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In Vitro Targeting of Strand Transfer by the Ty3 Retroelement Integrase[*][S]

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Background: Retroviruses and retrotransposons integrate with poorly understood preferences.
Results: Depending on *in vitro* reaction conditions, Ty3 retroelement integrase was targeted by a transcription factor or by Ty3 inverted-repeat terminal sequence.
Conclusion: Retroelement targeting can be reproduced in a defined system.
Significance: Biochemical models will enable improved understanding of retrovirus integration preferences and gene therapy vector safety.

The *Saccharomyces cerevisiae* long terminal repeat retrotransposon Ty3 integrates within one or two nucleotides of the transcription initiation sites of genes transcribed by RNA polymerase III. In this study the minimal components required to re-constitute position-specific strand transfer by Ty3 integrase are defined. Ty3 integrase targeted by a synthetic fusion of RNA polymerase III transcription factor IIIB subunits, Brf1 and TBP, mediated position-specific strand transfer of duplex oligonucleotides representing the ends of the Ty3 cDNA. These results further delimit the TFIIIB domains targeted by the Ty3 element and show that IN is the Ty3 component sufficient *in vitro* to target integration. These results underscore the commonality of protein interactions that mediate transcription and retrotransposon targeting. Surprisingly, in the presence of $MnCl_2$, strand transfer was TFIIIB-independent and targeted sequences resembling the Ty3 terminal inverted repeat.

Retroviruses display preferential patterns of integration in eukaryotic genomes, reflecting influences of host transcription factors and effects of chromatin components and DNA modification, sequence, and structure on activity of the preintegration complex of integrase (IN)[2] and cDNA known as the intasome (1–3). Understanding these integration biases, particularly in the case of retroviruses, is complicated both by the complexity of the target and the animal genomes themselves. For example, although it is known that lens epithelium-derived growth factor (LEDGF) is required for efficient integration of HIV-1 (4–5) and that the interaction between IN and LEDGF

maps to the C-terminal end of the catalytic core domain (6), mechanistic details of how LEDGF tethers the intasome to the target DNA remain elusive.

The relatively subtle integration preferences of retroviruses contrast with the striking preferences of some retrotransposons in lower eukaryotes and plants (7–9). For example, in *Saccharomyces cerevisiae*, the copia-like LTR retrotransposon Ty5 is targeted to heterochromatic DNA by interactions between the IN C-terminal domain and the Sir4 silencing protein (10–11) and copia-like Ty1 and gypsy-like Ty3 LTR retrotransposons target the 5′-flanking regions of Pol III-transcribed genes (12–13). In *Dictystelium discoideum*, the non-LTR retrotransposon TRE5 targets 5′-flanking regions of tRNA genes and the TRE5 ORF1 protein interacts with components of the Pol III transcription factor TFIIIB (14). The *Schizosaccharomyces pombe* gypsy-like Tf1 interacts with a subset of transcription factors to target RNA Pol II promoters (15).

Ty3 is distinguished by the precision of its integration within a few bases of Pol III TSS (13). However, despite this unusual insertion specificity, Ty3 has substantial structural and functional similarity to retroviruses (16). For example, cells expressing Ty3 accumulate VLPs containing processed Ty3 proteins and cDNA and the Ty3 IN has a conserved core domain that contains residues conserved among retroviral integrases, including the D, $DX_{35}E$ catalytic motif of polynucleotide esterases. Although the amino- and carboxyl-terminal domains of IN proteins are generally less-well conserved, they contain a zinc finger and a GPY/F motif, respectively. These motifs are also found in the Ty3 IN protein (17). Similar to retroviral cDNA, the Ty3 cDNA has LTRs and terminates with two "extra" bp at each end, which are endonucleolytically removed from the 3′-ends by Ty3 IN prior to strand transfer (18). Based on the retrovirus model, the resulting 3′-hydroxyls mediate S_N2 nucleophilic attacks at staggered positions in the duplex chromosomal DNA. These positions are offset by 5 nts so that concerted strand transfer generates the characteristic 5-bp direct repeats flanking the ends of Ty3 insertions. These similarities to retroviruses coupled with precise targeting make Ty3 an attractive model for probing the mechanisms by which targeting proteins might interact with the retroelement intasome. However,

a biochemically defined *in vitro* system that recapitulates the natural specificity of any retroelement including Ty3 has been lacking. We describe such a system here and use it to investigate Ty3 substrate and target sequences that influence integration.

