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The Brf and TATA-binding Protein Subunits of the RNA Polymerase III Transcription Factor IIIB Mediate Position-specific Integration of the Gypsy-like Element, Ty3*

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Ty3 integrates into the transcription initiation sites of genes transcribed by RNA polymerase III. It is known that transcription factors (TF) IIIB and IIIC are important for recruiting Ty3 to its sites of integration upstream of tRNA genes, but that RNA polymerase III is not required. In order to investigate the respective roles of TFIIIB and TFIIIC, we have developed an *in vitro* integration assay in which Ty3 is targeted to the U6 small nuclear RNA gene, *SNR6*. Because TFIIIB can bind to the TATA box upstream of the U6 gene through contacts mediated by TATA-binding protein (TBP), TFIIIC is dispensable for *in vitro* transcription. Thus, this system offers an opportunity to test the role of TFIIIB independent of a requirement of TFIIIC. We demonstrate that the recombinant Brf and TBP subunits of TFIIIB, which interact over the *SNR6* TATA box, direct integration at the *SNR6* transcription initiation site in the absence of detectable TFIIIC or TFIIIB subunit B″. These findings suggest that the minimal requirements for pol III transcription and Ty3 integration are very similar.

Integration site selection is a key step in the retroelement life cycle, potentially influencing both the effect of the insertion on the host genome and the expression of the element itself. Similar to retroviruses, yeast Ty elements transpose through reverse transcription of an almost full-length RNA copy into a full-length DNA copy, which is integrated into the host genome. Despite very similar molecular mechanisms of integration, budding yeast elements (Tys), both gypsy-like (Ty3) and copia-like (Ty1, 2, 4, and 5), differ from retroviruses in that they exhibit dramatic global integration site preferences (1–4). Ty1 elements, for example, integrate preferentially within a window of 750 bp,[1] upstream of genes transcribed by RNA polymerase III (pol III). Analysis of the yeast genome sequence shows that Ty2 and Ty4 also occupy this region upstream of a fraction of tRNA genes. Ty5 integrates into regions of silenced DNA, including the silent mating type loci and telomeres. Hence Ty1, Ty2, Ty4, and Ty5 exhibit regional integration specificity. Despite similarities with these other elements, Ty3 differs in that it integrates within a highly defined window, one or two base pairs (bp) upstream of pol III transcription initiation sites. Targeting of integration appears to be directed by the cooperative actions of Ty3 element- and cell-encoded factors. For example, in addition to the element-encoded integrase protein, Ty3 and Ty1 targeting requires the presence of a transcriptionally competent pol III promoter (5, 6), and Ty5 targeting requires factors involved in the establishment of silent chromatin (7).

Class III genes, including plasmid-borne U6, 5S, and tRNA genes, are used *in vivo* for position-specific Ty3 integration. Comparison of integration sites suggests that Ty3 integration preference is not a direct function of specific local sequences. Each class of pol III-transcribed genes differs from the others in composition of promoter elements (8) and distances of common motifs from the integration site (5). Pol III promoter mutations that affect transcription factor binding at positions distant from the integration site also block Ty3 integration (5, 9). These results lead to the hypothesis that interactions with the promoter-bound pol III transcription complex determine the selection of Ty3 integration sites.

Pol III genes use transcription factors (TF) IIIC and IIIB to assemble pol III at the transcription initiation site (reviewed in Ref. 8). TFIIIC is composed of six subunits and interacts with the *box A* and *box B* promoter elements. TFIIIB is composed of the TATA-binding protein (TBP), a 68-kDa protein (B″ or Tfc5, also referred to as TFIIIB90), and a 67-kDa protein (Brf, also called TFIIIB70). TBP and Brf are tightly associated in a complex referred to as B′, which is chromatographically separable from B″. The contributions of these transcription factors to the specific integration of Ty3 have been investigated using an *in vitro* integration assay. Reconstitution of specific integration into the initiation site of a tRNA gene requires Ty3 virus-like particles (VLPs), the plasmid-borne target gene, and DEAE-purified TFIIIB- and TFIIIC-containing fractions, consistent with DNA-bound TFIIIB, or TFIIIB and TFIIIC together, recruiting the Ty3 preintegration complex to its site of integration (10). Because TFIIIC is required to load TFIIIB onto DNA at the tRNA gene promoter, the respective roles of these factors could not be distinguished using the tRNA gene target.

The roles of TFIIIB and TFIIIC in pol III transcription have been examined in detail. *In vivo*, TFIIIC recruits TFIIIB to the region upstream of the transcription initiation site of all yeast (*Saccharomyces cerevisiae*) pol III genes (11, 12). TFIIIB then recruits pol III for initiation of transcription (13, 14). Two sets of observations show that TFIIIB is the central initiation factor

[1] The abbreviations used are: bp, base pair(s); pol III, polymerase III; TF, transcription factor; TBP, TATA-binding protein; VLP, virus-like particles; PCR, polymerase chain reaction.

of pol III: 1) TFIIIC can be removed from tRNA genes after TFIIIB is assembled at the promoter without loss of transcription activity (13). 2) *SNR6*, which does not require TFIIIC for loading TFIIIB *in vitro*, can be transcribed by pol III in the presence of TFIIIB only (15, 16); TFIIIB components have been demonstrated to contact pol III subunits directly (17–21).

The ability to bind TFIIIB directly to the *SNR6* gene in the absence of TFIIIC provides an *in vitro* system in which TFIIIB-bound DNA can be tested for TFIIIC-independent Ty3 targeting. In the experiments that are presented here, distinct roles for TFIIIB and TFIIIC in Ty3-specific integration have been investigated by developing a variation of a previously devised *in vitro* integration assay. The use of the three recombinant TFIIIB subunits, TBP, Brf, and B″ (22), allows a precise definition of the minimal protein requirements for specific integration of Ty3. We demonstrate that TFIIIB recruits the Ty3 preintegration complex to its site of integration. Recombinant B′ (*i.e.* TBP + Brf) is sufficient to direct specific integration of Ty3 *in vitro* and B″ contributes strongly to the efficiency of integration, conceivably through the DNA distortion that it generates near the integration site. TFIIIC contributes to targeting specificity and selection by determining the orientation of TFIIIB on the promoter.

