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13. A yeast 6.8-kb Sal I genomic DNA segment containing the *STE6* gene was inserted into the pUC19 vector at the Sal I site, yielding plasmid pUCSTE6-Sal. The coding sequence of STE6 was isolated from pUCSTE6-Sal as a 4.7-kb fragment by digestion with BstN I and Sst I. Full-length cDNA clones for wild-type and mutant *mdr3* genes have been described elsewhere (*11, 12*). Their coding sequence was excised from plasmid pGEM-7Zf as 4.1-kb fragments by digestion with Sph I and Cla I. Each resulting fragment was blunt-ended with T4 DNA polymerase and cloned into the Pvu II site of the yeast expression vector pVT101-U [T. Vernet, D. Dignard, D. Y. Thomas, *Gene* **52**, 225 (1987)]. JPY201 cells (*MAT***a** *ste6Δ::HIS3 gal2 his3Δ200 leu2-3,112 lys2-801 trp1 ura3-52*) were transformed by the lithium acetate method [H. Ito, Y. Fukuda, K. Murata, A. Kimura, *J. Bacteriol.* **153**, 163 (1983)]. Transformants were selected and maintained in selective synthetic dextrose medium lacking uracil (SD-URA).
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23. Similar quantitative mating experiments have been performed in two additional yeast *ste6* deletion strains generated in this report. For this purpose, a 5.2-kb Bgl II–Sac I genomic DNA fragment containing the *STE6* gene was cloned at the BamH I–Sac I sites of vector pGEM7Zf to give pSTE6-5.2. We engineered the *ste6* deletion *Δste6::HIS3* by substituting in pSTE6-5.2 a 3.4-kb Stu I–SnaB I segment of *STE6* with the 1.9-kb Sma I–Pvu II fragment of plasmid pJJ215 [J. S. Jones and L. Prakash, *Yeast* **6**, 363 (1990)] containing the *HIS3* gene. This deletion removes 87% of the STE6 coding sequence (amino acids 39 to 1168). The resulting *Δste6::HIS3* construction was excised by cleavage with Eco RI and Sac I and used for one-step gene replacement of the wild-type *STE6* gene in strains JPY200 *MAT***a** *his3 leu2 ura3 lys2 trp1* (*7*) and strain T109.6-3C *MAT***a** *his3 leu2 ura3* [M. Whiteway, R. Freedman, S. Van Arsdell, J. W. Szostak, J. Thorner, *Mol. Cell. Biol.* **7**, 3713 (1987)], yielding strains MRY4 and MRY5, respectively. The structure of the resulting deletion alleles in strains MRY4 and MRY5 was confirmed by Southern (DNA) analysis. Strains MRY4 and MRY5 were transformed with the four pVT-based plasmids listed in Table 1 and the mating efficiency of the transformants was quantified by filter assay [G. F. Sprague, Jr., *Methods Enzymol.* **194**, 77 (1991)]. Experiments were performed in duplicates and averaged results of the duplicates are as follows:

Plasmids	Mating frequency	
	MRY4 transformants	MRY5 transformants
pVTSTE6	1.1×10^{-1}	4.6×10^{-1}
pVT-MDR3S	2.5×10^{-4}	1.2×10^{-3}
pVT-MDR3F	$<5.2 \times 10^{-6}$	$<1.0 \times 10^{-7}$
pVT	$<5.0 \times 10^{-6}$	$<1.5 \times 10^{-7}$

24. Exponentially growing cells in selective SD-URA- medium were harvested by centrifugation and rinsed twice in TNE [10 mM tris (pH 7.0), 150 mM NaCl, 1 mM EDTA]. Cells resuspended in TNE were disrupted by two successive passages through a French pressure cell at 20,000 pounds per square inch. Unlysed cells were removed by centrifugation (1000*g*, 10 min) and crude membranes concentrated by centrifugation (100,000*g*, 30 min). Membranes were then resuspended in TNE containing 45% sucrose (w/v), loaded into a discontinuous sucrose gradient (60, 45, 35, and 30%), and centrifuged (100,000*g*, 3 hours). Membrane fractions at the 30 and 35% interface were pooled, washed in TNE, concentrated by centrifugation (100,000*g*, 30 min), and stored frozen in TNE containing 30% glycerol. All steps of the extraction were performed at 4°C in the presence of a cocktail of protease inhibitors (phenylmethylsulfonyl fluoride at 1 mM; leupeptin, pepstatin A, and trasylol, each at 5 μg/ml).
25. We thank J. McGrath and A. Varshavsky for providing the pUCSTE6-Sal plasmid and JPY201 strain and T. Vernet for providing the pVT101-U vector. Supported by a grant to D.Y.T. from the National Research Council and the Medical Research Council (MRC) of Canada and to P.G. from the National Cancer Institute. M.R. is supported by a postdoctoral fellowship from MRC.