EXPERIMENTAL PROCEDURES

Recombinant DNA Constructions—Plasmids were constructed using standard molecular biology procedures (19) unless otherwise noted. Details of plasmid constructions, plasmids and sequences of oligonucleotides used for constructions are provided in supplemental Experimental Procedures and supplemental Tables S1 and S2, respectively. Constructs were verified by DNA sequence analysis (Genewiz Inc., La Jolla, CA).

Proteins—*S. cerevisiae* strain BY4741 was induced to express galactose-regulated Ty3 from pDLC201 (20), and VLPs were harvested as previously described (21). Triple fusion protein ($Brf1_{1-382}$-TBP_{61-240}-$Brf1_{439-596}$, TFP) was expressed in bacterial strain Rosetta (DE3) pLysS (EMD Biosciences. San Diego, CA) and was purified essentially as described (22).

Recoded Ty3 IN (23) was cloned to allow expression of a C-terminal His_6-tagged protein under control of the *lac* promoter (pKN2412). Expression was induced in Rosetta (DE3) pLysS according to standard procedures. Extracts were enriched for IN by affinity chromatography using His60 Ni Superflow. IN was further purified using anion exchange chromatography over DEAE Sephadex A-25. Details of protein purifications are provided in supplemental Experimental Procedures.

In Vitro Integration and Strand-Transfer Assays—*In vitro* integration using VLPs was performed as described previously (24). Either TFIIIB or TFP were mixed with target plasmids on ice for 30 min before VLPs were added, and samples were incubated at 16 °C for 15 min. Strand-transfer reactions were performed in buffer R (20 mM HEPES pH 7.5, 70 mM NaCl, 0.1% Nonidet P-40, 7.5% DMSO, 5 mM DTT) supplemented with $MgCl_2$ or $MnCl_2$ cofactors. Generally, samples contained 50 fmol of target plasmid, 250 fmol of duplex DNA, 250 fmol of TFP, and 1000 fmol of IN in a total volume of 40 μl. Reactions were incubated at 24 °C for 1 h, and DNA products were extracted as described previously (24).

PCR was performed to amplify fragments diagnostic of strand transfer. For VLP integrations, one tenth of the DNA products were combined with primers 242 and 411, which anneal within the *SNR6* gene and at the downstream end of the internal domain of Ty3, respectively (25). In the PCR reactions monitoring strand-transfer products of duplex DNA substrates, primer HH1707, which anneals at the first half of the DNA substrates, was substituted for primer 411. Control PCR reactions amplified a segment of the target plasmid. Products were resolved by electrophoresis on non-denaturing 8% polyacrylamide gel or 1.5% agarose gel and visualized by staining with ethidium bromide. To determine strand-transfer sites, DNA fragments were extracted from the gel, cloned into pCR2.1 and sequenced.

Protein DNA Binding Assay—A 57-bp ^{32}P-labeled TATA-containing DNA probe was labeled, and EMSA was performed as described previously (22).

RESULTS AND DISCUSSION

A Synthetic Brf1 and TBP Fusion Protein Supports Ty3 Position-specific Integration—*SNR6* is transcribed by Pol III, but is distinguished in yeast from some other Pol III templates by the presence of an upstream TATA box. TFIIIB composed of TBP, Bdp1, and Brf1 functions to dock Pol III and enhance duplex opening at the position of transcription initiation (26). *In vitro* TFIIIB binds DNA via interactions between TBP and the *SNR6* TATA element and these interactions are sufficient to support TFIIIC-independent transcription initiation (27–28). On a template containing heteroduplex DNA at the transcription initiation site, Brf1 and TBP alone are sufficient to support transcription initiation (29). Function of TFIIIB subunits Brf1 and TBP in transcription initiation can be substituted by a structure-based fusion of the conserved domain of TBP flanked by segments of Brf1 ($Brf1_{1-382}$-TBP_{61-240}-$Brf1_{439-596}$) referred to as TFP (22).