<div align="center">EXPERIMENTAL PROCEDURES</div>

Strains and Plasmid Constructions—Standard methods were used for culturing and transforming *Escherichia coli* and *S. cerevisiae* and for recombinant DNA constructions (23). All plasmids were amplified in, and prepared from, *E. coli* HB101. Single-stranded DNA for site-directed oligonucleotide mutagenesis was prepared from *E. coli* RZ1032. Plasmid pU6LboxB (24) was the pol III transcription template and target for Ty3 integration *in vitro*. Plasmids pDLC370 (5) and pLY1842 served as PCR controls for integration into r-U6 and l-U6, respectively. Plasmid pDLC370 has a Ty3 insertion with Ty3 sequence beginning at position −5 relative to the transcription start site r-U6. Plasmid LY1842 was constructed by cloning a PCR fragment generated with primers 242 and 411 (see below) and pU6LboxB containing a Ty3 insertion at bp −2 relative to the l-U6 TATA transcription start site (Fig. 2*A*) into pCRII-TOPO. The pLY1855 plasmid was generated by removing the δ TATA box from pU6LboxB by site-directed mutagenesis using an oligonucleotide with sequence 5′-GCTGGAGATACAGAAC-TATTATGG-3′. Plasmid pU6LboxB-G56 was constructed by changing the conserved C56 of the *SNR6 boxB* to G using an oligonucleotide with sequence 5′-GGGGGGGAGTC**C**AACGCCCGATTGC-3″. Mutations were confirmed by DNA sequence analysis.

Proteins—Ty3 VLPs were prepared from *S. cerevisiae* strain AGY9 (pEGTy3-1) cells as described (25). Highly purified TFIIIC (oligobox B⁺ fraction) and pol III (MonoQ fraction), rTBP, rBrf, and rB″, were prepared and quantitated as described or referenced (13, 16, 26, 27) and are specified as active molecules in specifically initiating transcription (pol III) or specific DNA binding (TBP, Brf, B″, TFIIIC). TBP and B″ were fully active; Brf was ~20% active.

In Vitro Integration into SNR6 Targets—Under standard conditions (Figs. 2–4), samples for *in vitro* integration contained, in 50 μl of reaction buffer (40 mM Tris-HCl, pH 8, 7 mM MgCl₂, 3 mM dithiothreitol, 100 μg/ml bovine serum albumin, and 50 mM NaCl), 1.0 nM TBP, 0.7 nM Brf, 1.5 nM B″, and 3.6 nM target plasmid DNA. These components were preincubated for 30 min at 23 °C, shifted to 15 °C, and then 5 μg (protein) of Ty3 VLP fraction was added for 15 min. When purified TFIIIC was also present, it was preincubated with DNA for 30 min before adding TFIIIB components. Samples contained 10.5 nM TFIIIC for the experiment shown in Fig. 3. For Fig. 4, samples contained the noted multiples of 0.5 nM TFIIIC. For Fig. 5*A*, factors were preincubated with DNA for 1 h at 23 °C prior to adding VLPs. For Fig. 5*B*, B′-DNA complexes were allowed to form for 30 min prior to an additional 15-min incubation with subsequently added B″. Reactions were stopped by adding proteinase K, SDS, and EDTA, pH 8.0, to final concentrations of 0.2 mg/ml, 0.2% (w/v), and 20 mM, respectively, and incubating at 37 °C for 30 min. Reaction products were extracted with phenol/chloroform, and DNA was precipitated and redissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

PCR was performed essentially as described (28), with the following changes to make detection specific to the *SNR6* target: primer 242 (5′-GGAACTGCTGATCATCTCT-3′) (200 ng) and primer 411 (5′-

CGAAACACAAGACAACCC-3′) (164 ng) were used for amplification of 40 ng (18 fmol) of DNA from the integration reactions (first incubation for 2.5 min at 95 °C, followed by 40 cycles of denaturation at 94 °C for 1 min and renaturation/extension at 65 °C for 1 min, followed by 5 min at 72 °C). The standard amplification reactions in a total of 40 ng of target plasmid DNA yielded products equivalent to those of reactions containing 1.6 to 8 fg of Ty3-positive target plasmid in a total of 40 ng of target plasmid. Thus, the products are highly specific to positive plasmid templates. To control for consistent DNA recovery from the integration reaction and for consistent operation of the above PCR, primers 679 (5′-ACTCCCCGTCGTGTAGATAACTACG-3′) and 680 (5′-AAGCCATACCAAACGACGAGC-3′) were used to amplify the β-lactamase gene carried by the target plasmids. PCR amplification of 100 pg of DNA (5 μl of 1:200 reaction dilution) was performed using 200 ng of each primer (first denaturation for 2.5 min at 95 °C, followed by 18 cycles of denaturation at 94 °C for 45 s, renaturation at 55 °C for 45 s, and extension at 72 °C for 30 s, with a final 5 min at 72 °C). PCR products were resolved by electrophoresis on nondenaturing, 8% polyacrylamide gel and visualized by staining with ethidium bromide. The fluorescence video image was quantified using a gel documentation program.

Cloning of Specific Integration Products—PCR fragments representing specific integrations were separated as described above. Bands containing specific fragments were excised from the gel, DNA was eluted, ligated into the vector pCRII-TOPO according to the supplier's instructions, and transformed into *E. coli*. Individual transformants, selected on LB + ampicillin, were picked for analysis. DNA was prepared from five transformants per band and sequenced with oligonucleotide 242 primer.

Immunoblot Analysis—Recombinant B″ (5–80 fmol), the crude pol III transcription-competent fraction BR500 (7 μg; BRα (29)), and Ty3 VLPs were resolved on 9% polyacrylamide-SDS gel and transferred to polyvinylidene difluoride membrane. Polyclonal rabbit antiserum directed against B″ (40–487) (22) and ¹²⁵I-protein A were used to probe the blot, essentially as described (30). Bands were quantified by PhosphorImager analysis.

