Identification and Analysis of a Hyperactive Mutant Form of Drosophila *P*-Element Transposase

Eileen L. Beall, Matthew B. Mahoney and Donald C. Rio[1]

Department of Molecular and Cell Biology, University of California, Berkeley, California 94720-3204

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ABSTRACT

Transposition in many organisms is regulated to control the frequency of DNA damage caused by the DNA breakage and joining reactions. However, genetic studies in prokaryotic systems have led to the isolation of mutant transposase proteins with higher or novel activities compared to those of the wild-type protein. In the course of our study of the effects of mutating potential ATM-family DNA damage checkpoint protein kinase sites in the Drosophila *P*-element transposase protein, we found one mutation, S129A, that resulted in an elevated level of transposase activity using *in vivo* recombination assays, including *P*-element-mediated germline transformation. *In vitro* assays for *P*-element transposase activity indicate that the S129A mutant exhibits elevated donor DNA cleavage activity when compared to the wild-type protein, whereas the strand-transfer activity is similar to that of wild type. This difference may reflect the nature of the *in vitro* assays and that normally *in vivo* the two reactions may proceed in concert. The *P*-element transposase protein contains 10 potential consensus phosphorylation sites for the ATM family of PI_3-related protein kinases. Of these 10 sites, 8 affect transposase activity either positively or negatively when substituted individually with alanine and tested *in vivo*. A mutant transposase protein that contains all eight N-terminal serine and threonine residues substituted with alanine is inactive and can be restored to full activity by substitution of wild-type amino acids back at only 3 of the 8 positions. These data suggest that the activity of *P*-element transposase may be regulated by phosphorylation and demonstrate that one mutation, S129A, results in hyperactive transposition.

THE mobility of transposable elements is controlled to minimize the level of DNA damage that may result in lethality of the host cell. Thus, many transposons have evolved elaborate regulatory strategies to modulate the level of their movement. In many cases, the "wild-type" transposase protein works inefficiently and with a low turnover number as an enzyme, yet like other enzymes, works with exquisite specificity (Craig 1997; Mizuuchi 1997). In prokaryotic systems, where powerful genetic screening procedures are available, novel mutations have been obtained in transposase proteins that yield either hyperactive forms or proteins with novel activities or specificities (Kennedy and Haniford 1996; Sakai and Kleckner 1996; Stellwagen and Craig 1997; Reznikoff *et al.* 1999). In some cases, such as with V(D)J recombination, pseudorandom *in vitro* mutagenesis has yielded mutant recombinases with novel or altered activities (D. R. Kim, *et al.* 1999; Landree *et al.* 1999; Fugmann *et al.* 2000; Qiu *et al.* 2001; Yarnell Schultz *et al.* 2001). Using *in vivo* screening assays in *Escherichia coli*, hyperactive mutants of the mariner family member Himar 1 were identified by random mutagenesis (Lampe *et al.* 1999). All of these studies suggest that recombinase proteins, in their current form, have undergone selective pressure to limit their activities.

Control of the cell cycle, as well as the response of cells to DNA damage, involves cascades of protein phosphorylation (Norbury and Nurse 1992; Elledge 1996; Zhou and Elledge 2000). Control of cellular proliferation and repair of genotoxic damage to cellular DNA is critical for proper cell development and function. Normally, when cells experience exposure to DNA-damaging agents, checkpoint pathways become activated and result in a halt in the cell cycle to allow for the repair of the DNA damage (Zhou and Elledge 2000; Khanna and Jackson 2001). Analysis of DNA damage response components in many different organisms has revealed that one common class of proteins is a family of protein kinases that are related to the family of lipid kinases that use phosphatidylinositol-3 phosphate (PI_3) as a substrate (Hunter 1995; Zakian 1995). Members of the PI_3 kinase family function in both signal transduction and growth control processes when lipid substrates are used (Hunter 1995; Zakian 1995), whereas some members function in DNA damage checkpoint control (Zakian 1995; Jeggo *et al.* 1998; Smith and Jackson 1999; Durocher and Jackson 2001) and in a pathway of DNA repair conserved among eukaryotes, termed nonhomologous end joining (NHEJ), when protein substrates are used. It is known that *P* elements can use the

[1]*Corresponding author:* 401 Barker Hall, University of California, Berkeley, CA 94720-3204. E-mail: don_rio@uclink4.berkeley.edu

NHEJ pathway for DNA repair at the donor site following transposition (Staveley *et al.* 1995; Beall and Rio 1996; Gloor *et al.* 2000).

The ATM family of protein kinases appears to have evolved from lipid kinases and functions physiologically to regulate cell cycle progression and DNA damage responses (Hunter 1995; Elledge 1996; Zhou and Elledge 2000). A downstream effector of the DNA damage response in mammalian cells is the p53 tumor suppressor protein. While p53 is not essential for viability, it is required for induction of cell cycle arrest or cell death (apoptosis) that results following genotoxic DNA damage (Ko and Prives 1996; Giaccia and Kastan 1998). p53 is a substrate for three members of the PI_3 kinase family, the nuclear DNA-dependent protein kinase (DNA-PK), the ataxia telangiectasia-mutated (ATM) kinase, and the ATM-related kinase, ATR (Lees-Miller *et al.* 1992; Shieh *et al.* 1997; Banin *et al.* 1998; Canman *et al.* 1998; Jimenez *et al.* 1999; Tibbetts *et al.* 1999). DNA-PK, which consists of a heterotrimeric complex containing a 465-kD catalytic subunit (DNA-PKcs) and a regulatory component composed of the Ku 70- and 80-kD proteins, is involved in NHEJ repair following treatment with ionizing radiation and in the repair of immunoglobulin V(D)J recombination intermediates (Blunt *et al.* 1995; Lieber *et al.* 1997; Critchlow and Jackson 1998). While Ku 70 and Ku 80 as well as the NHEJ pathway of DNA double-strand-break repair are conserved in yeast, *Caenorhabditis elegans*, and Drosophila, no DNA-PKcs subunit gene appears to be present in the smaller genomes of these eukaryotes (Adams *et al.* 2000; Sekelsky *et al.* 2000). In Drosophila are two ATM-related genes, mei-41 and dATM. mei-41 functions in meiotic recombination and DNA damage checkpoint control (Sekelsky *et al.* 1998). The function of dATM is not known. Several substrates for the ATM family of protein kinases in mammals have been identified using *in vitro* kinase assays. The specific sequence motif phosphorylated by human DNA-PK was determined for the substrate, p53, and was found to be serine or threonine residues adjacent to glutamine (S/TQ or QS/T; Lees-Miller *et al.* 1992). However, recent studies indicate that all three kinases, DNA-PK, ATM, and ATR, can act on a minimum site of S/TQ, but that the nature of flanking amino acids can allow substrate discrimination by the different kinases (S. T. Kim *et al.* 1999).

