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Mutational Analysis of the Transcription Factor IIIB-DNA Target of Ty3 Retroelement Integration*

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The Ty3 retrovirus-like element inserts preferentially at the transcription initiation sites of genes transcribed by RNA polymerase III. The requirements for transcription factor (TF) IIIC and TFIIIB in Ty3 integration into the two initiation sites of the U6 gene carried on pU6LboxB were previously examined. Ty3 integrates at low but detectable frequencies in the presence of TFIIIB subunits Brf1 and TATA-binding protein. Integration increases in the presence of the third subunit, Bdp1. TFIIIC is not essential, but the presence of TFIIIC specifies an orientation of TFIIIB for transcriptional initiation and directs integration to the U6 gene-proximal initiation site. In the current study, recombinant wild type TATA-binding protein, wild type and mutant Brf1, and Bdp1 proteins and highly purified TFIIIC were used to investigate the roles of specific protein domains in Ty3 integration. The amino-terminal half of Brf1, which contains a TFIIB-like repeat, contributed more strongly than the carboxyl-terminal half of Brf1 to Ty3 targeting. Each half of Bdp1 split at amino acid 352 enhanced integration. In the presence of TFIIIB and TFIIIC, the pattern of integration extended downstream by several base pairs compared with the pattern observed *in vitro* in the absence of TFIIIC and *in vivo*, suggesting that TFIIIC may not be present on genes targeted by Ty3 *in vivo*. Mutations in Bdp1 that affect its interaction with TFIIIC resulted in TFIIIC-independent patterns of Ty3 integration. Brf1 zinc ribbon and Bdp1 internal deletion mutants that are competent for polymerase III recruitment but defective in promoter opening were competent for Ty3 integration irrespective of the state of DNA supercoiling. These results extend the similarities between the TFIIIB domains required for transcription and Ty3 integration and also reveal requirements that are specific to transcription.

Ty3 is a gypsy-like retroelement in *Saccharomyces cerevisiae* (1). Despite similarities between the proteins encoded by Ty3 and other gypsy-like elements and retroviruses, Ty3 has the unusual property of inserting within a few nucleotides of the transcription start site of genes transcribed by pol III,[1] including the tRNA, U6, and 5 S RNA genes. Mutations in the box A and box B promoter elements of the tRNA and U6 RNA genes that interfere with transcription also diminish transposition *in vivo*, suggesting that active targets *in vivo* must be able to bind pol III transcription factors (2).

Formation of the pol III transcription initiation complex (reviewed in Refs. 3–5) and Ty3 integration occur in close proximity to one another on DNA (2). The box A and box B promoter elements of the tRNA and *SNR6* genes serve to bind transcription factor (TF) IIIC through sequence-specific interactions with two of the six TFIIIC subunits. The TFIIIC complex acts in turn to load the transcription initiation factor TFIIIB (6–8). TFIIIB is comprised of three subunits: Brf1 (TFIIB-related factor 1), TBP, and Bdp1 (previously referred to as "B" and now designated Bdp1 for consistency with gene nomenclature (9)). *In vitro*, as described in more detail below, TFIIIB can bind to *SNR6* independently of TFIIIC (10, 11). The positions of the TFIIIC and TFIIIB subunits in promoter complexes have been mapped downstream and upstream, respectively, of the initiation site by cross-linking analysis (12, 13). Ty3 strand transfer occurs at a site that is located between the positions occupied by the TFIIIC 120-kDa subunit (Tfc4) on the downstream side and by the TFIIIB Bdp1 and Brf1 subunits on the upstream side. The strand transfer of the Ty3 3′ end to the transcribed strand is typically between bp +1 and −1, whereas the strand transfer on the non-transcribed strand is between bp −5 and −6 (14, 15), within the DNA segment that is strand-separated in the pol III open promoter complex (16).

The *in vitro* requirements for Ty3 integration into tRNA genes have been probed using TFIIIC and TFIIIB. In the *in vitro* integration reaction, virus-like particles formed in yeast cells overexpressing Ty3 act as the source of integrase and full-length, extrachromosomal Ty3 DNA (17). The level of Ty3 integration into a plasmid-borne target in the presence of various test proteins is monitored by PCR. Transposition into a tRNA gene type target was shown to require TFIIIC and TFIIIB. Integration was negatively affected by pol III, indicating that Ty3 might resemble pol III in its requirements for target access but that transcription initiation *per se* is not required (18).