Photochemical Cross-linking—Photochemical cross-linking was performed with an 88-bp DNA probe containing the *SNR6* TATA box (31) with 5-[*N′*-(*p*-azidobenzoyl)-3-aminoallyl]dUMP incorporated at bp −39 and −38 and [α-³²P]dCMP at bp −37 (26). The TATA box was modified to TGTAAATA to provide a unique orientation of TFIIIB-DNA complexes in conjunction with the TBP mutant TBPm₃ (24). Protein-DNA complexes were formed as described for *in vitro* integration but in a 20-μl reaction volume, with β-mercaptoethanol in place of dithiothreitol, 10 fmol of photoprobe, 200 ng of poly(dG-dC)·poly(dG-dC), and NaCl at 70 mM. Where indicated, B′-DNA complexes were formed with 400 fmol of TBPm₃ and 144 fmol of Brf for 60 min at ~20 °C. B″, in the indicated amounts, 5 μg of VLPs or B″ and 5 μg of VLPs (preincubated together for 15 min at 0 °C) was added for an additional 20 min, followed by 2 min of UV irradiation. Reaction mixtures were treated with nucleases and resolved on 9% polyacrylamide-SDS gel as described (32).

<div align="center">RESULTS</div>

Ty3 in Vitro Integration Upstream of the SNR6 Gene Is Detectable by PCR Amplification—In order to investigate whether TFIIIB suffices for Ty3 position-specific integration, a previously used *in vitro* tRNA gene integration assay (10) was modified to utilize *SNR6* as a target. Integration reactions contained Ty3 VLPs, the *S. cerevisiae SNR6* gene in plasmid pU6LboxB (24), and combinations of TFIIIB and TFIIIC. Pol III, previously shown to inhibit Ty3 integration (33), was omitted. TFIIIC was supplied either as a DEAE column fraction or as highly purified protein (27); TFIIIB was added either as a DEAE fraction or as recombinant protein (rTFIIIB).

Integration of Ty3 into the target plasmid was detected using a polymerase chain reaction (PCR)-based assay (Fig. 1*A*). One PCR primer anneals to the *SNR6* gene and the other to a unique sequence in Ty3. A diagnostic PCR template sequence is created when Ty3 integrates into the *SNR6* target in one of the two possible orientations, and the length of the corresponding PCR-amplified fragment specifies the position of integration. This assay differs from the previously described tRNA gene assay (10) in that the target primer does not overlap with the
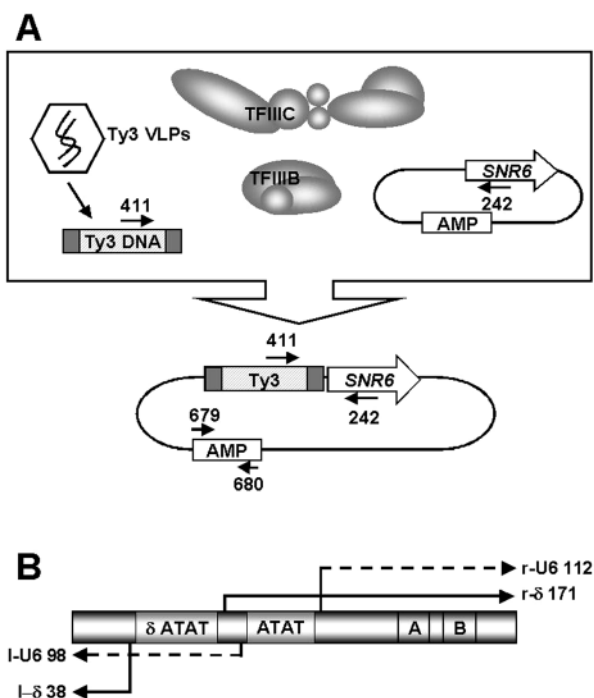
A

B

FIG. 1. **The Ty3 *in vitro* integration assay.** *A, in vitro* integration reactions contained Ty3 VLPs, TFIIIB, and/or TFIIIC, and a U6 snRNA gene (*SNR6*) plasmid target. Integration into the plasmid target (in one orientation only) was detected by PCR amplification using oligonucleotides 242 and 411 (*arrows*) to amplify integration products. Primers 679 and 680 were used to amplify a fragment containing the β-lactamase gene, which provides an internal control for the PCR and for the amount of plasmid DNA in the reaction. Following amplification, PCR products were separated on a nondenaturing 8% polyacrylamide gel. The size of each PCR product is characteristic of the position of integration. *B,* the promoter-Ty3 insertion region of pU6LboxB. Transcription in the presence of rTFIIIB and RNA polymerase III is bidirectional from two TATA elements. Transcripts dependent on the *SNR6* TATA box (l-U6, r-U6) are shown as *broken lines*, and transcripts dependent on the δ TATA box (l-δ, r-δ) are shown as *solid lines*. Transcript lengths in nucleotides are also shown.

Ty3 insertion, so that detection of integration is not limited to a single location; additional rounds of PCR are used to increase assay sensitivity.

The integration assay uses the plasmid pU6LboxB, which contains a modified *SNR6* gene with altered flanking DNA sequence (24) as its Ty3 target. A duplication of the *SNR6* transcriptional start site and terminator in pU6LboxB allows TFIIIB-DNA complexes oriented to promote transcription away from the *SNR6* gene (*leftward* in Fig. 1*B*) to be monitored in a transcription assay. In constructing pU6LboxB, the *SNR6* TATA box was inverted to preferentially orient TFIIIB for leftward transcription, and the *boxB* promoter element was moved closer to the natural *SNR6 boxA* promoter element in order to optimize the ability of TFIIIC to direct TFIIIB binding at the *SNR6* TATA box. TFIIIC shifts the preferred direction of transcription from leftward to rightward (Fig. 1*B*) on this template. In constructing pU6LboxB, genomic sequence upstream of the *SNR6* TATA box containing a Ty1 LTR (or δ) element was deleted, positioning a second TATA box (which originally flanked the *SNR6* gene-distal end of the δ element) between the *SNR6* TATA box and the terminator for leftward transcription. This "δ TATA box" (identical in sequence and orientation to the *SNR6* TATA box in pU6LboxB) generates its own pair of divergent transcription units. Thus, TFIIIB binding autonomously to either TATA box of pU6LboxB in either orientation generates four primary transcripts (l-U6, r-U6, l-δ, and r-δ in Fig. 1*B*). By selecting the site and orientation of TFIIIB bind-

ing, TFIIIC favors the formation of the r-U6 transcript and restricts the formation of the others (Ref. 24 and data not shown).