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Specific Binding of Chromosomal Protein HMG1 to DNA Damaged by the Anticancer Drug Cisplatin

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The mechanism of action of the anticancer compound *cis*-diamminedichloroplatinum(II) (cisplatin) involves covalent binding to DNA. In an effort to understand the tumor-specific cytotoxicity of such DNA damage, the interactions of these lesions with cellular proteins have been studied. One such protein has been identified as the high-mobility group protein HMG1. Recombinant rat HMG1 binds specifically (dissociation constant $3.7 \pm 2.0 \times 10^{-7}$ molar) to DNA containing cisplatin d(GpG) or d(ApG) intrastrand cross-links, which unwind and bend DNA in a specific manner, but not to DNA modified by therapeutically inactive platinum analogs. These results suggest how HMG1 might bind to altered DNA structures and may be helpful in designing new antitumor drugs.

Recent cancer statistics reveal the annual ratio of deaths to incidence of new solid tumors of the testes to be nearly zero (*1*). This result is thought to be largely the consequence of chemotherapy afforded by the anticancer drug cisplatin. The antitumor activity of cisplatin is generally accepted to involve binding to DNA, a process about which there is substantial chemical and structural information (*2*). The biological activity of the drug cannot be explained solely on the basis of its ability to damage DNA, however, because the geometric isomer *trans*-diamminedichloroplatinum(II) (*trans*-DDP) binds DNA and can block replication (*3–5*) but is ineffective as a chemotherapeutic agent (*6*). Stereochemical differences in the adducts formed by the two isomers imply that the antitumor activity of cisplatin arises from the formation of a specific structural motif on DNA which, in turn, triggers a cellular response leading to cell death. To investigate this possibility, attention has turned to identifying cellular proteins that recognize cisplatin-DNA lesions with the ultimate aim of understanding how such interactions might lead to the selective toxicity of the drug to tumor cells.

The existence of proteins in mammalian cellular extracts capable of binding to cisplatin adducts with high affinity and specificity has been established by gel mobility shift and Southwestern blot assays (*7–11*). Until recently, however, none of these proteins has been identified. The Southwestern blot analyses revealed the existence of two classes of proteins that recognize cisplatin-damaged DNA having molecular masses of ~28 kD and 80 to 100 kD (*8, 11, 12*). A gene that encodes an 81-kD cisplatin-DNA structure-specific recognition protein (SSRP1) has been cloned (*8*) and

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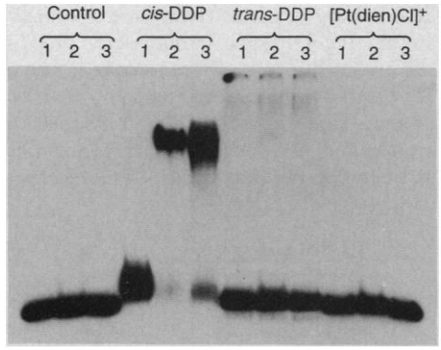

Fig. 1. Gel mobility shift assay of recombinant rat HMG1 binding to 123-bp DNA probes modified at a drug-to-nucleotide ratio (r_b) of 0.023 with cisplatin (*cis*-DDP), *trans*-DDP, or chloro(diethylenetriamine)platinum(II) ([Pt(dien)Cl]$^+$). Lane 1 in each case contains probe only, lane 2 contains probe and HMG1, and lane 3 contains probe, HMG1, and nonspecific chicken erythrocyte competitor DNA (*16*). Cisplatin-modified probes migrate slower than unmodified probes as discussed previously (*9*). Higher molecular weight material in the *trans*-DDP lanes is attributed to interstrand cross-linked probe.