A particulate fraction containing Ty3 VLPs isolated from yeast extracts by sucrose gradient centrifugation can provide active IN and substrate cDNA (30). Ty3 VLP-mediated cDNA strand transfer differs from Pol III transcription initiation in that it can be targeted by Brf1 and TBP without introduction of heteroduplex DNA at the TSS (24). However, the requirement of Ty3 for TFIIIB and TFIIIC for integration at most tRNAs and for TFIIIB or even Brf1 and TBP at *SNR6* complicates identification of interactions key to targeting. In order to better define the activities required for Ty3 strand-transfer targeting, we examined whether TFP could replace TBP and Brf1 as was found for Pol III transcription. The TATA box upstream of *SNR6* can bind TBP in either orientation and thus mediate bidirectional transcription initiation at upstream (*SNR6* distal) and downstream (*SNR6* proximal) sites (31). A related variant on plasmid pLY1855 supports Ty3 integration at both initiation sites (25). This target plasmid was combined with bacterially-expressed TFIIIB (TBP, Brf1, and Bdp1) or TFP and Ty3 VLPs were added as the source of integration activity and cDNA (25). Strand transfer was assayed using a PCR primed by cDNA- and target plasmid-specific oligonucleotides (Fig. 1A). Products consistent with TFIIIB bound to the TATA box in each orientation were observed in positive control reactions containing TFIIIB and VLPs (Fig. 1B, *lane 1*) (25) and in a test reaction containing TFP (Fig. 1B, *lane 2*), but not in reactions containing only VLPs (Fig. 1B, *lane 3*). Therefore, non-conserved TBP residues 1–60 and Brf1 region 383–438, which contains HR I and the HRI-II spacer, both of which are lacking in TFP, are dispensable for targeting Ty3 strand transfer to Pol III TSS.

Recombinant IN Is Sufficient to Mediate Position-specific Integration—A remaining major limitation in defining the Ty3 components required for targeting was the requirement for a complex VLP fraction as the source of both IN and cDNA. Although it might be anticipated that IN would directly mediate specificity, recent findings in the retrovirus system indicate that domains within some retroviral Gag proteins have the capacity to influence integration patterns (2). A system was therefore developed in which recombinant IN and duplex oligonucleotides were substituted for VLPs. These strategies were previously used to reconstitute the retroviral strand-transfer
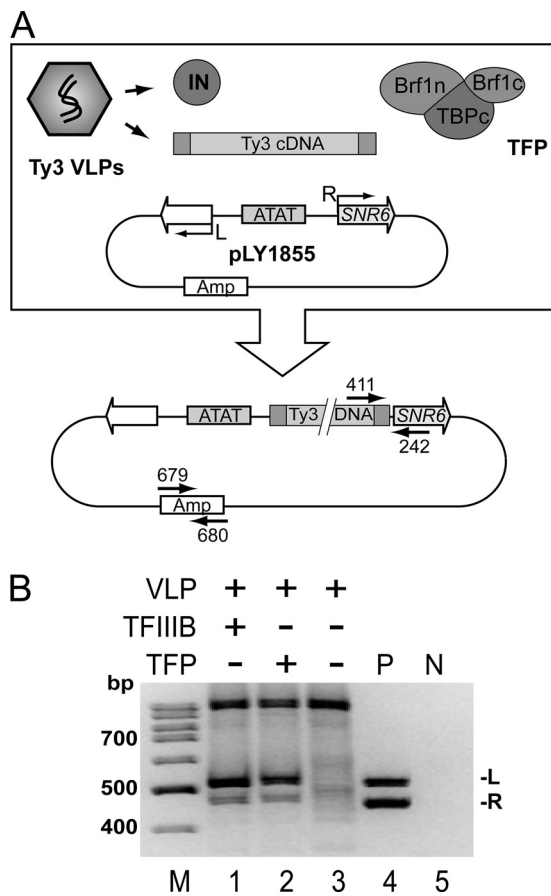
FIGURE 1. **Identification of minimal *in vitro* targeted strand transfer components.** *A, in vitro* strand-transfer assay. Reactions contained VLPs as a source of Ty3 cDNA and IN and TFIIIB or TFP (Brf1$_{1–382}$-TBP$_{61–240}$-Brf1$_{439–596}$). A functionally symmetric TATA box (ATAT) binds TBP in either orientation allowing bidirectional transcription initiation (*L* and *R* arrows). Strand-transfer products were detected in PCR primed from within Ty3 and *SNR6* (primers 411 and 242, respectively). DNA recovery was monitored with PCR using primers annealed to the backbone of the target plasmid (primers 679 and 680). *B,* TFP substitutes for TFIIIB in targeting Ty3 strand transfer. Strand-transfer reactions with test reactants (*lanes 1–3*), positive control (*P*) plasmids with a Ty3 LTR at leftward (pLY1842) and rightward TSS (pDLC370) (*lane 4*), or negative control (*N*) containing water alone (*lane 5*) were used to template PCR using primers 411 and 242. PCR products were separated on a nondenaturing 8% polyacrylamide gel. Products amplified from leftward (*L*) and rightward (*R*) TSS are indicated.