The ability to reconstitute specific integration of Ty3 upstream of *SNR6 in vitro* was first tested using DEAE column fractions enriched for TFIIIB and TFIIIC. Plasmid pDLC370 ("Experimental Procedures"), which contains a Ty3 insertion, was used as a template for primers 411 and 242 in PCR to amplify a 442-bp fragment that served as a positive control for integration into the r-U6 initiation site (9). As a control for the quantity of DNA in each integration reaction, primers 679 and 680 were also used to amplify the β-lactamase gene on pU6LboxB. PCR products were separated by nondenaturing polyacrylamide gel electrophoresis. Reconstitution of integration at the *SNR6* gene in these preliminary trials (data not shown) depended on the presence of Ty3 VLPs, as PCR amplification of reactions lacking VLPs was equivalent to amplification of the target plasmid alone. Integration reactions performed with TFIIIB and TFIIIC generated a predominant PCR product, whose length was consistent with specific integration close to the r-U6 transcription initiation site (Fig. 2*A*). A random (or at least complex and dispersed) pattern of integration was observed with Ty3 VLPs alone (Fig. 2*B, lane 2*).

Recombinant TFIIIB Directs Specific Integration of Ty3 in Vitro—The advantage of the relatively complex pU6LboxB construct is that it permits a determination of whether TFIIIB alone suffices to mediate Ty3-specific integration, while also examining whether TFIIIC exerts an effect on integration site selection. The binding of rTFIIIB to the target plasmid was verified by its ability to direct transcription by pol III *in vitro*. The four expected transcription products were generated, with preferential production of the l-U6 transcript, as expected (data not shown). Integration at the four transcription initiation sites is predicted to produce four different classes of PCR products (Fig. 2*A*). Integrations were performed under conditions tested for transcription activity, and with comparable concentrations and proportions of rTFIIIB and plasmid DNA (Fig. 2*B* and data not shown). The combination of rTFIIIB and TFIIIC yielded a single PCR-amplified integration product (Fig. 2*B, lane 3*). Integration in the presence of rTFIIIB alone generated three major PCR products (*lane 4*) each consistent in size with integration at one of the four transcription initiation sites (Fig. 2*A*); the size of the smallest PCR fragment corresponded with an integration event near the initiation site of r-U6 transcription (Fig. 2*B*, compare *lane 4* to *lanes 3* and *5*). The sizes of the remaining products corresponded to PCR templates generated by specific integration very close to the l-δ (*lane 4*, largest fragment, ∼552 bp), and r-δ and l-U6 (middle size fragment, ∼492 and ∼501 bp) transcription initiation sites, respectively.

To determine whether these PCR products indeed report specific integration events, DNA fragments contained in the bands marked at the side of Fig. 2*B* were recovered from a gel (*cf.* Fig. 2*B, lane 4*) and cloned into the vector pCRII-TOPO. Sequences were determined for the Ty3-*SNR6* junction in five clones from each fragment preparation using the *SNR6* internal PCR primer (Fig. 2*A*). Sequencing of these clones demonstrated that the smallest fragment corresponds to Ty3 sequence beginning at positions −6 and −7 relative to the r-U6 transcription start site (Fig. 2*C*). Because Ty3 integrates by means of a staggered strand transfer that is repaired, resulting in repeats of the intervening sequence on either end of the Ty3, this corresponds to a gene-proximal strand transfer between positions 1 and −1 and between −1 and −2, respectively, relative to the start site of transcription. The middle fragment appears to represent integration events at the l-U6 and/or r-δ transcription initiation sites. The identified integration sites in this region showed Ty3 sequence positioned at −2 and +5
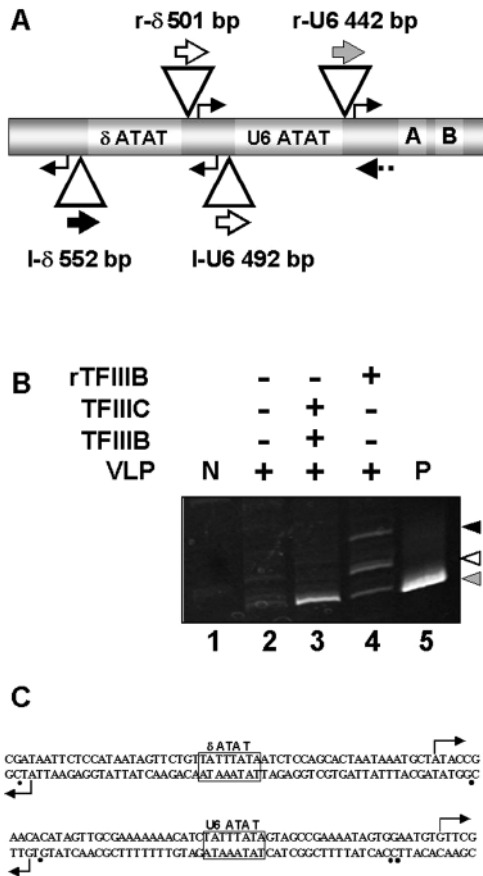
FIG. 2. **Recombinant TFIIIB mediates specific integration in the absence of TFIIIC.** *A,* Ty3 integration into pU6LboxB. Four positions of specific Ty3 integration mediated by rTFIIIB are indicated with *triangles* and labeled according to the transcription start site used as a target. The location of the Ty3 PCR primer that was used to amplify each of these integration events (in one orientation only) is represented by a *pattern-filled arrow*. The common primer in the U6 sequence is shown by a *dashed line*. The predicted sizes of PCR products reporting each integration event are indicated. *B,* determination of integration sites in pU6LboxB by analysis of PCR products. Products of integration reactions were used to template PCR; input proteins are specified *above lanes 2–4*. PCR fragments were separated on a nondenaturing 8% polyacrylamide gel and stained with ethidium. *Lane 1 (N,* the negative control), pU6LboxB target plasmid alone was used as the PCR template; *lane 5 (P,* the positive control), pDLC370 containing *SNR6* with a Ty3 insertion at the r-U6 start site was used as the PCR template. The *patterned arrows* indicate the correspondence with positions of insertion shown in *A. C,* fragments indicated by *arrowheads* in *B* were cloned, sequences determined, and Ty3 insertion sites deduced. *Dots* indicate the first base of Ty3 sequence in sequence primed from the *SNR6* gene. (Note that the gene-proximal strand transfer is located 5 nucleotides downstream of the beginning of Ty3 sequence because of a 5-base offset in the initiating, staggered two-strand attack and its repeat-generating repair.) *Bent arrows* indicate transcription initiation sites.

relative to the l-U6 transcription initiation site, but cannot be correlated unambiguously with a specific partner TFIIIB-DNA complex because these two initiation sites are located very close together. Analysis of clones derived from the largest product yielded Ty3 sequence at each transcription initiation site, including +3, relative to l-δ. We interpret this as due to contamination in the region of this weaker and slower migrating band in the gel by shorter PCR products. However, the fragment size, together with the sequence of one of the five analyzed clones (Fig. 2*C*), suggests that Ty3 integration was also associated with the l-δ transcription start site.

