Reprogramming the purine nucleotide cofactor requirement of *Drosophila* P element transposase *in vivo*

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Guanosine triphosphate (GTP)-binding proteins are involved in controlling a wide range of fundamental cellular processes. *In vitro* studies have indicated a role for GTP during *Drosophila* P element transposition. Here we show that P element transposase contains a non-canonical GTP-binding domain that is critical for its ability to mediate transposition in *Drosophila* cells. Moreover, a single amino acid substitution could switch the nucleotide binding-specificity of transposase from GTP to xanthosine triphosphate (XTP). Importantly, this mutant protein could no longer function effectively in transposition *in vivo* but required addition of exogenous xanthine or xanthosine for reactivation. These results suggest that transposition may be controlled by physiological GTP levels and demonstrate that a single mutation can switch the nucleotide specificity for a complex cellular process *in vivo*.

Keywords: GTP-binding protein/guanosine triphosphate/ P elements/transposition/xanthosine triphosphate

Introduction

GTP-binding proteins are critical regulators of a large variety of cellular processes, such as protein biosynthesis, signal transduction, cytoskeletal organization and intracellular membrane trafficking (Bourne *et al.*, 1990, 1991; Noel *et al.*, 1993; Sprinzl, 1994). In general, the functionally active conformation of a G protein requires interaction with GTP and Mg²⁺ ions. Most, but not all, G proteins studied possess a regulated, intrinsic GTPase activity that switches the active conformational state of the proteins to an inactive guanosine diphosphate (GDP)-bound form. Reconversion to the active state occurs via exchange of GDP for GTP, which can be facilitated by exchange factors (Bourne *et al.*, 1990, 1991; Noel *et al.*, 1993; Sprinzl, 1994). Previous biochemical experiments have suggested that GTP plays a role as a cofactor for mobilization of P transposable elements in the fruitfly *Drosophila melanogaster* (Kaufman and Rio, 1992).

P elements move via a non-replicative cut-and-paste mechanism that is catalyzed by an 87 kDa P element-encoded transposase protein (Berg and Howe, 1989). Transposase binds specifically to sequences internal to both ends of the transposon (Kaufman *et al.*, 1989) and is believed to excise the donor P element as a double-strand DNA intermediate. Following excision, the free 3'-hydroxyl groups of the released P element DNA are

thought to make a nucleophilic attack on transposase-activated 5'-phosphoryl groups at the target sequence (Kaufman and Rio, 1992). After insertion of the P element, host proteins presumably repair the single-stranded gaps flanking the element (Berg and Howe, 1989) and the double-strand breaks at the donor sites (Beall and Rio, 1996). *In vitro* studies using circular plasmids as donor and target DNAs indicated that optimal transposition efficiency requires GTP as well as Mg²⁺ (Kaufman and Rio, 1992). However, the molecular mechanism by which GTP can stimulate the *in vitro* transposition reaction, as well as the importance of GTP for transposition *in vivo* in *Drosophila* cells, have remained unclear. In order to address these issues, we generated mutant transposase proteins with altered GTP-binding sites in order to study the functional role of GTP in P element activity *in vivo*.

Results

Transposase binds GTP

Initially, we wanted to determine whether GTP interacts directly with P element transposase. Inspection of the transposase amino acid sequence revealed sequences in the central region of the protein that loosely match a consensus GTP-binding motif, conserved among many G proteins (Bourne *et al.*, 1990, 1991; Noel *et al.*, 1993; Sprinzl, 1994). In most G proteins studied to date, five conserved regions (G1 through G5) have been identified (Figure 1A). Subdomains G1, G2 and G3 are involved in phosphate binding and in coordination of Mg²⁺. Additionally, regions G4 and G5 form the guanosine-binding pocket. The most critical amino acids within this pocket are derived from G4, which is highly conserved and directly forms hydrogen bonds with substituents of the guanine ring. In P element transposase, subdomains G2, G4 and G5 are well conserved, whereas G1 and G3 deviate from the consensus, as indicated (Figure 1A). Some of the regions flanking the conserved core motif also seem to have some differences (Bourne *et al.*, 1990, 1991; Noel *et al.*, 1993; Sprinzl, 1994; data not shown). In order to test whether transposase actually binds GTP, recombinant transposase purified from transformed *Drosophila* Schneider L2 cells, was immobilized on a nitrocellulose filter and incubated with radiolabeled [α -³²P]GTP. This protein was purified by immunoaffinity chromatography (see Materials and methods). A large (1000-fold) molar excess of unlabeled ATP, which does not serve as cofactor for P element transposition *in vitro* (Kaufman and Rio, 1992), was added to the reaction as a non-specific competitor. After binding, the filter was extensively washed and subjected to autoradiography. Figure 1B shows that transposase efficiently binds GTP in this assay. By contrast, a negative control, consisting of mock-purified material from untransformed cells not expressing

