar doses of control virus (the

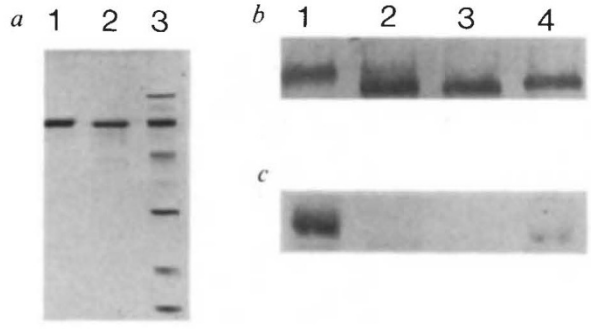

FIG. 2 Analysis of JHE purified by affinity chromatography from the haemolymph of fifth instar larvae of *H. virescens* and from the growth medium of cells of *S. frugiperda* infected with AcRP23.JHE. *a*, Sodium dodecyl sulphate polyacrylamide gel electrophoresis on a 12% gel stained with Coomassie Blue reveals slight differences in relative molecular mass (M_r). Lane 1, 2 μg of JHE expressed by the recombinant baculovirus (66K); lane 2, 2 μg of JHE from haemolyph (62K); lane 3, M_r standards of 97, 67, 43, 31, 22 and 14K. The data from a western blot (*b*) and a glycan blot (*c*) demonstrate that the difference in M_r is due to differential glycosylation of JHE. The central lanes show the effects of digestion with an endoglycosidase. *b*, *c*, Lanes 1, undigested JHE from recombinant baculovirus; lanes 2, digested JHE from recombinant baculovirus; lanes 3, digested JHE from haemolymph recombinant baculovirus; lanes 4, undigested JHE from haemolymph. In (*b*), the two digested preparations showed shifts to identical mobility. The immunologic similarity between the two purified preparations was revealed when the filter was probed with polyclonal antibodies developed against JHE from haemolymph[7]. In (*c*), the digested proteins lost their glycan-staining ability when they were treated according to the instructions of a glycan-staining kit[17], showing that all oligosaccharides had been removed.
METHODS. The two purified preparations were digested with an endoglycosidase selective for *N*-linked oligosaccharides (Peptide:N-acetylglycosidase F, Boehringer Mannheim Biochemicals) and analysed by immunoblotting (*b*) and blotting specifically for glycans (*c*) on proteins transferred to nitrocellulose filters by standard procedures. Gels b and c were run for 90 rather than 60 min and the bands of interest were enlarged to emphasize differences in M_r.