The patterns of integration directed by rTFIIIB alone (Fig. 2B, *lane 4*) and TFIIIB plus TFIIIC (*lane 3*) were quite
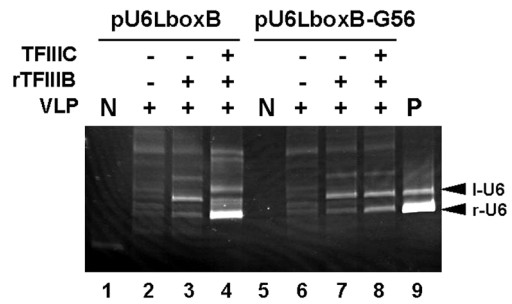

FIG. 3. **TFIIIC is not present in significant amounts in Ty3 VLPs.** Products of *in vitro* Ty3 integration into the reference pU6LboxB plasmid and into pU6LboxB-G56. The C56-GboxB mutation in the latter plasmid disrupts TFIIIC binding. Integration products were analyzed as specified in the legend to Fig. 2. PCR of *in vitro* integration products: *lanes 2–4*, pU6LboxB; and *lanes 6–8*, pU6LboxB-G56. Plasmids used for PCR controls: *lanes 1 and 5 (N* controls), pU6LboxB and pU6LboxB-G56, respectively; *lane 9 (P* control), a mixture of pDLC370 and pLY1842. Reaction components are indicated *above lanes 2–4* and *6–8*. *Arrowheads* indicate PCR products reporting insertion at the I-U6 (*top*) and r-U6 transcription initiation sites (*bottom*).

distinctive. The rTFIIIB-mediated integration products represented each of the four initiation sites, as just described, but TFIIIB and TFIIIC together generated PCR products consistent with integration primarily at the r-U6 transcription start site.

TFIIIC Affects Ty3 Integration by Influencing the Orientation of TFIIIB Binding to the TATA Box—Although the preceding experiment indicates that TFIIIB is the only pol III factor that is absolutely required for specific integration of Ty3, it is necessary to eliminate the possibility that the Ty3 VLP fraction contains and contributes TFIIIC. This seems unlikely because different integration patterns are generated in the presence and absence of TFIIIC (Fig. 2*B, lanes 3* and *4*) and because the l-δ integration site is over 120 bp away from *boxA*. Nevertheless, the possible dependence of integration on TFIIIC contributed by the Ty3 VLP fraction was addressed by introducing a $C^{56} \rightarrow G$ mutation into the *boxB* sequence of pU6LboxB to create plasmid pU6LboxB-G56. The *boxB* element is the primary, high affinity TFIIIC-binding site of pol III-transcribed genes, and the $C^{56} \rightarrow G$ mutation has been shown to abrogate TFIIIC binding to tRNA and U6 gene promoters (34–36). Thus, TFIIIC-dependent integration into pU6LboxB should be decreased by the $C^{56} \rightarrow G$ mutation, but TFIIIC-independent integration should not be affected.

Integration into the reference target in pU6LboxB (Fig. 3, *lanes 2–4*) and its mutated derivative in pU6LboxB-G56 (*lanes 6–8*) were compared. Similar patterns of specific integration mediated by rTFIIIB (compare *lanes 3* and *7*) and of nonspecific integration in the absence of TFIIIB (compare *lanes 2* and *6*) were seen with the wild-type and mutated targets. Addition of TFIIIC changed the pattern of integration into the reference target, as already noted (Fig. 2*B*), but left the pattern of Ty3 integration into the $C^{56} \rightarrow G$ mutant target essentially unchanged from integration mediated by TFIIIB alone (Fig. 3, compare *lane 8* with *lanes 3* and *7*). If sufficient TFIIIC had been present in Ty3 VLPs to affect integration of Ty3 into the wild-type target, then the pattern of integration in the presence (*lane 4*) and absence (*lane 3*) of additional TFIIIC should have been similar, and the patterns of integration into the reference and $C^{56} \rightarrow G$ mutant DNA (*lanes 3* and *7*, respectively) should have been entirely different. In each instance, the observed pattern was entirely consistent with the conclusion that TFIIIC was not significantly contributed by the VLP fraction.

Previous experiments showed that TFIIIC loads TFIIIB in the rightward orientation on the U6 promoter (24). In pU6LboxB, TFIIIB alone binds the TATA box preferentially in
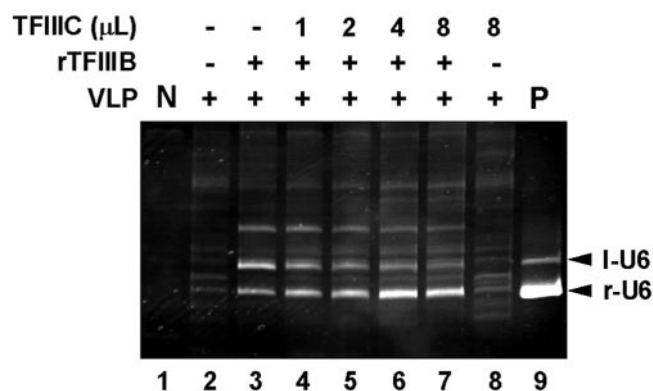
FIG. 4. **TFIIIC affects Ty3 integration by directing the assembly of TFIIIB on the U6 promoter.** Integration reactions were performed with 0.7–1.5 nM rTFIIIB subunits and 0.5–4.0 nM TFIIIC (shown as multiples of 0.5 nM). PCR analysis of the integration products was performed as described in the legend to Fig. 2. Reactants are indicated at the *top* of the panel. Negative (*N*) and positive (*P*) controls are as described in the legend to Fig. 3.