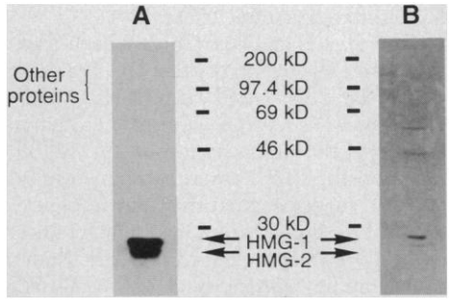

Fig. 3. Southwestern (**A**) and Western (**B**) blot analyses of HeLa nuclear extract. The extract (~80 μg per lane) was resolved by 12% SDS–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose, which was then divided for use in a Southwestern blot with a cisplatin-damaged 123-bp DNA probe (r_b = 0.023) or in a Western blot with rabbit serum antibodies to rat HMG1 and HMG2. The sizes and locations of molecular weight markers are indicated. Higher molecular weight proteins that are faintly visible in the Southwestern blot include SSRP1 (*13*).

sequenced (*13*). In studying the predicted peptide sequence of this clone, we found a run of 75 amino acids sharing 47% identity with a similar stretch of amino acids in the high-mobility group protein HMG1 (*14*). This result suggested that HMG1 might itself be a protein recognizing cisplatin-modified DNA.

To test this hypothesis, protein purified from an expressed rat HMG1 clone (*15*) was used in a gel shift assay with cisplatin-damaged DNA (*16, 17*). The purified HMG1 protein binds specifically to a 123-bp probe modified with cisplatin, altering its mobility (Fig. 1). Under identical conditions, the protein does not shift unmodified probe or probes platinated by the inactive compounds *trans*-DDP or chloro(diethylenetriamine)platinum(II).

To characterize further the nature of the platinated DNA recognition motif, gel mobility shift experiments were carried out with synthetic duplex oligonucleotides containing defined cisplatin adducts (*18, 19*). The results indicate that oligonucleotides containing either *cis*-[Pt(NH$_3$)$_2${d(GpG)-*N7*(1), -*N7*(2)}] or *cis*-[Pt(NH$_3$)$_2${d(ApG)-*N7*(1), -*N7*(2)}], intrastrand cross-links react specifically with HMG1, whereas unmodified oligonucleotides or an oligonucleotide containing *cis*-[Pt(NH$_3$)$_2${d(GpTpG)-*N7*(1), -*N7*(3)}] intrastrand adducts do not (Fig. 2). This recognition specificity is identical with that previously observed for proteins in crude cellular extracts (*9*).

Additional information about the interaction between HMG1 and cisplatin adducts was obtained by constructing a 100-bp DNA fragment containing a single, site-specific *cis*-[Pt(NH$_3$)$_2${d(GpG)-*N7*(1), -*N7*(2)}] intrastrand cross-link (*20*). From studies of this probe and its unplatinated control in gel mobility shift assays with purified HMG1 protein, and, assuming that one molecule of HMG1 binds to each retarded probe molecule, we measured dissociation constants K_d of $3.7 \pm 2.0 \times 10^{-7}$ M for binding to the platinum-modified probe and of $3.4 \pm 1.2 \times 10^{-5}$ M for nonspecific binding to the unmodified control probe (*20*). The K_d for nonspecific binding is similar to that previously observed for the binding of chicken HMG1 to chicken erythrocyte DNA (*21*).

When HMG1 is resolved by SDS–polyacrylamide gel electrophoresis, it migrates at a position identical to that of the 28-kD band previously observed by Southwestern blotting of cellular extracts from mammalian sources (*8*). Parallel Western and Southwestern blots of proteins in HeLa nuclear extracts were used to determine whether the 28-kD band is indeed HMG1 (*22*). The antiserum used in the Western blot was raised against denatured rat HMG2 but recognizes both HMG1 and HMG2 (*23*). Accordingly, two major bands appear in the Western blot, corresponding to HMG1 and HMG2 (Fig. 3B). In the Southwestern blot (Fig. 3A), which was probed with DNA containing cisplatin adducts, two bands are observed that migrate at the same positions as HMG1 and HMG2 in the Western blot. In addition, the relative amounts of probe bound to HMG1 versus HMG2 in the Southwestern blot correlate with the observed intensities of the respective bands in the Western blot. Finally, by using an antibody raised against recombinant rat HMG1, we were able to immunoprecipitate a protein that comigrates with the upper band, confirming that the protein in this band is indeed HMG1. From these results we identify the previously reported 28-kD protein as a mixture of HMG1 and HMG2, which comigrate on the 8% acrylamide gels used in earlier experiments (*8*).