Fig. 1. P element transposase is a GTP-binding protein. (A) Comparison of the consensus GTP-binding motif (Bourne *et al.*, 1990, 1991; Noel *et al.*, 1993; Sprinzl, 1994) to the amino acid sequence of P element transposase, p21ras and mammalian G proteins such as Go from rat brain. Presented are regions G1–G5. Residues conserved among GTP-binding proteins are indicated in bold. G5 has distinct consensus sequences among the different GTPase subfamilies. The conserved Ala, which directly contacts guanine, is usually flanked by specific polar amino acids (Z) that stabilize the side chains of Asp and Asn in G4 (Bourne *et al.*, 1990, 1991; Noel *et al.*, 1993; Sprinzl, 1994). Residues in transposase that deviate from the consensus motif sequence are marked with an asterisk. (B) Autoradiograph of radiolabeled GTP bound to transposase (Tnp) purified from a transformed *Drosophila* cell line, mock-purified protein (–) from untransformed cells not expressing transposase and purified recombinant SAR1p, a small GTP-binding protein from yeast involved in transport vesicle formation (Nakano and Muramatsu, 1989; Barlowe *et al.*, 1993).

transposase (see Materials and methods), did not bind a significant amount of GTP. In this assay, comparison with another G protein, recombinant yeast SAR1p (Nakano and Muramatsu, 1989; Barlowe *et al.*, 1993; Figure 1B), suggested that the GTP-binding affinity of transposase may be at least 3-fold lower than SAR1p based on the amount of radiolabeled material bound per mole of protein (see Materials and methods). However, this assay is only meant to be a qualitative measure of GTP binding, particularly since we do not know how much endogenous GTP is bound to the protein we purify (Goody *et al.*, 1991). The presence of the non-canonical amino acids in the transposase GTP-binding motif, specifically in the phosphate and Mg²⁺ binding regions (Figure 1A), could influence the affinity of transposase from GTP when compared with other GTP-binding proteins. Because there is perfect conservation in the guanine interaction site of transposase (Figure 1A), nucleotide binding should still be specific, in full agreement with the preference for GTP versus ATP as a cofactor *in vitro* (Kaufman and Rio, 1992).

Amino acid substitutions in transposase abolish both GTP binding *in vitro* and transposition *in vivo*

In order to test the functional importance of GTP binding by transposase for transposition in *Drosophila* cells, we developed an *in vivo* assay to test the catalytic activities of wild-type and mutant proteins. *Drosophila* Schneider L2 cells were transiently transfected with a plasmid that expresses either wild-type or mutant transposase protein (Figure 2A). In addition, a reporter plasmid was introduced in order to monitor the transposase-induced P element excision frequency. The reporter DNA contains a non-autonomous P element inserted directly downstream of the start codon for the bacterial kanamycin (Kan) resistance gene (Figure 2A). Only precise excision of the P element would place the translation start codon in frame with the rest of the kanamycin resistance coding sequence. Following recovery of plasmid DNA from the L2 cells, the DNA was introduced into *Escherichia coli* and excision events were measured by genetic selection for kanamycin

resistance. Correct excision of the P element was then verified by restriction endonuclease cleavage analysis of the recovered reporter plasmids. This assay allowed for a quantitative measurement of transposase activity *in vivo*. The *Drosophila* cells were transfected with the reporter plasmid in the presence or absence of vector DNA expressing wild-type transposase. The cells were harvested after 24 h and the plasmid DNA was recovered and analyzed. In *Drosophila* cells expressing wild-type transposase, we found that correct excision of the P element occurred at a frequency of 4×10^{-5} , as was evident from the appearance of kanamycin-resistant bacterial colonies (Table I) and from analysis of restriction endonuclease cleavage products (Figure 2A and B). By contrast, in the absence of transposase, no excision products could be detected (Table I) and restriction endonuclease cleavage analysis confirmed that reporter DNA recovered from the Amp^RKan^S colonies was identical to the input plasmid (data not shown).