reaction (32), although the greater size of the Ty3 IN complicated direct adoption of those protocols. The portion of *POL3* encoding the 61-kDa Ty3 IN was tagged with 6× His and recoded for bacterial expression (23). Wt IN and a catalytic site mutant (D225E/E261D) derivative (18) were expressed in *Escherichia coli*. These recombinant IN proteins were purified by nickel affinity chromatography. A duplex oligonucleotide containing 23 nt with complementarity to a PCR primer followed by 20 nt representing the downstream (U5) end of the unprocessed Ty3 LTR and a non-transferred, complementary strand of 45 nt were introduced into the *in vitro* strand-transfer reaction to substitute for unprocessed VLP cDNA. An identical substrate lacking two nts from the U5 3′-end ("pre-processed") was also tested (Fig. 2*A*). IN, duplex pre-processed substrates, *SNR6* DNA and TFP were combined in the strand-transfer reaction. Products of this reaction were used to template PCR primed with oligonucleotides complementary to the substrate

and plasmid target. The reactions including wt IN generated fragments of the size expected for Ty3 strand transfer at the divergent TSS (Fig. 2*B*, *lane 4*; Fig. 2*C*, *lanes 1* and *2*); the D22E/E261D mutant IN failed to generate these products (data not shown). Sequence analysis of four independent reactions identified eleven distinct joints of targeted strand transfers. The majority of joints were distributed within one or two nt of the TSS on the template strand or offset upstream by five nt on the nontemplate strand (Fig. 2*D*). A similar amount of product was generated in reactions using unprocessed duplexes (Fig. 2*C*, compare *lanes 1* and *2*). In addition, sequence analysis of strand-transfer products of the blunt substrate showed that the junction occurred at the terminal CA, so that strand transfer was preceded by removal of two nt from the 3′-end of the duplex (data not shown). These assays demonstrated for the first time that Ty3 IN is the sole Ty3 protein required to process 3′ extra nts and target strand transfer to the Pol III transcription initiation site.

Ty3 IN Strand Transfer Is Sensitive to Mutations in the cDNA Inverted Repeat Dinucleotide—Terminal IR are a signature feature of integrated transposons and retroviruses with TG/CA being virtually universally conserved. Upstream of the conserved dinucleotide the two ends can have distinct sequences and *in vitro* evolution of IN substrates has shown that additional variation is possible in the absence of requirements for replication (33). As discussed above, in the cDNA the IR copies are flanked on the outside ends by 2 "extra" bp which are removed during integration. Ty3 has a terminal 8-bp IR and 2 extra bp (plus strand, 5′-gaTGTTGTAT-3′ . . . ATACAA-CAcc-3′). U5 oligonucleotide substrates substituted in the outside ends of the IR (CA, wt; **T**A, C**G**, **TG,** mutants) and a duplex oligonucleotide in which the terminal Ty3 sequence was randomized, were assayed for strand transfer (Fig. 2*C*). This assay showed little difference in activity among reactions using blunt or pre-processed substrates with IR sequences ending in wt CA or mutant TA (Fig. 2*C*, *lanes 1–4*). Strand-transfer products were not generated from the randomized oligonucleotide substrate (Fig. 2*C*, *lane 9*). Significantly less strand transfer was observed for processed and blunt substrates with IR ending in G, rather than wt A (Fig. 2*C*, *lanes 5–8*). In addition, among the latter templates, more strand transfer was observed for preprocessed substrates indicating that processing was sensitive to mutations of the terminal "A" (Fig. 2*C*, *lanes 5–8*). In the case of *in vitro* relative rate assays of HIV-1 and Ty1 and Tf1 retrotransposon IN proteins, 3′-end processing of the two extra nt was blocked by mutations in the IR terminal "A" and was greatly reduced by changes in the conserved penultimate IR "C" (34) for Ty1 (35) and Tf1 (36). Although these strand-transfer assays combined with PCR detection are unlikely to be as sensitive to perturbation as real time enzymatic assays, they showed that Ty3 IN activity is sensitive to changes in the terminal IR.