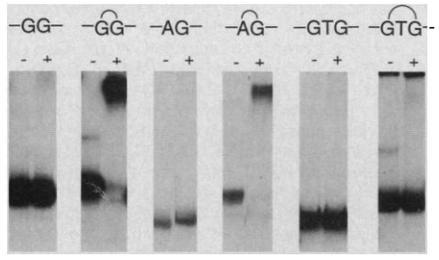

Fig. 2. Analysis of HMG1 binding to 105-bp duplex oligonucleotides containing *cis*-{Pt(NH$_3$)$_2$}$^{2+}$ 1,2-intrastrand cross-linked d(GpG) or d(ApG) adducts, or 1,3-intrastrand cross-linked d(GpTpG) adducts (*18*). The probes used in this gel mobility shift assay are site-specifically platinated 21-mer pentamers. All lanes contain nonspecific chicken erythrocyte competitor DNA. Plus and minus signs denote the presence or absence of HMG1 protein, respectively, and the curved lines above the bases indicate the presence of platinum.

The fact that HMG1 binds more strongly to DNA damaged with cisplatin than to unplatinated B-form DNA, or to DNA modified with therapeutically inactive platinum analogs, implies that a particular DNA structure dictates its binding specificity. In particular, the protein recognizes d(GpG) and d(ApG) 1,2-intrastrand cross-links, which comprise 90% of the cisplatin adducts formed in vivo (*24, 25*). These adducts bend the helix by 34° in the direction of the major groove and unwind it by 13° (*19, 26*), providing a signal for HMG1 binding. Additional insight into the DNA structural motif recognized by HMG1 comes from a comparative analysis of platinum adducts that do not react with HMG1. The 1,3-intrastrand d(GpTpG) cross-link bends the helix to a similar degree but unwinds it by 23° (*19, 26*), *trans*-DDP unwinds the DNA by 9° and allows bending in more than one direction (*26, 27*), and chloro(diethylenetriamine)platinum(II) adducts do not bend DNA (*26*) but unwind it by 5° to 7° (*27*). These observations suggest that the degree to which DNA is unwound is an important determinant for HMG1 binding. Because HMG1 and HMG2 have been identified as possible general transcription factors (*23*), their ability to recognize unwound DNA may be fundamental to such a role. The role of DNA bending in the recognition mechanism is unclear; whereas HMG1 is known to bind cruciform DNA, it does not bind to

hairpin-like structures (*17*).

The term HMG box (*28*) has been used to describe the region of homology between the 81-kD SSRP1 and HMG1 (*13*). This HMG box occurs in a variety of proteins, including the transcription factor hUBF (*28*) and the LEF-1 transcriptional regulator (*29*), suggesting that they may recognize similar DNA motifs. Interestingly, studies of the DNA-binding domain of LEF-1 indicate a binding specificity of 20- to 40-fold greater than that for nonspecific DNA and reveal that the protein contacts DNA in the minor groove (*29*). These results are consistent with the properties of HMG1 binding to cisplatin-modified DNA. HMG1 binds with 100-fold greater affinity to a 100-bp probe containing a single *cis*-[$Pt(NH_3)_2${d(GpG)-*N7*(1), -*N7*(2)}] intrastrand cross-link than to an unmodified control probe. Moreover, substitution of the ammines of cisplatin with bulky ligands does not eliminate HMG1 binding to platinum-modified DNA. Because these ligands lie in the major groove of DNA, it is likely that HMG1 binds to cisplatin-modified DNA from the minor-groove side.

The biological role of HMG1 binding to cisplatin adducts in vivo is currently unknown. The present results reveal that the protein preferentially recognizes adducts of the therapeutically active compound cisplatin, in particular, the d(GpG) and d(ApG) 1,2-intrastrand cross-links that comprise the majority of adducts formed on DNA in vivo. This observation is consistent with previously stated hypotheses (*9*) by which proteins might potentiate the cytotoxicity of cisplatin. Binding of HMG1 to platinum-modified DNA, which structurally resembles a natural substrate, may prevent recognition of the adducts by the cellular repair machinery, potentiating the inhibition of replication and leading to cell death. In this respect it is interesting to note that other investigators have observed a protein of a size similar to HMG1, the level of which is diminished in cisplatin-resistant cells (*11*). Alternatively, if HMG1 or HMG-box proteins are required for active transcription in rapidly dividing tumor cells, then titrating away these factors might lead to internal cellular disarray and, eventually, cell death. Similarly, cisplatin may effect the binding of HMG1 to regions of the genome where such binding does not normally occur, which may alter the transcription of critical genes. Finally, because cisplatin cross-links HMG1 to DNA in cells (*30*), its mechanism of action may involve covalent linking by free drug of the HMG1 protein to platinated DNA at the site of recognition.