The promoter structure of the U6 RNA gene *SNR6* differs from that of most tRNA genes in that it contains an upstream TATA element (7, 19). Although *SNR6* expression *in vivo* requires TFIIIC, *in vitro* the strong *SNR6* TATA box can directly mediate binding of TFIIIB through its TBP subunit. *SNR6* can then be transcribed by pol III, independently of TFIIIC (11, 20).

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[1] The abbreviations used are: pol III, RNA polymerase III; TF, transcription factor; TBP, TATA-binding protein; r-, rightward; l-, leftward.

In the latter context, TFIIIB binds to the nearly symmetric *SNR6* TATA element in either orientation, and this can be monitored by transcription of a plasmid construct containing divergent transcription units (21, 22). When TFIIIB and TFIIIC are present together, TFIIIC orients TFIIIB so that initiation occurs predominantly at the *SNR6*-proximal site. Using a modification of the *in vitro* integration assay described above, it was shown that, similar to what is observed for transcription initiation, TFIIIB is sufficient to support Ty3 integration at the *SNR6* transcription initiation site. In the presence of TFIIIC, Ty3 integration is specified by the predominant TFIIIB orientation (23).

The ability to assemble TFIIIB sequentially on the *SNR6* gene, together with knowledge of the TBP-DNA crystal structure and the availability of recombinant wild type and mutant proteins, has made it possible to delineate the roles of specific TFIIIB domains in pol III transcription initiation. TBP binds through sequence-specific interactions, sharply kinking DNA at both ends of its binding site (24, 25). Amino- and carboxyl-terminal halves of Brf1 interact with the carboxyl- and amino-terminal lobes of TBP, respectively, and contact the DNA on either side of the TBP-binding site to form the B′ complex (26–29). Bdp1 binds primarily through contacts with the carboxyl-terminal half of Brf1, stabilizes the complex, and probably brings DNA segments flanking the TATA element into closer proximity of one another. In the case of templates bound by TFIIIC, evidence suggests that both Brf1 and Bdp1 interact with TFIIIC (3, 13, 30, 31). Brf1 and Bdp1 each contact pol III, although the primary specific contacts appear to occur through Brf1 (32–35). The apparently secondary role of Bdp1 is underscored by the observation that minimal transcription complexes supporting pol III initiation can be formed from TBP and Brf1 alone on DNA that is premelted at the initiation site (36). These results support the model that Bdp1 plays a primarily post-recruitment role in formation of the open transcription complex. Indeed, pol III transcription initiation complexes formed with certain Bdp1 mutants are defective at the promoter opening step (4, 37). Using the *in vitro* integration system, it was previously shown that detectable *SNR6* transposition targeting occurs with B′ alone but that the level of transposition is significantly increased by the addition of Bdp1 (23). Whether Bdp1 plays a significant role by stabilizing the transcription complex for targeting or by producing a local DNA structure that is conducive to integration was not determined.

Insights concerning the roles of specific TFIIIB subunits and domains in pol III transcription provide a useful backdrop for designing experiments to probe the mechanism of the Ty3 integration reaction and explore the extent to which it resembles pol III recruitment and transcription initiation. The current study was undertaken using *SNR6*, highly purified TFIIIC, and recombinant wild type and mutant TFIIIB subunits to address the following questions: Are the same domains in Brf1 and Bdp1 required for integration and transcription? Is the Bdp1 post-recruitment function in promoter opening required for Ty3 integration? How do interactions between TFIIIB and TFIIIC affect the pattern of Ty3 integration sites *in vitro*? The results of these studies extend the similarities in protein-DNA complex requirements between Ty3 integration and pol III transcription initiation but identify interesting distinctions as well.

MATERIALS AND METHODS

Strains and Growth Conditions—Standard methods were used for culturing and transforming *Escherichia coli* and *S. cerevisiae* (38). All plasmids were amplified in and prepared from *E. coli* HB101 (*F− hsdS20 (r_B^- m_B^-) recA13 leuB6 ara-14 proA2 lacY1 galK2 rpsL20 (sm^r) xyl-5 mtl-1 supE44*λ^-). Ty3 was expressed in *S. cerevisiae*, NOY384, a gift from M. Nomura (University of California, Irvine) and transformed with the high copy galactose-inducible plasmid, pEGTy3-1 (39).

Plasmid Constructions—Recombinant DNA constructions and methods followed standard procedures (38). Plasmid pEGTy3-1 was used for galactose-inducible expression of Ty3 (39). Plasmid pLY1855 (23) was the target for Ty3 integration *in vitro*. Plasmids pDLC370 (2) and pLY1842 (23) served as PCR controls for integration into r-U6 and l-U6, respectively. Plasmid pDLC370 contains a Ty3 insertion upstream of *SNR6* at r-U6, and plasmid pLY1842 is a clone containing an amplified fragment templated from a Ty3 insertion at l-U6.