The Drosophila *P*-transposable element is 2.9 kb in length and encodes an 87-kD transposase protein that mediates the cleavage and strand-transfer steps of the transposition reaction (Rio *et al.* 1986). *P*-element transposition occurs through a cut-and-paste mechanism (Engels *et al.* 1990; Kaufman and Rio 1992) and, *in vivo*, requires double-stranded DNA break-repair functions to repair broken DNA left following transposition (Banga *et al.* 1991; Beall and Rio 1996; Kusano *et al.* 2001). Because ionizing radiation can induce cell cycle checkpoints in Drosophila (Sekelsky *et al.* 1998) and mei-41 shows a defect following DNA breaks induced by *P*-element transposase (Banga *et al.* 1991), it was interesting to note that the transposase protein coding sequence contains 10 potential ATM-family protein kinase phosphorylation sites (Figure 1). Eight of the potential ATM-family phosphorylation sites are clustered within the first 144 amino acids of the transposase protein, directly adjacent to the site-specific DNA-binding domain (amino acids 1–88; Lee *et al.* 1998). *P*-element transposition is thought to be regulated during the cell cycle, occurring in late S or G2, since repair of the gap following excision is greatly facilitated by the presence of a sister chromatid (Engels *et al.* 1990). Phosphorylation might be one mechanism for regulating transposase activity throughout the cell cycle or in response to the environment in Drosophila.

Here we report that the *P*-element transposase protein contains 10 potential phosphorylation sites for ATM-family kinases. When the serine or threonine residues in these sites are changed to alanine, transposase activity is affected either positively or negatively using an *in vivo* recombination assay. Seven of the 8 sites at the N terminus of the protein show a reduced activity when individually substituted with alanine, while 1 site, serine 129 (S129), shows an elevated activity. None of the mutations affect the stability or site-specific DNA-binding activity of the transposase protein. The S129A mutant also showed elevated activity in embryo microinjection assays for *P*-element-mediated germline transformation. Finally, the S129A mutant exhibited activity *in vitro* higher than that of wild type in an assay for donor DNA cleavage but not in an assay for strand-transfer integration. These data identify a mutant *P*-element transposase with an elevated activity and suggest that phosphorylation by an ATM-family protein kinase might regulate activity of the *P*-element transposase protein *in vivo*.

MATERIALS AND METHODS

Recombinant DNA and *in vitro* mutagenesis: The wild-type transposase-encoding plasmid pBSKS(+)PAcNNTnpII (Mul and Rio 1997) was used to produce all of the mutant transposase-encoding plasmids. Single-stranded DNA was produced from the plasmid pRSETA-KP (Lee *et al.* 1996) by standard methods using an M13 helper phage (Ausubel *et al.* 1987). Mutations for the eight N terminally located potential DNA-PK phosphorylation sites were introduced by annealing oligonucleotides containing the desired nucleotide changes to the single-stranded KP DNA, extending with T4 DNA polymerase and transforming the duplex DNA into the *dut*$^-$/*ung*$^-$ *E. coli* strain CJ236. Incorporation of the desired mutation was confirmed by DNA sequence analysis using the ALF DNA sequencing system (Pharmacia, Piscataway, NJ) and Cy5-labeled T7 and reverse primers. Each KP mutant plasmid was cleaved with *Sca*I and *Xho*I to produce a 0.5-kb DNA fragment. pBSKS(+)PAcNNTnpII was cleaved with *Xho*I and *Xba*I to produce a 1.9-kb DNA fragment. Mutant KP DNA fragments and the 1.9-kb pBSKS(+)PAcNNTnpII DNA fragment were inserted into the pBSKS(+)PAcNNTnpII vector cleaved with

*Sca*I and *Xba*I (a 5.6-kb fragment generated by partial cleavage with *Sca*I) to generate the mutated transposase-encoding plasmids.

The plasmids encoding the two alanine substitutions in the C terminally located potential ATM-family protein kinase phosphorylation sites (T405 and S459) were generated as follows: Single-stranded DNA was produced from pBSKS(+)PAcNNTnpII as described above. Mutations were introduced by annealing oligonucleotides containing the desired nucleotide changes to the single-stranded DNA. Incorporation of the desired mutation was confirmed by DNA sequence analysis using Sequenase 2.0 as described by the manufacturer (United States Biochemical, Cleveland) and the primer 5′-CACAT TAATGTTCGATCGC. Each mutant was cleaved with *Sac*I and *Eco*RI to produce a 0.5-kb fragment. pBSKS(+)PAcNNTnpII was cleaved with *Eco*RI and *Xba*I to produce a 0.9-kb fragment and *Sac*I and *Sca*I to produce a 0.95-kb fragment. Mutant and wild-type DNA fragments were inserted into the pBSKS(+) PAcNNTnpII vector cleaved with *Sca*I and *Xba*I (a 5.6-kb fragment generated by partial cleavage with *Sca*I) to generate the mutated transposase-encoding plasmids.

The plasmid encoding the transposase protein with alanine substitutions at all eight N terminally located potential ATM-family member phosphorylation sites (-All) was generated as follows: pBSKS(+)PAcNNTnpII containing alanine substitutions at all sites except S32 was used to generate the all-sites mutated plasmid by double-stranded DNA mutagenesis as described (Mul and Rio 1997). The *Sca*I and *Xho*I fragment derived from the mutagenized plasmid was inserted back into pBSKS(+)PAcNNTnpII as described above for the mutants generated with the KP single-stranded DNA.

Tissue culture transfection assay: The reporter plasmid pISP-2/Km (10 μg) and wild-type or mutant transposase-encoding plasmids [pBSKS(+) PAcTnp (10 μg)] were transfected into the Drosophila Schneider L2 cell line by standard calcium-phosphate methods (Ausubel *et al.* 1987). After 24 hr at 25°, cells were washed with PBS and transferred to 1.5-ml Eppendorf tubes. Plasmid DNA was recovered as described from two-thirds of the total transfection (Rio *et al.* 1986; O'Brochta and Handler 1988) and resuspended in 10 mm Tris-HCl, 1 mm EDTA, pH 8.0. The remaining one-third of the transfected cells was resuspended in sample buffer and one-sixth of the sample immunoblotted using affinity-purified anti-KP polyclonal antibodies (Beall and Rio 1997). One-tenth of the recovered DNA was used to transform the RecA$^-$ *E. coli* strain AG1574 (Kaufman and Rio 1992) by electroporation using a Bio-Rad (Hercules, CA) Gene Pulser as described by the manufacturer. Cells were plated onto L-broth plates containing 100 μg/ml ampicillin or L-broth plates containing 100 μg/ml ampicillin and 50 μg/ml kanamycin. Colonies were allowed to develop for 12–16 hr at 37°. Because 17 nucleotides (nt) of sequence from each end of the *P* element are left behind at the donor site following excision (Beall and Rio 1997), at least 15 nt must be removed from the 5′ *P*-element end to generate a functional kanamycin resistance open reading frame. Therefore, the observed excision frequency was actually an underestimate of the actual excision frequency since the assay required the production of a functional kanamycin resistance gene following excision of the *P* element.