The availability of this genetic assay enabled us to test mutant forms of transposase, generated by site-directed mutagenesis (Deng and Nickoloff, 1992), for activity *in vivo*. First, a triple mutant that disrupted the GTP-binding consensus motif was created by changing the conserved residues Lys267, Asn376 and Asp379 into alanines (transposase K267A/N376A/D379A). The effect of these mutations was striking and virtually abolished all transposition function *in vivo* (Table I). Immunoblot analysis of cell extracts was used to verify that the expression level of the mutant protein was equal to that of the wild-type transposase, suggesting that the protein was not grossly misfolded (data not shown). To determine whether the lack of transposase activity correlated with reduced affinity for GTP, we assayed immunoaffinity-purified transposase K267A/N376A/D379A (see Materials and methods) for GTP binding (Figure 2C). As expected from the changes in the amino acid sequence, no significant GTP binding could be detected *in vitro* (Figure 2C). Based on studies of the structurally defined p21ras GTPase (Der *et al.*, 1986; Sigal *et al.*, 1986), we also generated a series

Fig. 2. A functional GTP-binding site is required for transposase activity *in vivo*. (A) Cartoon outlining the experimental strategy used. Input plasmid DNA and the products of the *in vivo* P element excision assay are represented schematically. (B) Restriction endonuclease analysis of reporter DNA before (input) or after (output) transfection of L2 cells. *PvuII* cleavage of the unmodified pISP-2/Km reporter plasmid gives rise to four fragments (see input DNA). Sizes are indicated on the left side of the panel in kb. The 1.1 kb fragment consists of the 0.6 kb P element and 0.5 kb of the flanking kanamycin resistance gene. Consequently, excision of the P element results in a 0.5 kb *PvuII* cleavage product instead of the 1.1 kb fragment. The cleavage pattern in lanes 1–7, showing *PvuII*-cleaved DNA that was isolated from Kan-resistant bacterial colonies, is characteristic of correct excision of the P element. (C) Two mutant proteins were purified from transformed Schneider L2 cell lines and tested for GTP binding as described in Figure 1. The amount of radiolabeled GTP bound to the mock-purified transposase (–), transposase D379N-1 and transposase K267A/N376A/D379A (KND) was <10% of the amount bound by wild-type transposase.

of single amino acid substitutions in transposase that are each predicted to reduce markedly the affinity of the protein for GTP (Table I). These substitutions were single D379A and K377E mutations in the guanine-binding motif NKSD (Figure 1A) and a mutation in the putative phosphoryl-binding region (K287N; Figure 1A). These mutants were then tested in the *in vivo* transposase excision assay. Similarly to transposase K267A/N376A/D379A, all mutant proteins altered to have reduced GTP-binding capacity were defective in supporting transposition *in vivo* (Table I). However, the magnitude of the reduction in transposase activity for the single amino acid changes was not as dramatic as for the triple K267A/N376A/D379A mutation, suggesting that GTP binding by the single mutant transposases is not reduced to the same extent as the triple mutant. The protein expression level for all the

Table I. Comparison of wild-type and mutant transposase activities

PTnp	Activity (% WT) ^a
WT	100
K267A/N376A/D379A	0.1 ± 0.1 (n = 3)
D379A	2.7 ± 1 (n = 2)
K377E	14 ± 6 (n = 6)
K267N	4.8 ± 7 (n = 4)
W266G/Q268S/V352D/D353V	0.0 ± 0 (n = 3)
W266G/Q268S	0.2 ± 0.3 (n = 2)
D379N-1	2.0 ± 0.4 (n = 4)
D379N-2	0.3 ± 0.2 (n = 2)
None	0.0 ± 0.0 (n = 30)

^aThe activity of wild-type transposase is set at 100% and is based on the ratio of Kan^R + Amp^R colonies/Amp^R colonies which controls for DNA recovery from the tissue culture cells (see Materials and methods). D379N-1 and -2 are independent isolates of the same mutation. The number of independent experiments is also indicated.

mutants assayed was equal to that of wild-type transposase, suggesting that loss of activity was not due to misfolding of the protein (data not shown). Taken together, these data demonstrate that GTP binding by P element transposase is a required and indispensable step during the pathway of P element transposition *in vivo*.