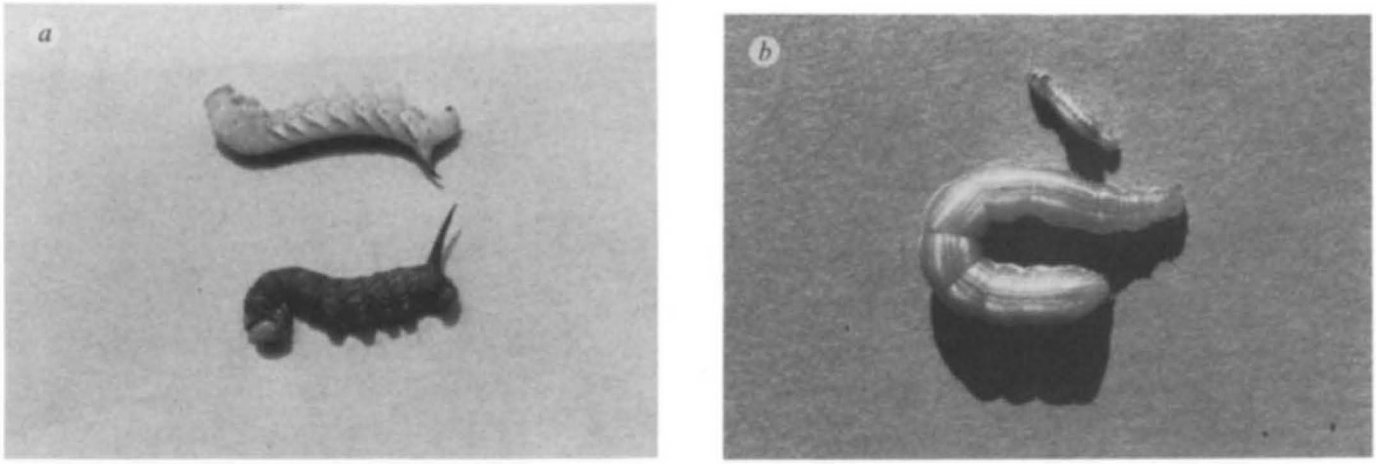

FIG. 3 The effects of expressed JHE on insects. *a*, The effects of JHE injected into mid-second instar larvae of *M. sexta*. *b*, The effects of infection of first instar larvae of *T. ni* with AcRP23.JHE.
METHODS. *a*, The top larva was injected with 5 μl of a 1 mg ml^{-1} solution of bovine serum albumin. The lower larva was injected with 50 pmol of purified JHE from AcRP23.JHE-infected *S. frugiperda* cells. The photograph was taken shortly after the molt to the third larval stadium, with the cuticular blackening in the lower animal clearly demonstrating the anti-juvenile hormone effects of the expressed enzyme. *b*, The smaller larva was infected with the engineered virus and development was halted in the third larval stadium whereas the larger insect of the same age was not infected. Insects treated with the control virus expressing the HIV coat protein were slightly heavier than untreated controls.