Embyro microinjection and transformation assay: Germline transformation of Drosophila strain w^{1118} was carried out using standard microinjection methods with bleach dechorionation (Spradling 1986). Plasmid DNA was prepared for injection by standard procedures and w$^+$ transformant lines were mated and used to prepare DNA for DNA blot hybridization using a *P*-element DNA probe.

Generation of Drosophila tissue culture cells expressing the S129A transposase: A stable Drosophila Schneider L2 cell line using plasmid pUChygMT-NNTnpII-S129A (in which the S129A mutation was transferred into pUChygMT-NNTnpII) was generated by transfection with calcium phosphate coprecipitation for 24 hr followed by selection with 200 μg/ml hygromycinB (Kaufman and Rio 1992).

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  1  MKYCKFCCKAVTGVKLIHVPKCAIKRKLWEQSLGCS
 37  LGENSQICDTHFNDSQWKAAPAKGQTFKRRRLNADA
 73  VPSKVIEPEPEKIKEGYTSGSTQTESCSLFNENKSL
109  REKIRTLEYEMRRLEQQLRESQQLEESLRKIFTDTQ
145  IRILKNGGQRATFNSDDISTAICLHTAGPRAYNHLY
181  KKGFPLPSRTTLYRWLSDVDIKRGCLDVVIDLMDSD
217  GVDDADKLCVLAFDEMKVAAAFEYDSSADIVYEPSD
253  YVQLAIVRGLKKSWKQPVFFDFNTRMDPDTLNNILR
289  KLHRKGYLVVAIVSDLGTGNQKLWTELGISESKTWF
325  SHPADDHLKIFVFSDTPHLIKLVRNHYVDSGLTING
361  KKLTKKTIQEALHLCNKSDLSILFKINENHINVRSL
397  AKQKVKLATQLFSNTTASSIRRCYSLGYDIENATET
433  ADFFKLMNDWFDIFNSKLSTSNCIECSQPYGKQLDI
469  QPDILNRMSEIMRTGILDKPKRLPFQKGIIVNNASL
505  DGLYKYLQENFSMQYILTSRLNQDIVEHFFGSMRSR
541  GGQFDHPTPLQFKYRLRKYIIARNTEMLRNSGNIEE
577  DNSESWLNLDFSSKENENKSKDDEPVDDEPVDEMLS
613  NIDFTEMDELTEDAMEYIAGYVIKKLRISDKVKENL
649  TFTYVDEVSHGGLIKPSEKFQEKLKELECIFLHYTN
685  NNNFEITNNVKDKLILAARNVDVDKQVKSFYFKIRI
721  YFRIKYFNKKIEIKNQKQKLIGNSKLLKIKL
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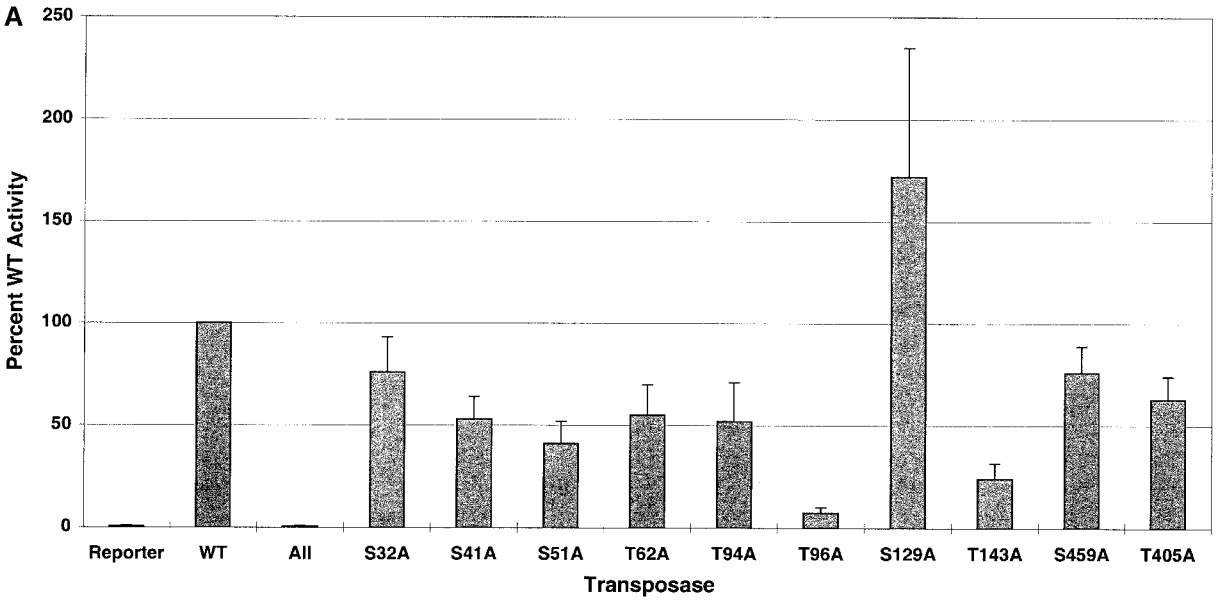
FIGURE 1.—Several potential ATM-family phosphorylation sites exist in the transposase coding sequence. The amino acid sequence of the *P*-element transposase protein is shown. The 10 potential ATM-family consensus phosphorylation site sequences (S or T adjacent to Q) are boxed in black. Eight of these sites are clustered in the first 144 amino acids of the transposase protein, directly adjacent to the site-specific DNA-binding domain (located in the first 88 amino acids; Lee *et al.* 1996).