TFP Mediates IN Association with Target DNA—Retroviral IN proteins display robust *in vitro* strand-transfer activity in the absence of host targeting factors. In the case of Ty3 IN, strand-transfer assays did not show evidence of a default nonspecific pathway. Nonetheless, this activity would yield more diffuse products in our assay and therefore be more difficult to detect than specific strand transfer. Therefore, the ability of IN to
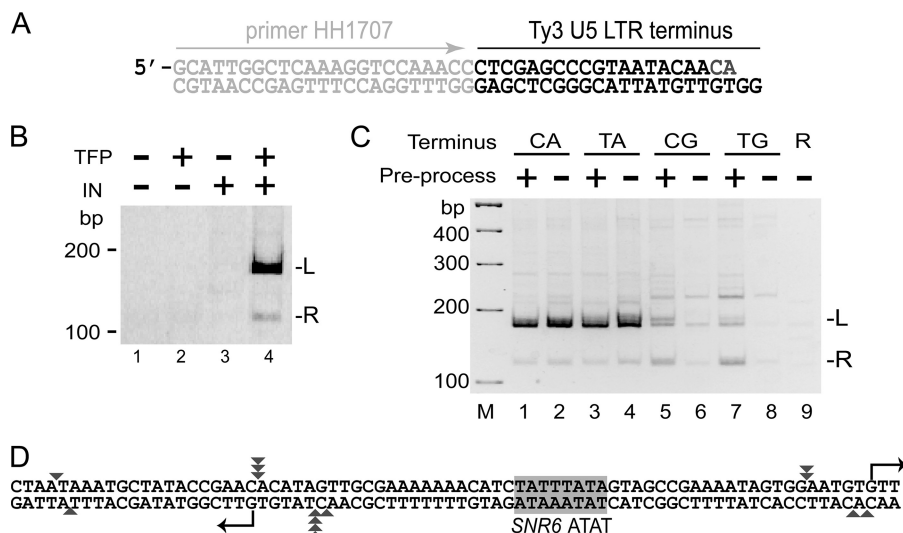
FIGURE 2. **Recombinant Ty3 IN mediates targeted strand transfer with duplex oligonucleotide substrates.** *A*, duplex oligonucleotide substrate. The duplex oligonucleotide substrate for strand transfer: left end (*light gray*): 23 bp sequence complementary to PCR primer HH1707; right end (*black*): 20/22 nt sequence of Ty3 LTR U5 terminus lacking the two 3′-nt removed by processing (preprocessed). *B*, recombinant IN mediates strand transfer of duplex Ty3 LTR U5 substrate. Duplex preprocessed oligonucleotide substrate (*A*) was incubated in the absence and presence of TFP and recombinant IN as described under "Experimental Procedures." PCR was performed on the products using primers HH1707 and 242 and products were fractionated as described in the Fig. 1 legend. *C*, recombinant IN mediates strand transfer of unprocessed and preprocessed substrates and is sensitive to substrate sequence. Strand-transfer assays were as in *B* except that substrates were either preprocessed (+) or blunt-end (−) and the terminal 2 nt of the Ty3 IR (5′-CA-3′) were varied as shown. *R*, DNA substrate in which random 22 nt replaced Ty3 U5 sequence (oligonucleotides HH1798 and HH1799). *D*, target sites of strand transfer. Products of four strand-transfer reactions (*B*, lane 4) were cloned and sequenced. Strand-transfer joints between Ty3 U5 and target sequence are shown (*triangles*). *Multiple triangles* at the identical site in all three cases represent two independent reactions. Eleven independent sites were identified.

interact with target DNA was reinvestigated using a more direct assay. A 57-bp duplex oligonucleotide DNA containing the *SNR6* TATA element was used to represent the target DNA. An identical duplex was previously used to measure binding of TFP specifically to TATA-containing DNA (22). Over a range of IN concentrations, no interaction between IN and the target DNA was observed (Fig. 3, *left panel*). In contrast, as reported previously, addition of TFP alone retarded mobility of the *SNR6* target duplex (22). In the presence of TFP, supershifting of the TATA-containing duplex was proportional to the amount of IN (Fig. 3, *middle panel*). However, this interaction was weak for both wt IN and a catalytic site mutant (data not shown). Overall, these results support a model in which the Ty3 intasome interactions with Pol III promoters is mediated by direct interaction of Ty3 IN with Brf1 and TBP components of TFIIIB. This model is similar to what has been proposed for targeting of Ty5 (37) and Tf1 (15) integration by IN tethering to target-bound proteins.