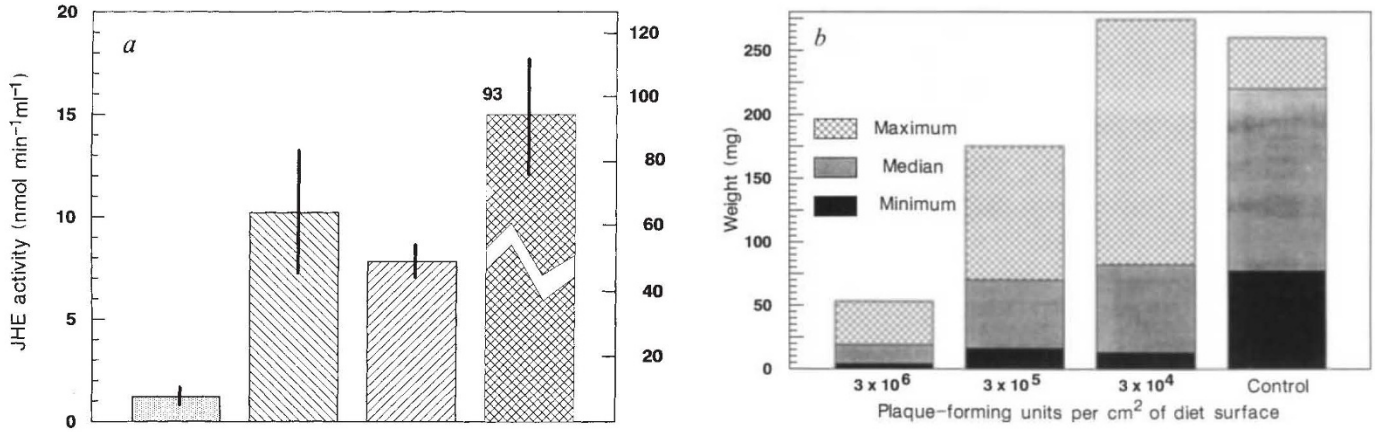

FIG. 4 The effects of AcRP23.JHE on larvae of *T. ni*. *a*, The effects of AcRP23.JHE infection on haemolymph JHE levels in *T. ni*. Following infection as described below, individual larvae were bled when the control larvae were in the third instar. The first bar indicates the low levels of JHE seen in the mid-third instar of feeding animals (or in larvae infected with wild-type AcNPV or AcNPV containing the gp120 gene[23]). The second bar indicates the much higher levels of JHE found in infected larvae, whereas the third bar shows the JHE levels in 10 control insects at 'bubble head' (the time between head capsule slippage and ecdysis). For comparison, the fourth bar (note different scale on right) shows the level of JHE present at peak levels in the normal fifth instar (the average rate of hydrolysis is indicated above it). *b*, Minimum, maximum and median weights of larvae exposed to AcRP23.JHE as first stadium larvae. Median weights are shown because some insects are not infected at lower doses. The mean weights of animals at the two higher concentrations are significantly different from the control population ($P=0.05$). Topical application of the juvenoid epofenonane (3 μg given once in mid-first stadium and again in mid-second stadium) or addition of methoprene (200 μg cm^2) or epofenonane (20 μg cm^2) to the surface of the diet led to a significant increase in the weights of larvae treated with AcRP23.JHE whereas there was no significant effect on early instars of control larvae or larvae treated with AcNPV.gp120[23] ($P=0.05$).
METHODS. Larvae were treated by placing the virus on the surface of the diet of a 30 ml diet cup (7 cm^2 surface). An egg sheet containing approximately 10 eggs laid during a carefully timed period was placed in the lid of the cup and the cup inverted over the sheet. For this experiment, each dosage was repeated three times with 10 larvae per replicate. After 4 days at 24°, the larvae were transferred to individual containers and monitored daily for 6 days.

identical polyhedrin minus AcNPV coding for the secreted gp120 coat protein of the human immunodeficiency virus (HIV)[23]). Larvae infected with this control virus were the same size or slightly larger than untreated control insects until they became moribund.

This reduction in feeding is an attractive aspect of this system, as one of the major problems involved with the use of baculoviruses for insect control is the feeding damage caused by infected insects[24]. However, these effects were only seen after treatment of first instar larvae. There are several possible explanations for this observation. In later stages, the low level of JHE produced under the control of a very late promoter is unable to overcome hormone biosynthesis. It also is likely that viral-induced production of an ecdysteroid UDP-glucosyl transferase reduces the effects of JHE[25]. This gene is present in both AcRP23.JHE and the control virus expressing the HIV coat protein. As yet, it is not known why *in vivo* JHE expression is so much lower than *in vitro* expression. Despite its stability in the face of a variety of reagents, both the natural JHE of *M. sexta*[21] and the JHE expressed from the vector are inactivated

rapidly *in vivo* (R. Ichinose, B. F. McGutchen and T.N.H., unpublished observations). If these problems of *in vivo* instability and low and late expression can be overcome, the JHE gene offers a very attractive tool for the development of genetically engineered insecticides and, at the very least, it will serve as a useful reporter gene in expression systems. □

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Binding of the *Drosophila Sex-lethal* gene product to the alternative splice site of *transformer* primary transcript

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SOMATIC sexual differentiation in *Drosophila melanogaster* is accomplished by a hierarchy of genes[1-5] of which one, *Sex-lethal* (*Sxl*)[6,7], is required for the functional female-specific splicing of the transcripts of the immediately downstream regulatory gene, *transformer* (*tra*). The first exon of the *tra* primary transcript is spliced to one of two acceptor sites. Splicing to the upstream site yields a messenger RNA which is neither sex-specific nor functional, but that produced after splicing to the downstream acceptor site yields a functional female-specific mRNA. Here we address the question of how the *Sxl* gene product determines the alternative splicing of *tra* primary transcripts. One suggestion is that non-sex-specific splicing to the upstream acceptor is blocked in female flies by sex-specific factors[8], but neither the identity of the female-specific factors nor the mechanism of the blockage has been specified. We have now performed co-transfection experiments in which *Sxl* complementary DNA and the *tra* gene are expressed in *Drosophila* Kc cells. Moreover, we find that female *Sxl*-encoded protein binds specifically to the *tra* transcript at or near the non-sex-specific acceptor site, implying that the female *Sxl* gene product is the *trans*-acting factor that regulates the alternative splicing.