Transposase protein purification: Wild-type and S129A *P*-element transposase proteins were partially purified from the Drosophila Schneider L2 stable cell lines following $CuSO_4$ induction as described (Kaufman and Rio 1992; Beall and Rio 1997). Briefly, nuclear extracts were prepared and precipitated with ammonium sulfate and fractions containing transposase were pooled, dialyzed, and chromatographed on heparin-agarose as described. The heparin flow-through fraction contains the active form of transposase. This fraction contained 10–15 mg/ml of total protein as assayed using Bradford dye binding. All buffers contained 50 mm NaF to inhibit phosphatase activity.

***In vitro* cleavage and strand-transfer assays:** Assays for *P*-element donor DNA cleavage and strand transfer were carried out exactly as described previously with the same DNA substrates (Kaufman and Rio 1992; Beall and Rio 1997, 1998). The protein fractions used were diluted serially in HGKED buffer and compared for the amount of transposase using immunoblotting, as indicated in Figure 5A.

RESULTS

Putative ATM-family phosphorylation site mutations affect transposase activity *in vivo*: To examine a possible role for ATM-family kinase phosphorylation in *P*-element transposition, we expressed mutant transposase proteins that contain individual alanine substitutions at each potential ATM-family protein kinase site in Dro-



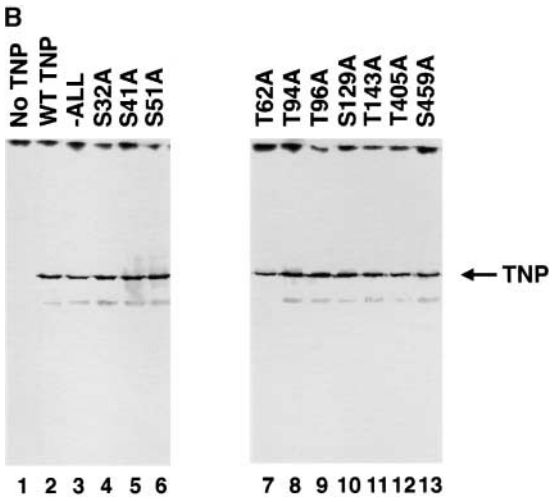


FIGURE 2.—*P*-element transposase excision activity *in vivo* is affected both positively and negatively by mutations in potential ATM-family phosphorylation sites. (A) Each of the indicated mutant transposase proteins was tested for activity as described (MUL and RIO 1997). Plasmids encoding wild-type or mutant transposase proteins along with a kanamycin-sensitive reporter plasmid containing a small nonautonomous *P* element were transfected into Drosophila L2 cells. Twenty-four hours after transfection, the cells were harvested and the plasmid DNA recovered. Transposase activity was detected following electroporation of the recovered plasmid DNA into *E. coli* by the gain in kanamycin resistance. Wild-type activity was determined from the following ratio: $(Amp^R Kan^R/Amp^R)\times 10^5$, which refers to the number of excision plasmids/the total amount of plasmid DNA recovered from the transfected cells. Percentage of wild-type activity refers to the excision frequency obtained for each mutant relative to the excision frequency determined for the wild-type protein (×100). For the histogram shown, the wild-type activity was set at 100%. Reporter, assay performed in the absence of the transposase-encoding plasmid. All, assay performed with the transposase-encoding plasmid containing alanine substitutions at all eight N-terminal sites. Shown are the average activity and standard deviations derived from multiple independent experiments in which each mutant was tested in duplicate in each experiment. The variance of each sample and the number of times the experiment was performed (n) Reporter = 0.34, n = 8; WT, 3.86, n =7; All = 0.12, n = 12; S32A = 389, n = 4; S41A = 154, n = 5; S51A = 146, n = 7; T62A = 285, n = 5; T94A = 433, n = 5; T96A = 39, n = 14; S129A = 4649, n = 7; T143A = 69, n = 6; S459A = 215, n = 4; T405A = 154, n = 4. (B) Immunoblot analysis of transposase proteins expressed by transfection of L2 cells. Protein extracts were prepared from a fraction of the transfected cells used for the recombination assay and subjected to electrophoresis and electroblotting, and transposase was detected with a polyclonal rabbit antibody to the KP protein (LEE *et al.* 1998).

sophila tissue culture cells. These experiments were prompted by the finding that *P*-element transposase is a phosphoprotein (as assessed by *in vivo* labeling with ^{32}P and phosphoamino acid analysis; E. L. BEALL and D. C. RIO, unpublished results) and the presence in the transposase sequence of 10 putative ATM-family kinase recognition sites (Figure 1). The mutant transposase proteins were tested for activity using a plasmid-based *P*-element excision assay (BEALL and RIO 1997; MUL and RIO 1997). The effect of each mutation on transposase activity was determined by comparing the number of excised plasmids recovered to the total amount of DNA recovered from each transfection. When the activities of the alanine substitution mutants were examined, varying effects on transposase activity were observed (Figure 2A). Substitution of alanine at all 8 N-terminal potential ATM-family phosphorylation sites resulted in a complete loss of transposase activity (Figure 2A, All). This inactivity does not appear to be due to a loss of site-specific DNA binding, since a transposase derivative, the KP protein that carries the N-terminal DNA-binding domain, with or without these alanine mutations, binds to the transposase binding site as determined by DNase I footprint protection (E. L. BEALL and D. C. RIO, data not shown). Immunoblot analysis of the transfected cell extracts revealed that the level of each mutant transposase protein was comparable to the wild-type transposase protein expression level (Figure 2B). The most severe

TABLE 1

***P*-element S129A transposase yields a higher frequency of *P*-element-mediated germline transformation**

	Transposase Helper DNA concentration	No. of fertile adults	No. of transformant vials	% transformed[b]	No. of different transformants by Southern blot hybridization	Total no. of transformants
Wild type50-1	50 μg/ml[a]	46	3	6.5	3	10
S129A 50-1	50 μg/ml	53	9	18.9	10	21
Wild type 250-1	250 μg/ml	20	1	10.0	2	2
S129A 250-1	250 μg/ml	83	22	48.2	40	78
Wild type 50-2	50 μg/ml	107	6	10.3	11	22
S129A 50-2	50 μg/ml	74	13	27.0	20	37
Wild type 250-2	250 μg/ml	31	7	35.5	11	20
S129A 250-2	250 μg/ml	141	24	34.0	48	84

Data are shown from two experiments using the indicated actin-transposase helper plasmid DNA concentration and the transformation vector, pw8, at 500 μg/ml. The number of fertile adults, number of transformant vials, the percentage of transformed fertile G_0 adults, the number of different independent insertions as assayed by DNA blot hybridization (data not shown), and the total number of transformants obtained are indicated in the labeled columns.

[a] *P*-element transformation vector, pw8; concentration, 500 μg/ml.