The transposase GTP-binding domain has a non-canonical structure

Since the P element transposase GTP-binding domain appears to constitute a separate or divergent member among the G protein classes, we were interested in testing the effects of introducing a more canonical GTP-binding core motif on transposase function *in vivo*. Four different amino acid substitutions (Trp266 to Gly, Gln268 to Ser, Val352 to Asp and Asp353 to Val, see Figure 1A) were introduced to make transposase match the conserved consensus motifs (Bourne *et al.*, 1990, 1991; Noel *et al.*, 1993; Sprinzl, 1994). Surprisingly, when tested in the transposition assay, the mutant W266G/Q268S/V352D/D353V transposase, containing an optimized GTP-binding pocket, lacked any detectable activity (Table I). Similar results were obtained with the transposase mutant W266G/Q268S (Table I). Both proteins were expressed at levels comparable with wild-type, so the loss of activity does not reflect protein instability and suggests that the protein is not globally misfolded. These observations suggest that the residues in the transposase GTP-binding pocket that deviate from the consensus are critical to obtain a functional catalytic center in transposase. Apparently, P element transposase possesses a novel type of GTP-binding domain that may be similar to the common GTP-binding motif in its interaction with the guanine moiety, but different in binding to the rest of the GTP molecule and Mg²⁺ ions (see Discussion).

Reactivation of a mutant transposase by manipulation of the intracellular environment

Taken together, the results above clearly establish the essential role of GTP binding in P element transposition *in vivo*. We then wanted to test whether it would be possible to change the nucleotide specificity for this regulatory function. Biochemical studies on the translation elongation factor-Tu (EF-Tu) (Hwang and Miller, 1987; Schimmel, 1993; Weijland and Parmeggiani, 1993) sug-

Fig. 3. Changing the purine nucleotide specificity for activation of P element transposition *in vivo*. (A) Autoradiograph of radiolabeled XTP bound to wild-type and mutant transposase proteins (upper part). The amount of XTP that bound to transposase D379N was set at 100%. The relative binding values for the other proteins are presented in the bar graph. As a positive control, the same procedure was performed in parallel for GTP (see bar chart). (B) Reactivation of nucleotide-switch mutants. Wild-type transposase, transposase K267A/N376A/D379A (KND) and two independently generated transposase D379N mutants were tested in the *in vivo* excision assay. The bar chart presents the effect of providing the cells with xanthosine or xanthine (see Materials and methods). (C) Immunoblot presenting that the level of transposase protein expression is unaffected by addition of xanthosine or xanthine. (D) Product analysis was performed as in Figure 2B for transposase D379N-1 activated with xanthosine, showing correct excision products.

gested that the base specificity of transposase could be changed from guanine to xanthine by substitution of a single key amino acid residue in subdomain G4, Asp379 into Asn (transposase D379N). An analogous substitution in EF-Tu generated a mutant protein with an affinity for xanthosine triphosphate (XTP) that is comparable with wild-type EF-Tu affinity for GTP, whereas GTP was no longer bound by the mutant protein (Hwang and Miller, 1987; Schimmel, 1993; Weijland and Parmeggiani, 1993). After generating the nucleotide-switch mutant transposase D379N, it was first tested for the ability to bind XTP or GTP. In contrast to wild-type transposase, no significant GTP binding could be detected by transposase D379N *in vitro* (Figure 2C). However, when both proteins were incubated with radiolabeled XTP, the opposite result was obtained: XTP was bound by the transposase mutant D379N but not by the wild-type protein (Figure 3A). The GTP-binding-defective mutant transposase (K267A/N376A/D379A) failed to bind either XTP or GTP in this assay (Figures 2C and 3A). These results indicate that by amino acid substitution D379N, we had successfully switched the nucleotide-binding specificity of transposase from GTP to XTP.