With the identification of HMG1 and SSRP1 (*13*) as cisplatin-modified DNA binding proteins, research can now focus on determining whether these proteins mediate the tumor-specific activity of the drug or are involved in a mechanism of resistance. Any correlation between the levels of these proteins and the sensitivity of tissues to cisplatin might indicate such a role and may assist in the rational design of more effective platinum anticancer drugs.

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16. Recombinant rat HMG1 was purified under reducing conditions from pT7RNHMG1 (gift of M. Bianchi) containing *Escherichia coli* Bl21(DE3)pLysE lysate by substituting this lysate for calf thymus extract in a published protocol [L. N. Marekov, D. G. Demirov, B. G. Beltchev, *Biochim. Biophys. Acta* **789**, 63 (1984)]. The HMG1 thus prepared was further purified by passing over a 5-ml EconoPac heparin column (Bio-Rad) and elution from this column with a 0 to 1 M NaCl gradient. The resulting HMG1 was quantitated by the Bio-Rad Bradford assay and found to be homogeneous when assayed by SDS–polyacrylamide gel electrophoresis. The 123-bp probes were prepared from a commercially available ladder (*10*) and were platinated and ^{32}P-labeled as previously described (*9*). Gel mobility shift analysis was carried out as described (*17*). Reaction mixtures contained 100 ng/ml probe, of 20% glycerol, 10 mM $MgCl_2$, 150 mM NaCl, 10 mM Hepes-NaOH, pH 7.9, 1 mM EDTA, 0.05% Nonidet P-40, 200 μg/ml of bovine serum, and, as indicated in the legend to Fig. 1, 2 μg/ml HMG1 and 200 μg/ml chicken erythrocyte nonspecific competitor DNA of heterogeneous length, in a 10-μl volume, and were incubated 10 min on ice prior to loading.
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18. Oligonucleotides 21 bases in length containing single 1,2-intrastrand d(GpG) or d(ApG), or 1,3-intrastrand d(GpTpG), adducts of cisplatin were prepared as described (*19*). These oligonucleotides, or unplatinated control oligonucleotides, were 5′-phosphorylated in the presence of adenosine 5′-triphosphate (ATP) and T4 polynucleotide kinase and annealed to complementary 21-base oligonucleotides that were end-labeled with [$\gamma^{32}P$]ATP to yield duplex DNA with two-base 3′ overhangs. This material was then treated with T4 DNA ligase, resolved on a native polyacrylamide gel, and probes 105-bp long were purified (*9*). Gel mobility shift experiments were carried out under the conditions described above (*16*).
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was replaced with 10 mM KCl, and the acrylamide concentration of the gel was increased to 10%. The amounts of shifted ([HMG · DNA]) and unshifted ([DNA]) probe were determined from quantitative analyses of dried gels on a Molecular Dynamics phosphorimager. The concentrations of free HMG1 ([HMG]) were obtained by subtracting [HMG · DNA] from the concentrations of HMG1 added to the binding reactions. The dissociation constant K_d was calculated at three different protein concentrations (9×10^{-8}, 1.8×10^{-7}, and 3.6×10^{-7} M) and at a DNA probe concentration of 6 to 9 nM by using the equation K_d = [HMG][DNA]/[HMG · DNA].

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Positive Control of Pre-mRNA Splicing in Vitro

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Positive control of the sex-specific alternative splicing of *doublesex* (*dsx*) precursor messenger RNA (pre-mRNA) in *Drosophila melanogaster* involves the activation of a female-specific 3′ splice site by the products of the *transformer* (*tra*) and *transformer-2* (*tra-2*) genes. The mechanisms of this process were investigated in an in vitro system in which the female-specific 3′ splice site could be activated by recombinant Tra or Tra-2 (or both). An exon sequence essential for regulation in vivo was shown to be both necessary and sufficient for activation in vitro. Nuclear proteins in addition to Tra and Tra-2 were found to bind specifically to this exon sequence. Therefore, Tra and Tra-2 may act by promoting the assembly of a multiprotein complex on the exon sequence. This complex may facilitate recognition of the adjacent 3′ splice site by the splicing machinery.