the orientation that directs leftward transcription. TFIIIC stabilizes TFIIIB binding in the opposite orientation, and transcription preference switches accordingly. The results shown in Fig. 3 suggest that, although TFIIIC is not absolutely required for Ty3 integration into DNA, it influences Ty3 integration site preference. In order to better assess this effect, highly purified TFIIIC was titrated into integration reactions performed at one concentration of rTFIIIB. In the presence of TFIIIB only, integration was seen to be distributed among the transcription initiation sites with a preference for integration at l-U6 (and/or r-δ) (Fig. 4, *lane 3*). Increasing amounts of TFIIIC (*lanes 4–7*) progressively shifted integration preference to the r-U6 transcription initiation region; specific integration was dependent on the presence of rTFIIIB (compare *lanes 2* and *8* with *lanes 3–7*). The change in integration pattern observed in Fig. 4 correlated directly with TFIIIC-dependent changes in transcription (data not shown**,** but see Ref. 37). Thus, TFIIIC affects Ty3 integration *in vitro*, by dictating the location and polarity of TFIIIB binding at a pol III promoter.

B' (TBP and Brf) Bound to DNA Is the Minimal Target for Ty3 Integration—Since recent experiments have shown that the B'-DNA complex is sufficient to mediate pol III transcription of templates with partly preopened promoters (38) it was of interest to explore whether any TFIIIB subunit or pair of subunits can also mediate Ty3 integration. In order to simplify the analysis, the δ TATA was deleted from pU6LboxB to create the *SNR6* target plasmid LY1855.

Subunits of TFIIIB bind to DNA in an ordered manner (14, 26, 39). In the absence of TFIIIC, TBP binds directly to the TATA box, Brf modestly stabilizes TBP binding (40) to form the B'-DNA complex, and B" addition generates the hyper-stable DNA complex that mediates pol III binding to duplex DNA and confers DNase I hypersensitivity at the transcription initiation site. Single TFIIIB subunits and pairs of subunits were tested for their ability to direct integration. At concentrations used in the standard assay, that is in the presence of the entire TFIIIB complex, specific integration was not detected (data not shown). Increased concentrations of protein were also tested, but none of the rTFIIIB components was individually able to reconstitute integration (Fig. 5*A, lanes 2–4*). However, the combination of TBP and Brf (B') sufficed to mediate specific integration (*lanes 5* and *6*) with a preference for the l-U6 gene start site. Integration mediated by B' required higher concentrations of TBP and Brf (compare *lanes 5, 6*, and *11*), as well as longer times of preincubation of recombinant protein with DNA than did integration mediated by TFIIIB. Other combinations
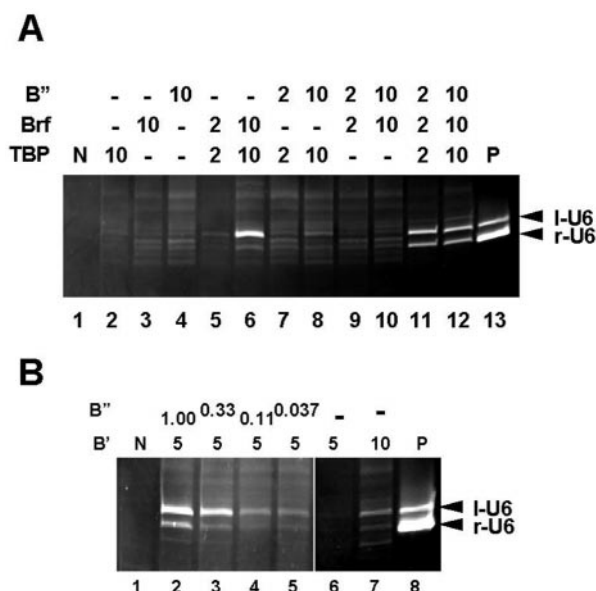

FIG. 5. **TBP and Brf bound to DNA constitute the minimal integration target for Ty3 and B" promotes efficient integration.** *A,* integration into plasmid pLY1855 (*SNR6* TATA only) as a function of TBP, Brf, and B" concentration (indicated at the *top* in multiples of the standard integration assay described under "Experimental Procedures"). VLPs were present in all reactions at the standard 5 μg/sample. Negative (*N*) plasmid control: pLY1855; positive (*P*) controls as described in the legend to Fig. 3. Specific integration sites are indicated at the *right side*. *B,* placing a limit on the contamination of VLPs with B". Integration into pLY1855. The concentrations of B' and B" are shown as multiples of concentrations in a standard reaction. Negative and positive controls (*lanes 1* and *8*) as in *panel A*. Lanes *2–5,* B' was preincubated with target DNA for 30 min prior to the addition of B"; *lanes 6* and *7,* only B' was added.

of subunits (*lanes 7–10*) and the individual subunits (*lanes 7–10*) were incapable of directing specific integration even at elevated concentrations.

B" Increases the Efficiency of Ty3 Integration—A firm conclusion that the B'-DNA complex recruits Ty3 to its integration site requires proof that VLPs do not constitute a significant source of B". It was necessary first to estimate the amount of B" that would have to be contributed to generate the observed integration. Integration in the absence of added rB" was first compared with different amounts of rB' (Fig. 5*B, lanes 6* and *7*). A significant level of integration was observed with 10-fold the standard amount of rB' (×10 = 500 fmol of TBP and 360 fmol of Brf) (Fig. 5*B, lane 7*). The rB" was then assayed over the range of 1 to 0.03 times the standard amount (75–2.8 fmol) with B' preincubated and B" added subsequently (*lanes 2–5*) to correspond with the effective order of addition that would prevail if B" were contributed by the VLP preparation. The yield of PCR products was quantified as a function of added rB". This analysis specified that, if B" were absolutely required for the observed Ty3 integration and contributed solely by the VLP fraction, 5 μg of VLPs (the amount in a standard assay) would have to contain 12.5 fmol of active B". The upper limit of B" in the VLP fraction was then established in three ways. The most direct, but least sensitive estimate was provided by immunoblot analysis. Two relatively sensitive functional and structural assays for B" were also used.