When a plasmid expressing the *tra* gene under the control of the promoter of the copia-element long terminal repeat (LTR)[13] (plasmid copia-tra) was transfected into Kc cells, the non-sex-specific mRNA was exclusively generated (data not shown). To analyse whether *Sxl* gene products affect *tra* pre-mRNA splicing, we co-transfected the copia-tra plasmid with male- or female-specific *Sxl* cDNA located downstream of the *Drosophila* heat-shock-protein 70 gene (*HSP70*) promoter[14] (plasmid hsp-Sxl). Only non-sex-specific splicing of *tra* transcripts was found when the copia-tra DNA was co-transfected either with the hsp vector or with the male-specific *Sxl* cDNA (hsp-Sxl M1) (Fig. 1*b*; lanes 1, 3). By contrast, female-specific splicing of *tra* transcripts was additionally observed on co-transfection of the copia-tra plasmid with the female-specific *Sxl* cDNA (hsp-Sxl F1) or with a mixture of hsp-Sxl M1 and hsp-Sxl F1 (Fig. 1*b*; lanes 2, 4). With hsp-Sxl F1, about 80% of *tra* RNAs were female-specific. That hsp-Sxl M1 did not interfere with the effect of hsp-Sxl F1 is consistent with earlier genetic analyses indicating that the product from male-specific *Sxl* transcripts is not functional[1-3]. With a plasmid containing a frame-shift mutation in the coding region of female-specific *Sxl* cDNA, the amount of female-specific *tra* mRNA was drastically reduced (Fig. 1*b*; lane 5). The fraction of female-specific *tra* mRNA decreases in proportion to the amount of transfected hsp-Sxl F1 (data not shown), indicating that female-specific splicing of *tra* pre-mRNA depends on the dosage of the *Sxl* gene product. When a *transformer-2 (tra-2)* cDNA[15,16] was co-transfected with the copia-tra plasmid, no female-specific *tra* mRNA was detected (Fig. 1*b*; lane 6), consistent with *tra-2* not acting upstream of *tra*[11].

To clarify how the female-specific *Sxl* gene product controls alternative splicing, we constructed two plasmids containing a mutant *tra* gene in which either one of the two acceptor sites had been deleted (Fig. 2*a*). When the mutant plasmid containing a deletion of the non-sex-specific acceptor site (plasmid copia-traΔN; mutation *traΔN*) was co-transfected with hsp-Sxl M1 or F1, *traΔN* pre-mRNA was efficiently spliced at the female-specific acceptor site irrespective of the presence of the functional *Sxl* gene product (Fig. 2*b*; lanes 1, 2). By contrast, with the mutant in which the female-specific acceptor site had been deleted (plasmid copia-traΔF; mutation *traΔF*), *traΔF* pre-mRNA was spliced efficiently at the non-sex-specific acceptor site only in the absence of a functional female-specific *Sxl* gene product (Fig. 2*b*; lanes 3, 4). Essentially similar results were reported when mutant *tra* genes were introduced into either male or female flies[8], but the product of *Sxl* was not shown to be directly responsible for the *tra* alternative splicing. Our results strongly indicate that the functional *Sxl* gene product acts at or near the non-sex-specific acceptor site to inhibit the non-sex-specific splicing of *tra* pre-mRNA.

If this interaction occurs, we would expect that the Sxl protein binds specifically to the transcript close to the non-sex-specific acceptor site. We tested the binding activity of the Sxl protein by north-western-blotting analysis of the female-specific *Sxl* gene product overproduced in *Escherichia coli* by the T7 promoter system[17]. All of the proteins from *E. coli* overproducing the Sxl protein were subjected to SDS-PAGE and then blotted onto nitrocellulose membrane. The blotted membrane was probed with various *tra*-derived RNAs synthesized *in vitro* (Fig. 3*a*). The *tra* RNA containing the non-sex-specific acceptor site bound strongly to a protein of relative molecular mass (M_r) 41,000 (Fig. 3*b*; lane 2), consistent with the size of the Sxl protein predicted to be encoded by the female-specific *Sxl* cDNA sequence. This protein was also detected by an anti-serum raised against the Sxl protein (data not shown). The *tra* RNA did not bind to any proteins from *E. coli* cells carrying the vector without the *Sxl* cDNA (Fig. 3*b*; lane 1). To determine which region of *tra* RNA binds to the Sxl protein, we tested *traΔN* and AF130, representing the deleted sequence of *traΔN*, RNA probes for binding activity (Fig. 3*a*). No binding was found with *traΔN* RNA, whereas AF130 RNA bound to the Sxl protein (Fig. 3*b*; lanes 3, 4). Binding of the protein to *tra* RNA was strongly inhibited by addition of poly(U), but not by poly(C) (Fig. 3*b*;

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