Somatic sex determination in *Drosophila melanogaster* involves a cascade of regulated splicing events (*1*). The last step in this hierarchy is the alternative splicing of *dsx* pre-mRNA. The *dsx* gene contains six exons. In females, *dsx* mRNA consists of exons 1, 2, 3, and 4 (*2*) (Fig. 1A) and encodes a protein that represses the expression of male-specific genes (*3, 4*). In males, exons 1, 2, 3, 5, and 6 comprise the alternative *dsx* mRNA (*2*) (Fig. 1A) whose translation product blocks female sexual differentiation (*3, 4*).

In *Drosophila*, the female-specific processing pathway of *dsx* pre-mRNA depends on the activities of both the *tra* and *tra-2* genes (*2, 5*). The polypyrimidine tract (6) of the female-specific 3′ splice site is interrupted by purine residues and is inefficiently recognized by the splicing apparatus (*5, 7–10*). When the purines are mutated to pyrimidines, the *dsx* female-specific 3′ splice site is constitutively used both in vivo (*9*) and in vitro (*11*). Genetic studies in flies (*7*) and transfection experiments with cells in culture (*8–10*) indicate that products of *tra* and *tra-2* serve as activators of the female-specific 3′ splice site. Male flies produce functional *tra-2* (*12*) but not *tra* gene products (*5*). As a result, *dsx* pre-mRNA undergoes default processing to generate the male *dsx* mRNA.

The primary structures of Tra (*13*) and Tra-2 (*14*) are consistent with their roles as splicing regulators; Tra-2, but not Tra, contains a ribonucleoprotein-consensus (RNP-CS) RNA binding domain, a 90-residue motif implicated in protein-RNA interactions (*15*). Recombinant Tra-2 produced in *Escherichia coli* binds specifically to a region in the female-specific exon of *dsx* pre-mRNA (*8*). This region contains six copies of a 13-nucleotide (nt) sequence and is required for female-specific splicing (*7–10*). Both Tra and Tra-2 contain arginine-serine–rich regions (RS domain) that are present in several splicing factors (*13, 14, 16–18*). Although the function of this motif is not known, recent studies indicate that it can direct proteins to speckles (*19*), subnuclear regions where several splicing components are preferentially localized (*20*).

To study the mechanisms involved in the positive control of *dsx* female-specific splicing by Tra and Tra-2, we established an in vitro splicing system that consisted of HeLa cell nuclear extracts and recombinant Tra and Tra-2 produced in a baculovirus expression system. Female-specific splicing was not observed when an in vitro–synthesized transcript of the *dsx* minigene was incubated under splicing conditions in HeLa cell nuclear extracts in the absence of Tra and Tra-2 (Fig. 1B). The low level of splicing observed resulted from the use of a cryptic 3′ splice site (*21*). Addition of recombinant Tra or Tra-2 (or both) (*22*) activated female-specific splicing (Fig. 1B, lanes 2 to 5 and 12 to 26). The highest level of female-specific splicing was observed when both Tra and Tra-2 were added (Fig. 1B, lanes 22 to 26). Activation of female-specific splicing by Tra or Tra-2 alone has also been observed in transfection studies with *Drosophila* tissue culture cells (*9, 10*). In flies, female-specific splicing requires both Tra and Tra-2. This difference may result from the use of minigene substrates and altered ratio of regulators and basic splicing components in vitro and in transfection systems.

The repeat region in exon 4 is required for female-specific splicing in vivo (*7–10*). The deletion of most or all of the repeat sequence from the minigene transcript severely impaired or abolished activation in vitro (Fig. 2). In addition, the splicing of both human β-globin (hβ-globin) (*23*) and *fushi taruzu* (*ftz*) pre-mRNA (*24*) was unaffected by Tra and Tra-2 (*11*). These results demonstrate that Tra and Tra-2 function in a sequence-specific manner in vitro.

To test the possibility that the repeat region is sufficient for regulation, we introduced this sequence downstream of the 3′ splice site of hβ-globin intervening sequence 1 (*23*). By analogy with the suboptimal female-specific 3′ splice site of *dsx* pre-mRNA, we used hβ-globin constructs that contained mutations that adversely affected 3′ splice site recognition. The polypyrimidine tract in G(py) (*25*) is interrupted by purines. The branchpoint sequence in G(bps) (*26*) deviates significantly from the consensus. Both mutations substantially decrease the efficiency of hβ-globin splicing in vitro (*25, 26*).

In splicing reactions with the hybrid substrates, the 3′ splice sites from G(py) and G(bps) were not utilized in the absence of Tra and Tra-2 (Fig. 3, lanes 2 and 13). Instead, splicing to cryptic splice sites was

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