The catalytic activity of the mutant transposase D379N was then assayed *in vivo*. Since the intracellular levels of XTP are very low when compared with GTP (Kornberg and Baker, 1992), we did not expect a high transposase activity from this mutant protein. Indeed, transposase D379N exhibited a severely reduced ability to activate P element excision *in vivo* (Table I), even though it was

expressed at a level similar to wild-type transposase (data not shown, and see below: Figure 3C). To investigate whether the transposase mutant D379N would now respond to XTP instead of GTP, we took advantage of the low intracellular XTP levels and the permeability of cells to both nucleosides and free bases. We reasoned that the intracellular level of XTP might be increased by addition of exogenous xanthosine or xanthine to the culture media of the transfected cells. Strikingly, the excision activity of mutant D379N transposase was strongly stimulated (up to 50-fold) by the addition of either xanthosine or xanthine (Figure 3B). By contrast, neither the wild-type nor the GTP-binding-deficient transposase K267A/N376A/D379A was stimulated by the addition of either xanthosine or xanthine (Figure 3B). Restriction endonuclease analysis of the recovered plasmid DNAs showed that mutant transposase D379N generated correct excision products (Figure 3D). Moreover, the level of protein expression was not influenced by addition of either the nucleoside or the free base (Figure 3C). Consistent with the *in vitro* binding experiments, these results indicate that the specificity of the purine nucleotide cofactor requirement in the mutant transposase D379N has been switched from GTP to XTP *in vivo*. In the absence of xanthosine or xanthine, a low level of activity for transposase D379N was detected (Figure 2C and data not shown). Presumably, the switch mutant transposase responds to a low amount of endogenous XTP metabolized in the GTP synthesis pathway. Alternatively, at normal intracellular GTP concentrations the severely reduced GTP-binding affinity might still be

sufficient to use GTP for a very low number of transposition events. Previous studies indicated that micromolar concentrations of GTP were sufficient to activate P element transposase activity *in vitro* (Kaufman and Rio, 1992). Taken together, these results indicate that the nucleotide cofactor specificity of P element transposition can be altered *in vivo* and the mutant protein reactivated by the exogenous addition of the XTP precursors, xanthine and xanthosine.

Discussion

These studies demonstrate that P element transposase is a GTP-binding protein. The transposase nucleotide-binding region has several conserved sequence motifs known to be specifically required for GTP binding in other proteins, as well as unique aspects distinct from the classical G protein family or the tubulins (Figure 1A). Selective mutations in these motifs abolish GTP binding *in vitro* (Figure 2C) and, most importantly, eliminate transposase activity *in vivo* (Table I). Interestingly, by introducing a single amino acid change, D379N, the purine nucleotide specificity for binding *in vitro* and activation of transposase activity *in vivo* was switched from GTP to XTP. While this mutation had been shown to change the ligand of other G proteins *in vitro*, our results are the first demonstration that this nucleotide specificity switch can be carried out *in vivo*.

The D to N mutation in the NKXD motif of other G proteins has been used for a variety of studies. Originally, in the translation factor EF-Tu, the GTP–XTP switch was used to demonstrate that two XTP molecules were hydrolyzed by EF-Tu during translation (Hwang and Miller, 1987; Weijland and Parmeggiani, 1993). The GTP–XTP switch was also used to demonstrate the reciprocal GTPase activating effects of the *E. coli* Ffh and FtzY proteins which are involved in membrane protein targeting (Powers and Walter, 1995). This amino acid substitution can also switch the GTP to XTP specificity of *E. coli* adenylosuccinate synthetase (Kang *et al.*, 1994), the Ha-ras p21 oncoprotein (Zhong *et al.*, 1995; Schmidt *et al.*, 1996) and the Rab5 protein, which is involved in endocytic membrane fusion (Rybin *et al.*, 1996). In the cases of Ha-ras and Rab5, no effect was observed for the mutant D to N proteins on the interactions with their effector or target proteins. The mutant D119N Ha-ras protein was tested for activity *in vivo* and found to give an oncogenic phenotype, due to its high dissociation rate for GDP (Schmidt *et al.*, 1996). The activity of the mutant Ha-ras protein, unlike P element transposase, could not be affected *in vivo* by addition of exogenous xanthine (Schmidt *et al.*, 1996). This result may reflect differences in purine metabolism between mammalian and *Drosophila* cells or differences in the biochemical properties of Ha-ras and P element transposase *in vivo*.