[b] Tranformation frequency has traditionally been given as the number of G_0 progeny producing one or more transformants/the number of fertile G_0 adults (Spradling 1986). However, since here we determined the number of different transformants by Southern blot hybridization, we used this number, rather than the number of G_0 progeny producing at least one transformant, to calculate the transformation frequency.

reduction in transposase activity was observed when alanine was substituted at threonine 96 (Figure 2A, T96A at ~7% activity), followed by threonine 143 (Figure 2A, T143A at ~24% activity) and serine 51 (Figure 2A, S51A at ~41% activity). Interestingly, the transposase mutant containing an alanine substitution at serine 129 showed increased activity (Figure 2A, S129A at ~172% activity), suggesting that phosphorylation at this site might be inhibitory. Additionally, this S129A mutant shows elevated levels of activity in embryo *P*-element injection/transformation experiments (see below; Table 1). These data suggest that phosphorylation at S51, T96, and T143 may be essential for a high level of transposase activity. One mutation, S129A, exhibits an elevated transposase activity *in vivo*, suggesting that a phosphorylation event at this site might be inhibitory.

Occasionally, serine or threonine phosphorylation can be mimicked by aspartate (D) or glutamate (E) substitution at the phosphorylated amino acid position (Johnson and O'Reilly 1996). On the basis of the relative size of each amino acid side chain, D is usually substituted for S, and E is usually substituted for T. Mutant transposase proteins that showed an average activity <50% of the wild-type transposase activity when substituted with alanine were also substituted with either D at the positions of potential serine phosphorylation or E at the positions of potential threonine phosphorylation. For T96, which showed the most severe reduction in transposase activity when substituted with alanine (Figure 2A), D, E, and S substitutions were made. If phosphorylation at S51, T96, and T143 is required for transposase activity, then substitution of acidic amino acids at these positions may result in an increased activity relative to the alanine-substituted proteins.

Substitution of D at S51 partially restored transposase activity (Figure 3, compare S51A at ~39% activity to S51D at ~76% activity), whereas substitution of E at either T96 or T143 caused even more reduction in transposase activity (Figure 3, compare T96A at ~10% activity to T96E at ~6% activity and T143A at ~25% activity to T143E at ~2% activity). Substitution of either D or S at position T96 showed a modest increase in transposase activity (Figure 3, compare T96A at ~10% activity to T96D at ~12% activity and T96S at ~12% activity). The acidic amino acid substitutions did not affect the expression levels of the mutant transposase proteins (data not shown). Among the cases examined, it appeared that there was, at most, a twofold increase in transposase activity when an acidic amino acid was substituted for a potential phosphorylation site. It is worth noting that substitution of serine at T96 did not restore transposase activity, suggesting that a threonine at position 96, possibly due to the different sizes of the side chains, is critical for transposase activity.

Finally, if phosphorylation at residue S129 inhibits transposase activity, then substitution of D at this position should result in reduced transposase activity. As shown in Figure 3, substitution of D at position S129 resulted in a nearly threefold reduction in transposase activity (compare S129A at ~172% activity with S129D at ~54% activity). These observations suggest that possible phosphorylation at S129 is inhibitory *in vivo*.

To further examine the critical nature of S51, T96, and T143 for transposase activity, we tried to reactivate

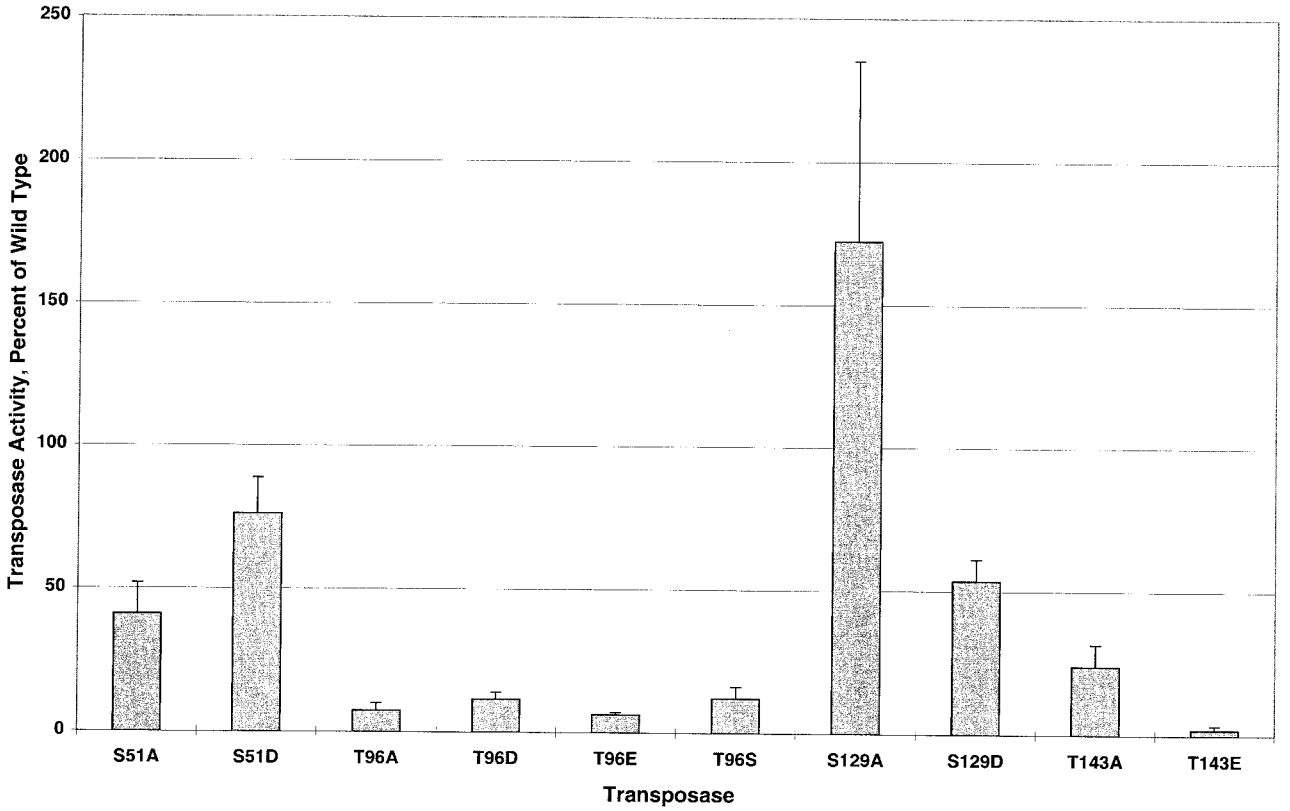


FIGURE 3.—*P*-element transposase excision activity *in vivo* can be altered by acidic amino acid substitutions at several potential ATM-family phosphorylation sites. Acidic amino acid substitutions were made at potential ATM-family phosphorylation sites in the transposase coding sequence that showed the most dramatic effect on transposase activity when substituted with alanine (S51, T96, S129, and T143). For each amino acid, aspartate (D) was substituted for serine or glutamate (E) was substituted for threonine, except for T96, in which all three substitutions were made. Each mutant transposase protein activity was calculated as described in Figure 2. Shown are the average activity and standard deviations derived from multiple independent experiments in which each mutant was tested in duplicate in each experiment. The variance of each sample and the number of times the experiment was performed (n) S51D = 210, n = 4; T96D = 6.9, n = 4; T96E = 0.79, n = 4; T96S = 21, n = 6; S129D = 111, n = 2; T143A = 4.1, n = 3.

