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 protein 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...
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 Mg\(^{2+}\) 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\(^{R}\)Kan\(^{S}\) 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
Reactivation of mutant P element transposase

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

<table>
<thead>
<tr>
<th>PTnp</th>
<th>Activity (% WT) a</th>
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<tbody>
<tr>
<td>WT</td>
<td>100</td>
</tr>
<tr>
<td>K267A/N376A/D379A</td>
<td>0.1 ± 0.1 (n = 3)</td>
</tr>
<tr>
<td>D379A</td>
<td>2.7 ± 1 (n = 2)</td>
</tr>
<tr>
<td>K377E</td>
<td>14 ± 6 (n = 6)</td>
</tr>
<tr>
<td>K267N</td>
<td>14.8 ± 7 (n = 4)</td>
</tr>
<tr>
<td>W266G/Q268S/V352D/D353V</td>
<td>0.0 ± 0 (n = 3)</td>
</tr>
<tr>
<td>W266G/Q268S</td>
<td>0.2 ± 0.3 (n = 2)</td>
</tr>
<tr>
<td>D379N-1</td>
<td>0.0 ± 0.4 (n = 4)</td>
</tr>
<tr>
<td>D379N-2</td>
<td>0.3 ± 0.2 (n = 2)</td>
</tr>
<tr>
<td>None</td>
<td>0.0 ± 0.0 (n = 30)</td>
</tr>
</tbody>
</table>

* 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.

None of the 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 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 transposase 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 protein for GTP (Table I). These substitutions were single

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. 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 transformed L2 cell lines and tested for GTP binding as described in Figure 1. The amount of radiolabeled GTP comparable with wild-type, so the 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.
suggested 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 1), 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 1). 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 Haras p21 oncprotein (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 Haras 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 Haras 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 Haras 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 Haras 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 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; Tutela 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- 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 transposes 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 pUChygMT-Tnp was generated by transfection by calcium phosphate co-precipitation with plasmid pUChygMT-Tnp for 24 h following by selection with 200 µg/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 10×103 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, 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 μCi/ml, ~3000 Ci/mmol) in the presence of 4 μM 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 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., 1993). pUChygMT-Tnp was derived from the same transposase cDNA and pUChygMT (a gift from C.Thummel, University of Utah). pISP-2.Kan contains a 0.6 kb non-autonomous P element derived from plasmid 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.Kan 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 (20 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 of 5 M NaCl overnight at 4°C. The plasmid DNA in the supernatant was extracted with phenol and chloroform, ether precipitated, resuspended in water, electrophoresed into E.coli strain AG1574 and selected for Kan resistance by plating on selective media. The excision frequency was calculated as [N Amp + Kan]/N Ampml in which N Amp + 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 ± 6.5×10−6. For the various transposase mutants, activity is given as the percentage of wild-type activity. Typically, the number of AmpR colonies was 33 ± 31×104 per 106 L2 cells, and the amount of DNA recovered from 2–4×105 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 μCi/ml, ~3000 Ci/mmol), prepared as described (Walseth and Johnson, 1979), in the presence of 1 µM unlabeled XDP for 8 h at 4°C. In the excision assay, the cells were provided with culture media either with or without (+) 100 µM xanthosine or xantine 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 xantine (−) 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 D379–5 transposase, respectively.
References


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