We have shown here that small molecules such as xanthine or xanthosine, added extracellularly, can modulate transposase activity *in vivo*. We assume that uptake of xanthine and xanthosine by the cells leads to an intracellular accumulation of XTP. Plausible pathways for XTP formation could be phosphorylation of xanthosine or phosphoribosyl transfer to the xanthine moiety (Kornberg and Baker, 1992). Possibly, kinases or phosphoribosyl

transferases in the nucleotide synthesis *de novo* or salvage pathways will have reduced substrate specificity due to the unusually high concentrations of xanthine or xanthosine. It is highly unlikely that any xanthine-related nucleotide other than XTP would activate transposase D379N, since only guanosine triphosphate or its non-hydrolyzable analogs, but not the monophosphate or diphosphate forms, can stimulate wild-type transposition *in vitro* (Kaufman and Rio, 1992). In addition, the affinity of P element transposase for GTP or XTP appears to be much higher than for GDP or XDP since the radiolabeled nucleotide preparations used in Figure 3A contained a 20-fold molar excess of the unlabeled diphosphate forms. The ability of transposase to use XTP as a cofactor for transposition may be useful for future biochemical experiments. Previous studies have implicated the *Drosophila* Ku p70 homolog, IRBP, in P element transposition (Beall *et al.*, 1994; Beall and Rio, 1996). The mammalian Ku antigen possesses intrinsic DNA-dependent ATPase and DNA helicase activities (Cao *et al.*, 1994; Tuteja *et al.*, 1994). The ability to make transposase use XTP as a cofactor may be useful in dissecting the effects of ATP-dependent Ku activities versus GTP or XTP-dependent effects of transposase *in vitro*. This approach was critical for deducing the role(s) of GTP in the early steps of ribosome assembly and translation (Schimmel, 1993; Weijland and Parmeggiani, 1993).

P element transposase is the first GTP-binding protein shown here to be directly involved in a DNA rearrangement reaction (see also Kaufman and Rio, 1992). Other DNA transposition reactions, such as those of phage Mu or the bacterial Tn7 transposon, require ATP as a cofactor, whereas retroviral integration does not require an exogenous nucleotide cofactor (Mizuuchi, 1992; Craig, 1996). In these cases, the nucleotide functions in conjunction with an accessory protein (MuB or TnsC, respectively) to undergo target DNA interactions, but does not function as an energy source for phosphodiester bond formation, as is the case for DNA ligases (Kornberg and Baker, 1992). The role of GTP in P element transposition is unclear. It is not yet known if transposase possesses GTPase activity, although earlier studies showed that purified transposase co-fractionated with a DNA-dependent ATPase activity on glycerol gradients (P.D. Kaufman and D.C. Rio, unpublished results). Other studies have shown that non-hydrolyzable GTP analogs can substitute for GTP during *in vitro* transposition reactions (Kaufman and Rio, 1992). These observations suggest that GTP might function as a regulatory molecule rather than a hydrolyzed cofactor for the transposition reaction. Alternatively, GTP hydrolysis might play a role later in the reaction pathway, for instance, in disassembly and/or recycling of the transposition complex. ATP hydrolysis is required for turnover of type II DNA topoisomerases, such as *E. coli* DNA gyrase (Maxwell and Gellert, 1986; Kornberg and Baker, 1992).

The mutagenesis studies described here suggest that P element transposase has a non-canonical GTP-binding motif. Other proteins that contain atypical GTP-binding motifs have been described, such as the α -, β - and γ -tubulins (Sage *et al.*, 1995) and *E. coli* ftzZ (de Boer *et al.*, 1992; RayChaudhuri and Park, 1992). Comparison of the amino acid sequences of these proteins to transposase