the transposase protein containing alanine substitutions at all of the eight potential ATM-family phosphorylation sites by replacing the alanine residues with the corresponding wild-type amino acid. Substitution of T96 back into the -All alanine mutant protein resulted in a nearly 24-fold increase in transposase activity (Figure 4, compare -All at ~0.5% activity to T96WT at ~12% activity). Even though substitution of T143 back into the -All protein resulted in only a 7-fold increase in transposase activity (Figure 4, T143WT at ~4% activity), substitution of both T96 and T143 back into the -All protein resulted in nearly a 70-fold increase in transposase activity (Figure 4, T96/T143WT at ~33% activity). Most strikingly, replacement of S51, T96, and T143 into the -All protein resulted in a nearly complete restoration of transposase activity (Figure 4, S51/T96/T143WT at ~85% activity). Note that all of these mutants contain alanine at the inhibitory site, S129. Because a stimulation in transposase activity above wild type was not observed with the S51/T96/T143WT mutant protein (containing S129A), additional potential ATM-family phosphorylation sites must contribute to maximal transposase activity. Together, these data suggest that modification at several potential ATM-family kinase sites may regulate transposase activity both positively and negatively *in vivo*.

The S129A mutant transposase showed an elevated frequency of *P*-element-mediated germline transformation: The finding that the S129A mutant transposase protein exhibited an elevated level of activity in the tissue culture cell recombination assay prompted us to ask if this modified protein would also increase the efficiency of *P*-element-mediated germline transformation. Following coinjection of w^{1118} preblastoderm embryos with plasmid containing either wild type or S129A transposase expressed from the strong actin 5C promoter, along with the standard *P*-element transformation vector pw8, normal matings were performed to identify germline transformants. As shown in Table 1, in two independent experiments using either 50 or 250 μg/ml of the indicated actin-transposase helper DNA,

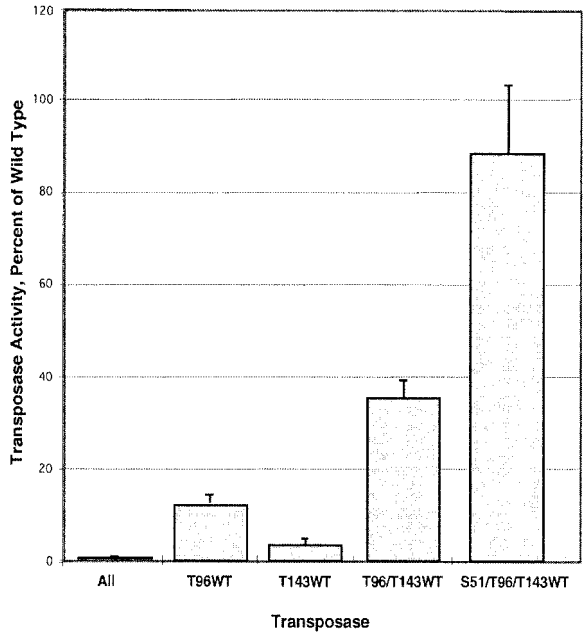


FIGURE 4.—*P*-element transposase excision activity *in vivo* is reactivated when wild-type potential ATM-family phosphorylation sites are restored. The sites that showed the most dramatic effect on transposase activity (S51, T96, and T143) were placed back to their wild-type sequence in the transposase mutant containing alanine substitutions at all eight N-terminal sites (All). Each mutant transposase protein activity was calculated as described in Figure 2. Shown are the average activity and standard deviations derived from several independent experiments in which each mutant was tested in duplicate in each experiment. Note that all of the mutants tested in this panel contain alanine at position S129. The variance of each sample and the number of times the experiment was performed (n) T96WT = 5.6, n = 10; T143WT = 1.9, n = 6; T96/T143WT = 49, n = 5; S51/T96/T143WT = 298, n = 8.

the S129A transposase yielded a three- to fivefold increase in the frequency of recovering w^+ transformants. Moreover, when these transgenic strains were assayed for the number of independent insertions using DNA blot hybridization, there was a general trend of a larger number of independent transformants being obtained with the S129A transposase helper (Table 1, last two columns). Indeed, in some cases using the higher (250 μg/ml) concentration of the S129A helper plasmid, multiple insertions were obtained in the same initial transformant, suggesting that multiple insertional events occurred in the same gamete. Thus, taken together, these data indicate that the S129A transposase can yield higher frequencies of germline *P*-element transformation when compared to the wild-type enzyme. This mutant form of *P*-element transposase may be generally useful to improve the efficiency of *P*-element-mediated germline transformation.

The S129A transposase exhibited elevated donor DNA cleavage but not elevated strand-transfer activities *in vitro*: Because we observed elevated activity with the S129A mutant in two different *in vivo* assays for transposase activity (see above) and because we had previously developed *in vitro* assays for both the donor DNA cleavage and strand-transfer activities of *P*-element transposase (BEALL and RIO 1997, 1998), we wanted to assay the S129A mutant transposase activities *in vitro*. To express the S129A protein in Drosophila tissue culture cells, we used the previously developed hygromycin selection and metallothionein promoter system we had developed for the wild-type enzyme (KAUFMAN and RIO 1992; BEALL and RIO 1997). Although the transformed Schneider L2 cells did express the mutant S129A protein, this protein did not accumulate to the same level as the wild-type protein. Moreover, when we attempted to purify the S129A mutant protein using our standard purification (KAUFMAN and RIO 1992; BEALL and RIO 1997), we found that the S129A protein behaved differently from the wild-type protein and importantly did not bind well to the DNA oligonucleotide affinity resin, containing three copies of the transposase consensus binding site, that had formed the basis for the purification of the wild-type enzyme (BEALL and RIO 1997; MUL and RIO 1997). However, it is possible to assay for the *P*-element transposase cleavage and strand-transfer activities in partially purified heparin-agarose chromatography fractions. Immunoblotting was used to determine the relative concentrations of the wild-type and S129A mutant proteins in these chromatographic fractions so that equivalent amounts could be directly compared in the activity assays (Figure 5A).