In the Presence of MnCl₂, Strand Transfer Is TFP-independent and Sequence Specific—*In vitro* substitution of the natural $MgCl_2$ metal cofactor with $MnCl_2$ in the case of HIV-1 IN reduces specificity for cDNA termini (34) and enhances activity in disintegration assays (38). To test the effect of $MnCl_2$ on the association of Ty3 IN with its target, $MgCl_2$ was either supplemented or substituted with $MnCl_2$ in the strand-transfer reactions. PCR analysis of products of $MnCl_2$-containing reactions showed surprisingly that strand transfer was dependent upon IN, but independent of TFP (Fig. 4*A*). Strand transfer was not observed for the randomized oligonucleotide substrate in the presence of $MnCl_2$, indicating that it required specific interactions with IN (data not shown). Reactions containing VLPs showed only a low level of non-targeted products in the pres-
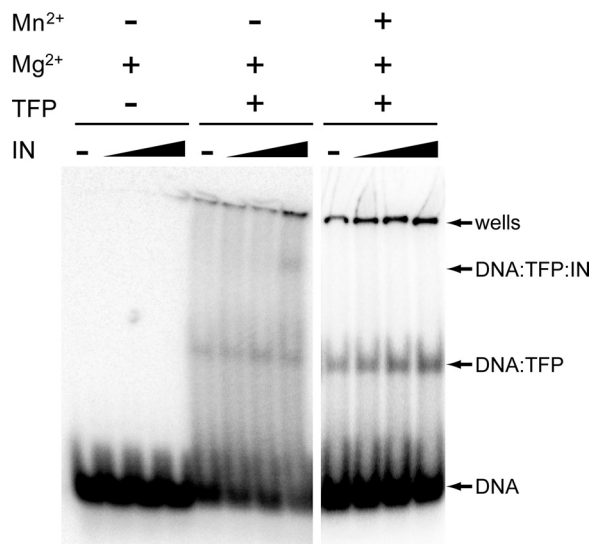

FIGURE 3. **Ty3 IN interacts with target DNA via interactions mediated by TFP.** Protein-^{32}P-labeled DNA complexes were formed with 0.3 nM 57-bp TATA box-containing probe (see "Experimental Procedures": EMSA), 12.5 nM TFP (*middle* and *right panels*), and increasing amounts of IN (0, 12.5, 25, 50 nM), and were analyzed on a 4% native polyacrylamide gel. Binding buffers contained 7 mM MgCl₂ (*all panels*), and MnCl₂-containing reactions were 10 mM (*right panel*). IN was titrated in each series (− and wedge).

ence of $MnCl_2$ (supplemental Fig. S1). In the presence of $MnCl_2$, TFP shifted the TATA-containing probe indicating that $MnCl_2$ does not produce TFP-independent strand transfer by disruption of TFP binding (Fig. 3, *right panel*). However, the IN supershift was no longer observed, suggesting that the presence of $MnCl_2$ affected the interaction between TFP and IN.