did not reveal any similarity to transposase. In β -tubulin, which both binds and hydrolyzes GTP, there are not any of the conserved motifs found in the ras superfamily (Sage *et al.*, 1995). There does appear to be a glycine-rich motif (GGGTGSG), perhaps analogous to the G1 region of the ras family, that may be involved in phosphoryl binding. However, mutagenesis studies failed to identify any other regions in tubulin analogous to the G3 and G4 regions in the GTPase superfamily (Sage *et al.*, 1995). In P element transposase, it seems that the guanine-binding region G4 (NKSD; Figure 1A) is conserved within the ras-like GTP-binding protein superfamily. The G1 and G3 regions of transposase deviate from the ras superfamily (Figure 1A). Also, some of the flanking sequences are different from the superfamily in amino acid sequence or length. Our data indicate that mutations that change transposase to be ras-like abolish formation of correct transposition products *in vivo*. However, it should be pointed out that the guanine nucleotide binding sites in ras and EF-Tu are composed of several loop regions at the ends of regions of secondary structure and that, even though ras and EF-Tu are only 17% identical in amino acid sequence, they fold to give virtually superimposable three-dimensional structures (Wittinghofer and Pai, 1991). Thus, it is possible that, although quite different in amino acid sequence, the guanine nucleotide-binding region of transposase might bear some structural similarity to the ras-like GTPase superfamily. Taken together, our data indicate that P element transposase possesses a non-canonical GTP-binding domain, part of which (the guanine-binding region) appears to be like ras, but which contains other regions that are quite distinct.

The observations reported here suggest that P element transposition may be regulated by physiological GTP concentrations, which would have to be at a specific level to activate the catalytic functions of transposase. At this point, we do not know whether regulation of transposase might occur by fluctuations in overall GTP concentration during the cell cycle, changes in local concentration or changes in the accessibility of the nucleoprotein complex performing the transposition reaction. Previous genetic studies have suggested that P elements transpose predominantly during the G₂ phase of the cell cycle (Engels *et al.*, 1990). Unraveling the molecular mechanism of the potential coordination of transposition with progression through the cell cycle will be the subject of future study. Additionally, these findings show that a relatively minor alteration of primary protein structure can affect the regulation of an intricate cellular process. It is also possible that the function and regulation of other G protein activities, e.g. in intracellular signaling pathways (Bourne *et al.*, 1990, 1991; Noel *et al.*, 1993; Sprinzl, 1994) can be studied *in vivo* by manipulation of their genes as well as the intracellular environment through the addition of small molecules extracellularly.

Materials and methods

Purification of transposase

A stable *Drosophila* Schneider L2 cell line pUChyMT-Tnp was generated by transfection by calcium phosphate co-precipitation with plasmid pUChyMT-Tnp for 24 h followed by selection with 200 μ g/ml hygromycinB (Rio and Rubin, 1985). At 20–22 h after induction of the

metallothionein promoter with 0.7 mM CuSO₄, the cells were washed with phosphate-buffered saline (PBS) and incubated for 30 min on ice in 50 mM Tris-HCl, pH 7.5, 200 mM LiCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP40, 0.1 mM DTT, 0.2 mM PMSF, 5 mM Na₂S₂O₅ and other protease inhibitors, followed by centrifugation at 100 000 g at 4°C. P element transposase was immunoaffinity-purified to 60–70% purity by incubation of the extract for 3 h at 4°C with the monoclonal antibody PG4 coupled to protein G-Sepharose. PG4 is a monoclonal antibody raised against the C-terminal 15 amino acids of transposase (P.D.Kaufman, K.Rusilas, S.Misra and D.C.Rio, unpublished results). The resin was washed with 6–10 $\times$ 10³ bead volumes of 20 mM HEPES-KOH, pH 7.6, 1 M LiCl, 0.5% NP40, 100 mM NaCl and eluted with 20 mM HEPES-KOH, pH 7.6, 100 mM NaCl, 0.1 mM DTT, 16% glycerol, 0.05% NP40, 3.5 M MgCl₂. A 'mock' purification was performed with cell extracts from untransformed Schneider L2 cells, which do not express P element transposase.

GTP binding

Approximately 1.8 μ g transposase (21 pmol), an equivalent amount of protein from the mock preparation and 225 ng SAR1p (9 pmol) were applied to nitrocellulose using the Bio-Rad dot-blot chamber and bound for 3 h at 4°C followed by washing of the wells several times with 20 mM HEPES-KOH, pH 7.6, 20% glycerol, 100 mM NaCl, 1 mM DTT, 0.2 mM PMSF and overnight incubation at 4°C. The filter was removed from the chamber and processed as described (Wagner *et al.*, 1992). Incubation with [α -³²P]GTP (4 μ Ci/ml, ~3000 Ci/mmol) in the presence of 4 μ M ATP was for 2 h at room temperature. The filter was then exposed to X-ray film and quantitated by phosphorimaging.