The assay for donor DNA cleavage by transposase uses a plasmid carrying a 0.6-kb *P* element, which, when released from the plasmid backbone, can be detected by DNA blot hybridization of the reaction products. As is apparent in Figure 5B, at equivalent concentrations of transposase the S129A mutant exhibits a significant increase (three- to fivefold) in donor DNA cleavage activity compared to wild type. At the lowest concentration of protein tested, no activity was observed for the wild-type transposase, whereas low activity was observed for S129A (Figure 5B; compare lanes 9 with lane 3). At higher protein concentrations, S129A activity was higher than that of wild type (Figure 5B; compare lanes 11 and 13 with lanes 5 and 7). All reactions required GTP as a cofactor to observe *P*-element excision. Thus, taken together, these results show that, at equivalent transposase concentrations, the S129A mutant shows an elevated activity for donor DNA cleavage compared to the wild-type protein.

The assay for strand transfer by transposase uses radiolabeled oligonucleotide substrates carrying the 3′ *P*-element end, including a transposase binding site, and an unlabeled plasmid DNA as an integration target (BEALL and RIO 1997). The strand-transfer product appears as a slowly migrating linear or nicked circular species following agarose gel electrophoresis and autoradiography. In contrast to the assay for donor DNA cleavage, the S129A mutant transposase displays activity that is

similar to the wild-type protein at the lowest concentration of protein tested (Figure 5C; compare lane 9 to lane 3). At higher protein concentrations, the S129A mutant showed slightly less activity than wild type (Figure 5C; compare lanes 11 and 13 with lanes 5 and 7). Thus, while the S129A transposase exhibited elevated donor DNA cleavage (Figure 5B), the strand-transfer activity of this mutant protein is comparable to that of wild type. This finding may not be surprising because the strand-transfer assay is not as stringent a test for transposase activity since the precleaved oligonucleotide substrates used bypass the initial synaptic complex assembly required for donor DNA cleavage at the *P*-element ends.

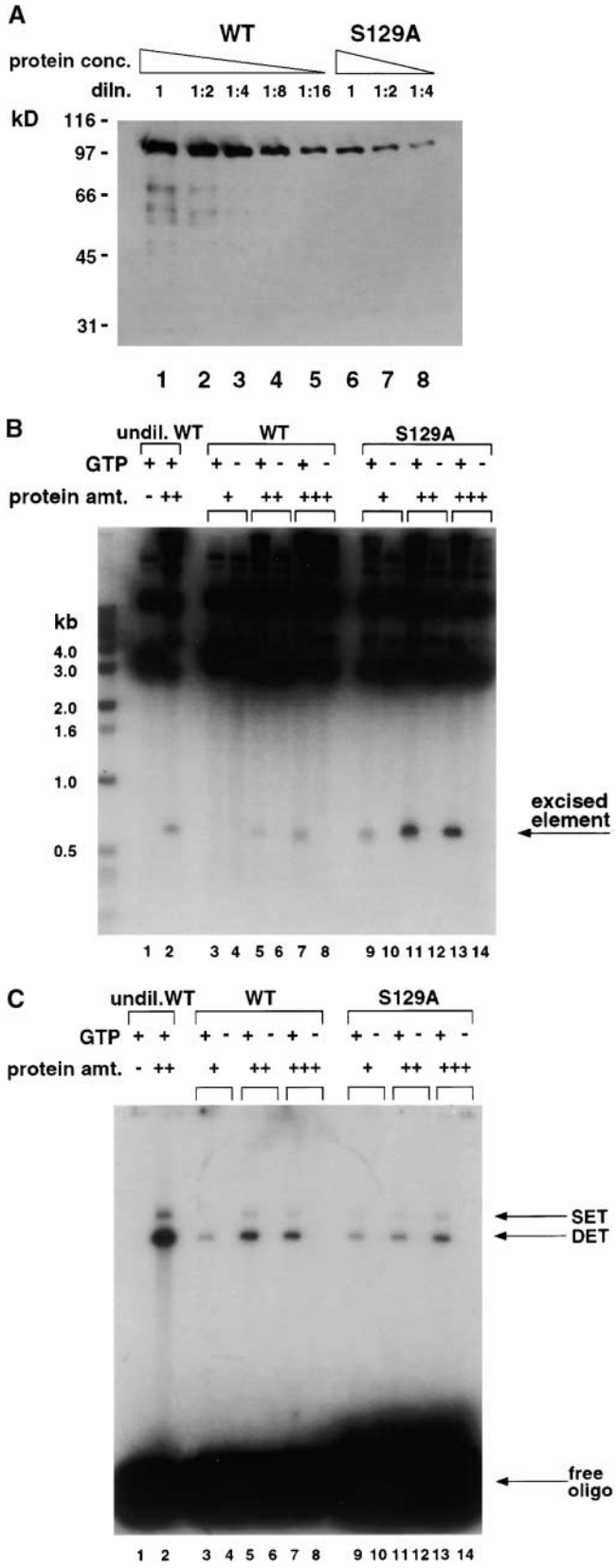


FIGURE 5.—The *P*-element S129A transposase exhibits elevated donor DNA cleavage and strand-transfer activities *in vitro*. (A) Immunoblot analysis of partially purified wild-type and S129A mutant heparin-agarose fractions. Various dilutions of the pooled H0.1 flow-through fraction were tested (fold dilution indicated above the lanes) for wild type (lanes 1–5) and S129A (lanes 6–8). Protein concentrations of these fractions are indicated in MATERIALS AND METHODS. (B) Assay for *P*-element donor DNA cleavage and an autoradiogram of a DNA blot hybridization filter containing donor DNA cleavage assays using pISP-2/Km as a donor DNA and probed with a radiolabeled *P*-element DNA fragment. Control reactions with wild-type purifed transposase (TdT0.3) are shown with (lane 2) or without (lane 1) GTP in the reaction. Reactions using increasing amounts of wild-type 1:16 diluted transposase with (lanes 3, 5, and 7) or without (lanes 4, 6, and 8) GTP and the increasing amounts of undiluted S129A mutant transposase with (lanes 9, 11, and 13) or without (lanes 10, 12, and 14) GTP are shown. An arrow indicates the mobility of the excised 0.6-kb *P* element from pISP-2/Km (BEALL and RIO 1997). (C) Assay for *P*-element strand transfer *in vitro* and an autoradiogram of an agarose gel containing the DNA strand-transfer products. Control reactions using wild-type transposase in the presence of GTP (lane 2) or in the absence of transposase (lane 1). Various concentrations of either wild-type transposase in the presence (lanes 3, 5, and 7) or absence (lanes 4, 6, and 8) of GTP or the S129A mutant transposase in the presence (lanes 9, 11, and 13) or absence (lanes 10, 12, and 14) of GTP are shown. Arrows indicate the mobility of either single-end strand-transfer (SET) or double-end strand-transfer (DET) products or the radiolabeled *P*-element strand-transfer oligonucleotide substrate (free oligo).