For immunoblotting, 10-μg aliquots of VLP (twice the amount used in the standard integration reaction) were fractionated by SDS-polyacrylamide gel electrophoresis together with His-tagged rB" (5–80 fmol) and crude fraction (BR500), containing native B", as standards, probed with B" antiserum and developed with ^{125}I-protein A (Fig. 6*A*). Radioactivity at the position of B" was quantified (data not shown). The 5 fmol
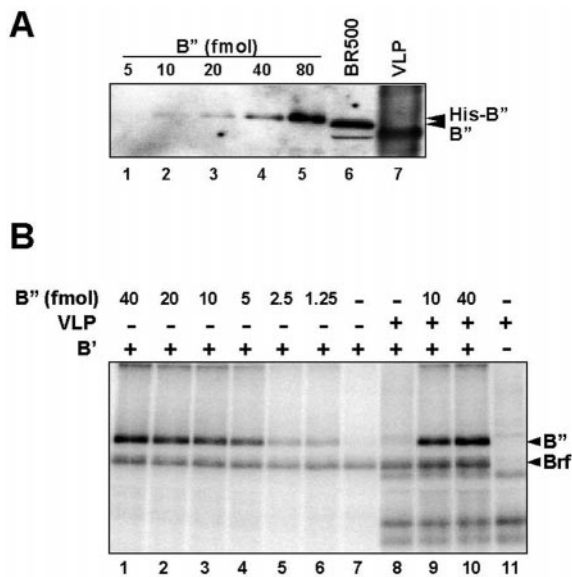
A

B

FIG. 6. **VLPs do not contain B″.** *A,* immunoblot analysis. To titrate the sensitivity of the immunoblot**,** 5, 10, 20, 40, and 80 fmol of B″ (*lanes 1–5,* respectively) were tested as described under "Experimental Procedures." Because rB″ is His tagged and therefore has slightly reduced mobility relative to native B″, BR500 was also used to provide an accurate B″ size marker (*lane 6*). Ten μg of VLP, twice the amount present in the standard reaction, was assayed for *lane 7. B,* VLPs do not contribute cross-linkable B″. B′-DNA complexes with an internally labeled oligonucleotide duplex representing *SNR6* were allowed to form, followed by the addition of 40–1.25 fmol of B″ (*lanes 1–6*), no additional protein (*lane 7*), 5 μg of VLPs (*lane 8*), or 5 μg of VLPs with B″ (*lanes 9* and *10*). The probe was also reacted in the absence of B′ with 5 μg of VLPs. Cross-linked B″ and Brf are indicated with *arrows*.

of B″ standard was barely detectable above background, and 10 fmol was readily detected. Significant cross-reactive protein in the VLP fraction (*lane 7*) migrated faster than the native B″ (*lane 6*). The PhosphorImager profile of *lane 7* did not display a peak above background that would be predicted for the presence of ≥10 fmol of native B″, limiting the B″ contribution from the VLPs in the integration assay to <5 fmol. Nevertheless, because the detection limit of the immunoblot analysis was close to the possible B″ threshold set by the Fig. 5 experiment, two further tests for the presence of B″ were devised.

The first test was based on the fact that entry of B″ into the B′-DNA complex generates a heparin-resistant TFIIIB-DNA complex that can be sensitively detected by electrophoretic mobility shift assay with a labeled TATA box-containing DNA probe (41). Addition of as little as 2.8 fmol of B″ to pre-formed B′-DNA complexes generated readily detectable heparin-resistant TFIIIB DNA complexes, and preincubation of B″ with VLPs did not prevent formation of these complexes. Addition of twice the amount of VLPs used in the integration assays to B′-DNA complexes failed to generate any heparin-resistant TFIIIB-DNA complexes, signifying that 5 μg of VLPs could at most contribute 1.4 fmol of complex-forming B″ (data not shown).

The second test detected B″ in the TFIIIB-DNA complex by site-specific photochemical cross-linking (32, 42, 43). B″ lies proximal to the major groove at bp −39 and −38 of the *SNR6* gene (31) such that incorporation of the photoactive nucleotide 5-[*N′*-(*p*-azidobenzoyl)-3-aminoallyl]dUMP at these positions with an adjacent radioactively labeled nucleotide readily detects 1.25 fmol of B″ upon TFIIIB-DNA complex formation, UV irradiation, nuclease digestion (transferring the radioactive DNA label to the cross-linked protein), and SDS-polyacrylamide gel electrophoresis (Fig. 6*B, lane 6*). Because the DNA-binding reaction mixtures contained only low levels of a non-

specific DNA carrier, the binding of 5 μg of VLPs alone to this DNA probe yielded cross-linking to four proteins (*lane 11*), one of which migrated slightly more slowly than recombinant B″ and one slightly faster than Brf. These cross-linking signals did not increase in the presence of B′-DNA or TFIIIB-DNA complexes (compare *lane 11* with *lanes 8* and *10*). Addition of 5 μg of VLPs to the B′-DNA complex generated a very weak signal at the position of the B″ band, corresponding in intensity (by PhosphorImager analysis) to less than 0.3 fmol of B″ (*lane 8*). However, summation of the PhosphorImager signals of the B′-alone (lane 7) and VLP-alone (*lane 11*) samples indicated that even this very weak apparent B″ signal derived solely from the B′-DNA complex and that it was made perceptible by the background density contributed by VLPs (analysis not shown). The VLP-dependent increase in cross-linking signal at the Brf band in *lane 8* derived from the same cause. Additional analysis of the extremely weak signal overlapping the B″ band in *lane 7* indicated that it was an extraneous, multicross-link product, highly sensitive to TBP concentration and UV dosage. Since preincubation of B″ with VLPs did not prevent B″ cross-linking (*lanes 9* and *10*), this analysis established that 5 μg of VLPs (conservatively) contained less than 0.3 fmol of B″. This is at most 40-fold less B″ than required to account for the Ty3 integration observed in Fig. 5*B* as a B″-requiring process. We conclude that specific Ty3 integration is not absolutely dependent on the B″ component of TFIIIB.