Plasmids

Plasmid pBSKS(+)-Pac-Tnp was derived from pBSKS(+) (Stratagene), the 2.6 kb actin 5C fragment from pPac (Ashburner, 1989), and the transposase cDNA with 25% of the N-terminus chemically resynthesized to alter the codon usage to the most frequently occurring codons in *Drosophila* (Lee *et al.*, 1996). pUChyMT-Tnp was derived from the same transposase cDNA and pUChyMT (a gift from C.Thummel, University of Utah). pISP-2/Km contains a 0.6 kb non-autonomous P element derived from pISP-2 (Rio *et al.*, 1986) and the kanamycin resistance fragment from plasmid pKm109-9 (Reiss *et al.*, 1984).

P element excision assay

Similar assays for P element excision from extrachromosomal plasmid substrates have been described previously (Rio *et al.*, 1986; Beall and Rio, 1996). *Drosophila* Schneider L2 cells were transfected by calcium phosphate co-precipitation with pBSKS(+)-Pac-Tnp for expression of transposase under control of the *Drosophila* actin 5C promoter and with pISP-2/Km as a reporter plasmid. To recover plasmid DNA from the Schneider L2 cells 24 h after transfection, the cells were harvested, washed four times with PBS and incubated in lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 0.6 % SDS) for 5 min at room temperature. Chromosomal DNA was precipitated with one-fourth volume 5 M NaCl overnight at 4°C. The plasmid DNA in the supernatant was extracted with phenol and chloroform, ethanol-precipitated, resuspended in water, electroporated into *E.coli* strain AG1574 and selected for Kan resistance by plating on selective media. The excision frequency was calculated as $[N_{\text{Amp}} + N_{\text{Kan}}]/N_{\text{Amp}}$ in which $N_{\text{Amp}} + N_{\text{Kan}}$ and N_{Amp} represent the number of bacterial colonies resistant to both Kan and ampicillin (Amp) or to Amp, respectively. Thus, the results are corrected for the recovery of the plasmid DNA from the L2 cells. The wild-type transposase activity for each individual assay is set at 100%, which equals a frequency of $38 \pm 6.5 \times 10^{-6}$. For the various transposase mutants, activity is given as the percentage of wild-type activity. Typically, the number of Amp^R colonies was $83 \pm 31 \times 10^6$ per 10⁶ L2 cells, and the amount of DNA recovered from 2–4 $\times$ 10⁶ cells was analyzed.

XTP binding and activation

Transposase proteins were tested for XTP binding as described above for GTP, except here the filter was incubated with [γ -³²P]XTP (4 μ Ci/ml, ~3000 Ci/mmol), prepared as described (Walseth and Johnson, 1979), in the presence of 1 μ M unlabeled XDP for 8 h at 4°C. In the excision assay, the cells were provided with culture media either with or without (–) 100 μ M xanthosine or xanthine 4–5 h after transfection. P element excision was analyzed as described above. The activity of the various transposases tested in the absence of xanthosine or xanthine (–) is set as 1.0 in the bar chart (Figure 3B). In this case, the relative enzymatic activities were 100%, 1.8%, 0.45% and 0.1 % for the wild-type, D379-1,

D379-2 and KND transposase proteins, respectively. From these basal levels in the absence of xanthosine or xanthine, activation of excision activity by the addition of exogenous xanthosine or xanthine is presented as fold stimulation. In the case of wild-type transposase, we observed a reduction in activity in the presence of xanthosine or xanthine so that the stimulatory effect was calculated to be 0.80 and 0.54, respectively. By contrast, both compounds could efficiently activate the transposase mutant D379N, as indicated. When the mutant activities were compared with the wild-type activity in the presence of the additives, xanthosine could activate up to 41% of the wild-type activity and xanthine up to 43%. At 24 h after transfection with the reporter, either in the absence (–) or presence of a vector expressing either wild-type transposase or the D379N-1 mutant, a portion of the cells harvested for DNA analysis were washed with phosphate-buffered saline and subjected to SDS–polyacrylamide gel electrophoresis and immunoblotting.

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