DISCUSSION

Mutation of potential ATM-family phosphorylation sites modulates *P*-element transposase activity: Here we report that multiple potential ATM-family phosphorylation sites are in the *P*-element transposase protein (Figure 1) and that mutation of eight of these sites modulates *P*-element transposase activity using an *in vivo* recombination assay. Alanine substitution at several of the potential ATM-family phosphorylation sites (most notably, S51, T96, and T143) resulted in a severe reduction in transposase activity, whereas alanine substitution at one site (S129) resulted in increased transposase activity, suggesting that phosphorylation at these sites might regulate transposase activity both positively and negatively. Using ligation-mediated PCR, we found that ala-

nine substitution at S51, T96, or T143 inhibits transposase at or prior to the donor DNA cleavage step of the transposition reaction (E. L. Beall and D. C. Rio, data not shown). The exact sites of transposase protein phosphorylation *in vivo* have yet to be determined. However, it should be noted that any particular site of phosphorylation *in vivo* may be difficult or impossible to detect if, for instance, the stoichiometry of phosphorylation is low, if phosphorylation is regulated during the cell cycle, or if phosphorylation has a rapid turnover of the phosphate at a given position.

A hyperactive mutant of *P*-element transposase: It is interesting to note that one mutant, S129A, shows increased activity in both the tissue culture transfection assay and by direct embryo microinjection for *P*-element-mediated germline transformation. The S129A mutant displayed elevated *in vitro* donor DNA cleavage, but not strand-transfer activity. This finding is not surprising since we know little about the complex nature of the steps involved in the assembly and activation of the donor cleavage synaptic complex, whereas the strand-transfer reaction using oligonucleotide substrates exhibits relaxed DNA substrate requirements. It is easily conceivable that the S129A mutation could somehow promote synaptic complex assembly and increase donor DNA cleavage without affecting the strand-transfer reaction.

The S129 site was initially recognized as a potential site for the ATM family of nuclear protein kinases (Figure 1). If S129 is phosphorylated *in vivo*, it is possible that phosphorylation at this site negatively regulates transposase activity by preventing assembly of a functional transposition complex at the transposon termini and/or by controlling the catalytic rate of the enzyme, so that transposition occurs only at a specific point in the cell cycle. The increased activity displayed by the S129A protein may be due simply to an uncoupling of the transposase activity from the normal controls that might operate during DNA damage checkpoints and the cell cycle (Elledge 1996; Zhou and Elledge 2000). Alternatively, phosphorylation at S129A may be required to prevent or inhibit transposition complex assembly to keep transposition frequencies low. Further analysis of the S129A mutant transposase protein may reveal how modification at this site could affect its activity.

A potential role for regulation of transposase activity throughout the cell cycle or in response to external stimuli: Because repair of the gap following *P*-element excision *in vivo* is greatly facilitated by homologous sequences, it is thought that transposase activity is modulated throughout the cell cycle such that the transposase protein is active only in the S or G2 phases (Engels *et al.* 1990). In mammalian cells, it appears that the DNA-PK activity may also be regulated throughout the cell cycle, since it has been reported that there is 5- to 10-fold more activity in extracts derived from cells synchronized in G2 (reviewed in Jin *et al.* 1997). However, for repair of V(D)J recombination intermediates, the activity of DNA-PK appears to be required in G1 of the cell cycle (Lee and Desiderio 1999). In Drosophila, it is possible that other ATM-family kinases (mei-41 and/or dATM; Sekelsky *et al.* 1998, 2000; Adams *et al.* 2000) may play a role in controlling *P*-element transposition and possibly be responsible for regulating transposase activity during the G2 phase of the cell cycle. It is known that mammalian ATM can be activated throughout the cell cycle by ionizing radiation (Pandita *et al.* 2000).

Previous phosphoamino acid analysis of *in vivo* radiolabeled transposase protein revealed that transposase is phosphorylated on serine, threonine, and tyrosine (E. L. Beall and D. C. Rio, unpublished results). A regulatory role for tyrosine phosphorylation is known for a number of proteins *in vivo* (Songyang *et al.* 1995). Interestingly, in mammalian cells, the c-Abl tyrosine kinase has been shown to associate with DNA-PKcs and the Ku subunits and this association allows phosphorylation of DNA-PKcs to downregulate its activity (Kharbanda *et al.* 1997). c-Abl is known to be activated in response to DNA-damaging agents that include ionizing radiation and methyl methanesulfonate (Kharbanda *et al.* 1995, 1997) and is one of the few known nuclear localized tyrosine kinases (reviewed in Wang 1993; Feller *et al.* 1994). Thus, if the Drosophila c-Abl protein kinase is regulated by DNA damage or in the cell cycle, it might provide a means to allow tyrosine phosphorylation of *P*-element transposase.

Several studies have suggested that external or environmental stimuli may trigger DNA rearrangements (McClintock 1984). The DNA rearrangements associated with V(D)J recombination are critical to lymphocyte differentiation and occur only at specific developmental stages and apparently at a prescribed time during the cell cycle (Chen and Alt 1993; Lee and Desiderio 1999). Recent studies indicate that the yeast retrotransposon, Ty1, is negatively controlled by signal transduction or DNA repair pathways (Curcio and Garfinkel 1999). In the case of *P* elements, there are conflicting reports regarding whether DNA damage induces transposition or whether *P*-element mobilization can cause the global mobility of other transposable element families (Gerasimova *et al.* 1984; Woodruff *et al.* 1987; Eggleston *et al.* 1988). Thus, it is an interesting possibility that transposase phosphorylation could activate *P*-element transposition in response to external stimuli. Identification of the sites in the transposase protein that are detectably modified by phosphorylation *in vivo* through phosphopeptide mapping and mass spectrometry will aid in the identification of the full spectrum of protein kinases that may play a role in regulating transposase activity.

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