The PCR amplicon from products generated in the presence of $MnCl_2$ concentrations greater than 10 mM in the presence or
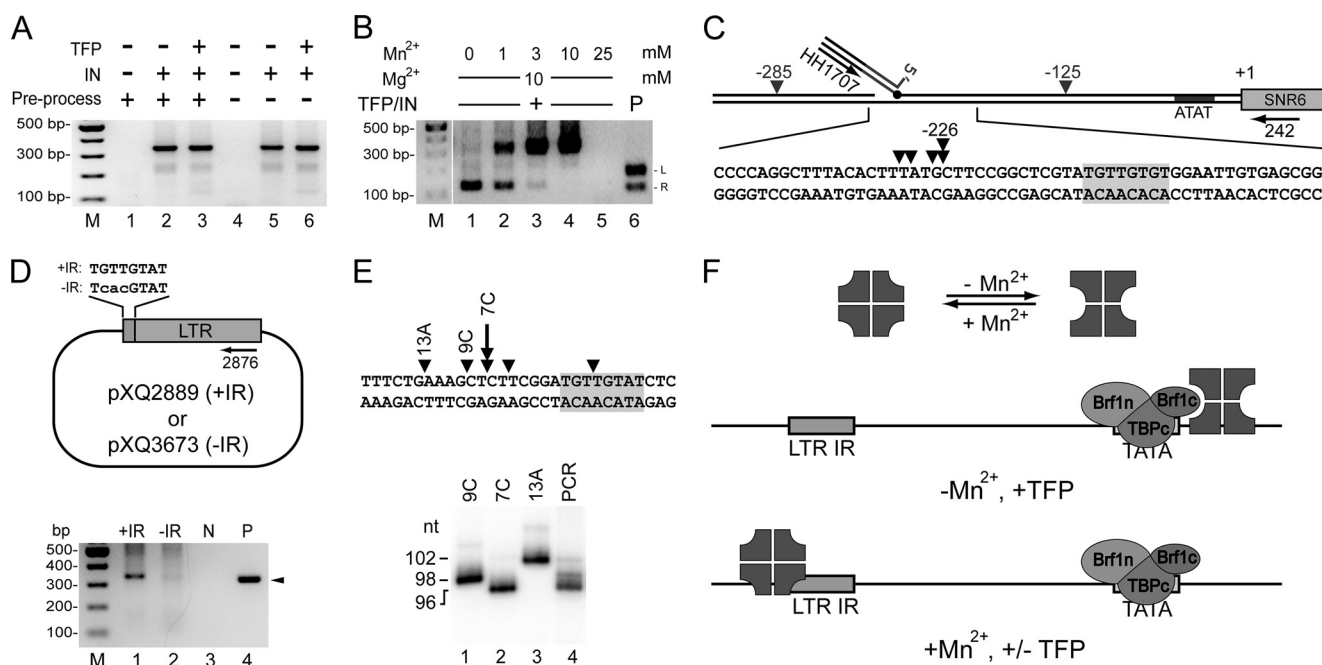
FIGURE 4. **Ty3 IN mediates sequence-specific targeting in the presence of MnCl$_2$.** *A*, Ty3 IN strand-transfer in the presence of MnCl$_2$ is TFP independent. Strand transfer reactions and analysis were performed as described in the Fig. 2 legend except that 10 mM MnCl$_2$ replaced MgCl$_2$ and PCR products were resolved on a 1.5% agarose gel. *B*, appearance of novel strand-transfer product is dependent upon MnCl$_2$. Reactions as described in *A* performed in the presence of 10 mM MgCl$_2$ and MnCl$_2$ concentrations from 0 to 25 mM (*lanes 1–5*). Plasmids pXQ3659 and pXQ3660 were used as control for strand-transfer positions under MgCl$_2$-containing conditions (*lane 6*). PCR products were analyzed as in *A*. *C*, MnCl$_2$-dependent strand transfer targets vicinity of sequences similar to Ty3 IR. PCR reactions were performed as described above (*A, lane 3*), and products were sequenced and labeled in diagram as described in the Fig. 2 legend. Target plasmid Ty3 IR (5′-TGTTGTAT-3′/5′-ATACAACA-3′)-like sequence, *shaded box*. *D*, MnCl$_2$-dependent strand-transfer targets Ty3 IR. *Upper panel*, plasmids containing Ty3 LTR lacking the downstream inverted repeat and wt (+IR) (pXQ2889) or mutated (−IR) (pXQ3673) IR were tested for targeting activity. *Lower panel*, reaction conditions and assay were as described above (*A, lane 3*) with pre-processed Ty3 U5 duplex oligonucleotide substrate. Insertions were analyzed using the same forward primer (HH1707) and a reverse primer (XQ2876) annealed downstream of the Ty3 IR. Control PCR used pXQ3661 containing a Ty3 U5 end upstream of the IR sequence as template (*P*). DNA recovery was monitored by PCR using primers XQ2876 and XQ2877 (data not shown). PCR products were analyzed as in *A*. *E*, strand transfer is imprecise and proximal to the outside end of the Ty3 IR. PCR amplicons (*D, lane 1*) were cloned and sequenced allowing identification of products with strand transfers at positions −7(C), −9(C), and −13(A). These clones (*lanes 9C, 7C,* and *13A*) were used to template ^{32}P-labeling PCR using primers XQ2876/XQ3135. Products were resolved on a 10% denaturing polyacrylamide gel. *F*, model for differential sequence or transcription complex targeting by Ty3 IN. Ty3 IN adopts different states in the presence or absence of MnCl$_2$ (*top*). In the absence of MnCl$_2$, TFP recruits IN (*middle*). IN conformation in MnCl$_2$ prevents interaction with TFP and allows association of IN subunits not interacting with substrate to interact with target (*bottom*).

absence of TFP was ~300 bp (Fig. 4, *A* and *B*). Experiments were performed in which MnCl$_2$ or MgCl$_2$ was increased in the absence or presence of the other metal cation and the products were amplified using PCR (Fig. 4*B* and data not shown). In high MgCl$_2$ and low MnCl$_2$ bands representing products of strand transfers flanking the TFP binding site were observed as previously described. However increasing MnCl$_2$ correlated with increasing amounts of higher molecular weight products including a major product of about 300 bp and decreasing amounts of lower molecular weight products (Fig. 4*B*). Since these products were clearly discrete from previously observed targeted strand-transfer products, products of three independent reactions were cloned and submitted for sequencing. This analysis showed strand transfer mainly within a small region. Among the six sites revealed by sequencing, four occurred within a 5-bp region from −231 to −226 upstream of *SNR6* and the others occurred at positions −285 and −125 (Fig. 4*C*). One possibility was that strand transfer at a secondary TFP-binding site occurred in the presence of MnCl$_2$. However, inspection failed to identify TATA-like sequences, and insertions were independent of TFP (Fig. 4*A*). Instead, the sequence (5′-TGT-TGTGT-3′/3′-ACAACACA-5′) resembling the terminal IR sequence of Ty3 (5′-TGTTGTAT-3′/3′-ACAACATA-5′) was

identified between −213 and −205 upstream of *SNR6*. The four clustered positions of strand transfer occurred 13 to 18 nt upstream of the 5′-end of this sequence (Fig. 4*C*).