However, B″ does increase the level of Ty3 integration and it can also change the distribution of integration sites. Preincubating B′ and B″ together with DNA before adding VLPs favored integration at the r-U6 site (Fig. 5*A*, compare *lanes 6* and *12*), which would be created by TBP bound in its less favored orientation. This is reminiscent of previous demonstrations that B″ can freeze the orientation of B′ (TBP + Brf) on DNA in non-equilibrium distributions (44). An order-of-addition experiment (based on Fig. 5*B*) was designed to determine whether the changes in the distribution of Ty3 integration events upon addition of B″ could be explained similarly. Integration reactions were performed using target pLY1855, constant B′, and varying quantities of B″. In one-half of the experiment, B′ was preincubated with the target DNA before addition of B″ (Fig. 5*B, lanes 2–5*). In the other half, B″ was added simultaneously with B′. The pattern of integration did depend on the order of addition: preincubating B′ with DNA before adding B″ favored integration into the l-U6 region (*lanes 2–5*); when B′ was not preincubated with DNA, integration occurred equivalently at the l- and r-U6 regions (Fig. 5*A, lanes 11* and *12* and data not shown). In all cases, higher levels of B″ generated more integration.

These results suggest that the left-right integration site selection of Ty3 reflects the bias in orientation of TBP on DNA. B″ does not influence the direction of B′ binding *per se*; rather, it stabilizes the B′ complex on DNA regardless of orientation. Parallel observations have been made on transcription polarity. Part of the enhanced efficiency of Ty3 integration that is contributed by B″ may be attributable to more efficient DNA complex formation, but it is likely that B″ also makes a direct structural contribution to Ty3 integration, as discussed below.

DISCUSSION

Ty3 transposition targets the pol III transcription machinery. In this work, we precisely define this connection by showing that TFIIIB is the essential cellular factor of Ty3 transposition. TFIIIC is dispensable for position-specific integration *in vitro* into a U6 RNA gene target, presented as bare duplex DNA.

In order to identify the contributions of components of the TFIIIB-DNA complex to Ty3 targeting, the abilities of individ-

ual and pairwise combinations of the transcription factor sub-units to mediate integration have been tested. TBP and Brf constitute the B′ component of TFIIIB and bind DNA as a complex. Together with VLPs, the B′-DNA complex generates a modest level of integration. The possibility that contamination of the VLP complex with B″ contributes to this basal activity has been excluded. It has been shown recently that specifically initiating, B″-independent pol III transcription occurs on *SNR6* templates containing a partially preopened promoter (made by creating a short heteroduplex loop upstream of the transcription initiation site) (38). These results argue that similar targeting interactions are central to position-specific Ty3 integration and pol III transcription.

The locations of transcription factor components on DNA and their protein-protein interactions provide clues to potential interactions between TFIIIB subunits and the Ty3 preintegration complex. Brf contacts DNA near the site of Ty3 integration (26, 42). The primary contacts between TFIIIB and pol III are mediated by Brf (17–19, 21). Within TFIIIB Brf is oriented so that a conserved C-terminal region interacts with TBP. The N-proximal, TFIIIB-like half of Brf interacts with the C34 and C17 subunits of pol III (17, 19, 26, 45). The C-terminal half of Brf also contacts C34 (19). Although there is no striking similarity between the sequences of Ty3 reverse transcriptase or integrase and C17 or C34 (analysis not shown), pol III inhibits Ty3 integration *in vitro* (33). A direct competition between the Ty3 preintegration complex and pol III for overlapping sites in the N-terminal region of Brf could help to explain this inhibitory effect of pol III.

The increased sensitivity developed for detecting Ty3 integration into the *SNR6* target has also made it possible to demonstrate that Ty3 VLPs produce integration products when incubated together with naked DNA. These products differ significantly from the specific integration products observed in the presence of pol III transcription factors. Thus, the Ty3 preintegration complex apparently retains the ability to target a range of sites in naked DNA. This is a characteristic of nonspecific retroelements.

Although B″ is not absolutely required for *in vitro* integration, it does strongly affect the efficiency of the integration reaction. Significant conformational changes occur upon addition of B″ to the B′-DNA complex, including the introduction of an additional bend between the TATA box and the transcriptional start site (46). B″ also generates increased DNase I sensitivity at the transcriptional start site, suggesting a role for B″ in facilitating promoter opening (31). Melting of duplex DNA at the start site appears to require interactions between Brf and B″, since mutations in either subunit can cause deficiencies in promoter opening (31). Ty3 introduces its strand transfers at nucleotides −5 and −1 (thus inserting Ty3 sequence beginning at bp −6), adjacent to the downstream end of DNA-bound Brf (42), and within the domain of the transcription bubble (39). Retroelements preferentially mediate integration into regions of DNA containing bends or kinks (47–50). The work that is presented here suggests that nucleophilic attack mediated by Ty3 integrase resembles the process mediated by retroviral integrase proteins in the sense that it favors positions with distorted base pairing or stacking but that, in the case of Ty3, protein-protein interactions play a dominant role, perhaps by converting a basal activity for nonspecific insertion to location-specific function. Further analysis will be required to know whether B″ affects the precision of Ty3 integration as well as its efficiency.

Targeting of Ty3 integration to the *SNR6* gene does not require TFIIIC in this *in vitro* system. However, TFIIIC exerts important effects on integration because it is required for load-ing TFIIIB onto tRNA genes and because it determines the binding orientation of TFIIIB on the *SNR6* gene (24). TFIIIC generates the same bias in Ty3 integration into the multiple TFIIIB docking sites of the pU6LBoxB construct (Fig. 2*B*) that it exerts on transcription. TFIIIC also facilitates access of TFIIIB to chromatin (51) and plays a key role in Ty3 integration *in vivo*; disrupting box B promoter elements greatly reduces integration (5). Thus, it is likely that, *in vivo*, proteins that make the target gene accessible for TFIIIB binding and stabilize TFIIIB-DNA interactions contribute to integration. This inference is supported by the recent isolation of a Ty3 transposition-defective yeast strain, which was shown to have a mutation causing truncation of a TFIIIC subunit.[2] Our initial analysis of sites of Ty3 integration in the absence of TFIIIC also leaves open the possibility that TFIIIC refines the precision of Ty3 targeting, perhaps by interfering with integration a few base pairs downstream of the start site of transcription (*cf.* Fig. 2*C* with Refs. 52 and 53).

In summary, the pol III transcription initiation factor TFIIIB plays a central role in Ty3 targeting. We show that B′ contributes to the targeting of Ty3 by acting as a protein-docking site for Ty3 on DNA. The binding of B″, which stabilizes B′, may also induce a structure that is conductive to Ty3 integration. The contributions of protein docking domains and DNA structure to Ty3 targeting can now be dissected, using the *SNR6* integration assay and characterized Brf and TBP mutants in conjunction with TFIIIB-independent DNA structures.

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