The strand-transfer products recovered in the vicinity of plasmid sequences resembling the Ty3 IR suggested that IN might confer sequence specificity to strand transfer under some conditions. To directly test whether Ty3 IN targeted Ty3 IR-like sequences, a plasmid containing an isolated Ty3 LTR truncated at the downstream end to remove one IR (pXQ2889) was used as a target (Fig. 4*D*). Strand-transfer assays were performed using MnCl$_2$ as the cation and the preprocessed U5 oligonucleotide duplex substrate. PCR templated by products of this reaction showed dominant fragments of about 300 bp (Fig. 4*D, lane 1*). The mixed PCR products were cloned and sequences of six clones were determined. This analysis showed strand-transfer joints at positions −13, −9, and −7 relative to the outside end of the target Ty3 LTR (5′-TGTTGTAT-3′). To assess the distribution of target sites more completely, cloned strand-transfer products at these positions were used as templates to obtain ^{32}P-labeled markers. Migration of these markers was compared with that of products of an independent strand-transfer reaction. Comparison of the distribution of PCR fragments templated by products of the total strand-trans-

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fer reaction to the sizes of the sequenced standards showed that there was a narrow distribution of strand transfers ~5–13 bp from the upstream end of the plasmid-borne LTR (Fig. 4*E*, *lane 4*). To further test the dependence of the strand-transfer reaction on the Ty3 IR, the target plasmid was modified in the Ty3 IR from TGTTGTAT to TCACGTAT to produce plasmid pXQ3673 (Fig. 4*D*). In contrast to PCR templated by the reaction using the wt IR target, PCR of the reaction containing the mutated IR failed to generate a product (Fig. 4*D*, *lane 2*). Control PCR reactions monitoring DNA recovery showed no difference in plasmid recovery between the two sets of samples (data not shown). Although it appears that in MgCl$_2$, in the absence of a targeting factor, strand transfer does not occur or is extremely inefficient, this may be because the PCR assay is less sensitive to detection of highly distributed products. In the presence of MnCl$_2$, strand transfer was independent of TFP and concentrated near Ty3 IR-like sequences. Thus, IN strand transfer activity *per se* does not depend upon the presence of a targeting transcription factor. U5 strand transfer was only observed upstream of the Ty3 IR. This is consistent with asymmetric targeting by the 8-bp sequence to regions which in a chromosomal context would lie outside of Ty3.

The experiments in which the position of TFP was shifted by IN in the presence of Mg^{2+} but not in the presence of Mn^{2+}, together with the redirection of strand transfer in the presence of MnCl$_2$ suggested that IN interacts directly with IR-containing DNA in the presence of MnCl$_2$. However, gel shift experiments similar to those which detected weak TFP-mediated IN association with TATA-containing target probe failed to identify detectable binding to IR-containing 50-mers (data not shown).

The possibility that the presence of MnCl$_2$ enhances weak sequence-specific interactions is intriguing. The crystal structure of the primate foamy virus intasome (39) showed a dimer of dimers with the catalytic site at the dimer-dimer interface; residues interacting with the donor IR mapped to the catalytic core and C-terminal domains of the interface. If we assume a similar structure for Ty3 IN, outer subunits might be available to participate in targeting. We speculate that in the presence of MgCl$_2$, they interact preferentially with the TFIIIB complex, whereas in the presence of MnCl$_2$, this interaction is disfavored and IR-interacting residues mediate interactions (Fig. 4*F*). Although this activity is interesting in terms of intasome structure-function, it may have minimal *in vivo* significance. *In vivo*, integration into Pol III initiation sites clearly dominates (40) and the concentration of MnCl$_2$ required for IR targeting was significantly greater than the reported physiologic concentration (41).

In summary, the involvement of multiple retroelement and host proteins and poorly-defined insertion preferences complicate elucidation of retroelement targeting. This study reconstitutes precise retroelement targeting *in vitro* for the first time and delimits the retroelement and host components responsible. Intriguingly, our studies showed that both protein-targeted and IR sequence-targeted modes of strand transfer can occur *in vitro*. We propose that outer intasome subunits not involved in strand transfer are available for target interaction.

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