Supercoiled target DNA was prepared by centrifugation twice over cesium chloride density gradients, followed by chromatography over Sepharose CL2B. DNA was extracted with isopropanol saturated with cesium chloride followed by precipitation using standard methods. Linear integration targets were prepared by digesting pLY1855 (purified as described above) with the restriction endonuclease *Hin*dIII. Digested DNA was extracted with phenol:chloroform and precipitated. DNA was resuspended in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. Linear DNA was checked for complete digestion with agarose gel electrophoresis.

Protein Preparations—Ty3 virus-like particles were prepared as described from yeast strain NOY384 transformed with pEGTy3-1 (40). Highly purified TFIIIC (oligobox B+ fraction) and pol III (MonoQ fraction) were purified as described (6). Purified wild type, recombinant proteins were quantified as active molecules in specifically initiating transcription (pol III) or specific DNA binding (TBP, Brf1, Bdp1, and TFIIIC) as described or referenced (41). TBP and Bdp1 were fully active; Brf1 was ~20% active. Amounts of Brf1 refer to total protein. The recombinant split Brf1 and Bdp1 used in these experiments were shown to have transcription activities on supercoiled and linear templates singly and in combination as previously reported or as indicated under "Results" (data not shown).

Wild type and internally deleted Bdp1 proteins were carboxyl-terminally His_6-tagged and purified under native conditions through nickel-nitrilotriacetic acid-agarose, Bio-Rex 70, and Superose 12 as described previously for Bdp1(138–596) by Kumar *et al.* (33). Bdp1(224–487), Bdp1(1–352), and Bdp1(352–594) were amino-terminally His_6-tagged and purified under native conditions through the nickel-nitrilotriacetic acid-agarose step. Wild type Brf1 (amino- and carboxyl-terminally His_6-tagged) and Brf1 deletion proteins (amino-terminally His_6- or His_7-tagged) were purified under denaturing conditions on nickel-nitrilotriacetic acid-agarose (and on Superose 6 for Brf1(1–282) and Brf1(284–596)), followed by stepwise dialysis out of urea as specified in Refs. 36 and 41. TBP was purified and quantified as described (11). Quantities of mutant Brf1 and Bdp1 are specified as fmol of protein determined by Coomassie staining against bovine serum albumin standards on gels.

In Vitro Integration into SNR6 Targets—*In vitro* integration with wild type proteins was performed as described previously (23) except where noted otherwise. Where added, TFIIIC (100 fmol) was complexed with 150 fmol of target DNA for 10 min in integration reaction buffer prior to addition of TFIIIB (50, 180, and 75 fmol of TBP, Brf1, and Bdp1 protein, respectively). The reaction volumes were 25 or 50 μl as noted. TFIIIB components were allowed to form complexes with target DNA for 60 min at 23 °C. At the end of this time, components were shifted to 15 °C, 2.2 or 5 μg (protein) of Ty3 virus-like particle fraction (depending on activity) were added, and the incubation was allowed to proceed for 10–15 min. The reaction samples were treated with proteinase K and extracted with phenol:chloroform. DNA was precipitated with ethanol.

PCR with primer 242, which anneals within the *SNR6* gene, and with primer 411, which anneals at the downstream end of the internal domain of Ty3, was used to amplify diagnostic fragments from one-fifth of the integration reaction volume (23). The reactions were initiated with a 95 °C polymerase activation step for 12 min, followed by 40 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 60 s. The 72 °C elongation step was extended by 3 s/cycle. The reaction was terminated with a 72 °C incubation for 5 min, after which the sample was brought to 4 °C. To control for consistent DNA recovery from the integration reaction and for consistent operation of the above PCR, primers 679 and 680 (23) were used to amplify the β-lactamase gene carried by the target plasmid (data not shown). This PCR amplification was performed with 0.03% of the content of each integration reaction and with 200 ng of each primer for 19 cycles of polymerization. PCR products were resolved by electrophoresis on a nondenaturing 8% polyacrylamide gel and visualized by staining with ethidium bromide.

Integration Reactions Using SNR6 Targets and Mutant TFIIIB Proteins—For integration reactions with the split Brf1 proteins and Brf1 Δ1–68Δ383–424, Brf1 Δ383–424, wild type Brf1 (1 pmol), TBP (200 fmol), and Bdp1 (200 fmol) were used in 25-μl reactions. Each of the mutant Brf1 proteins was used at 200 fmol. Integration reactions con-

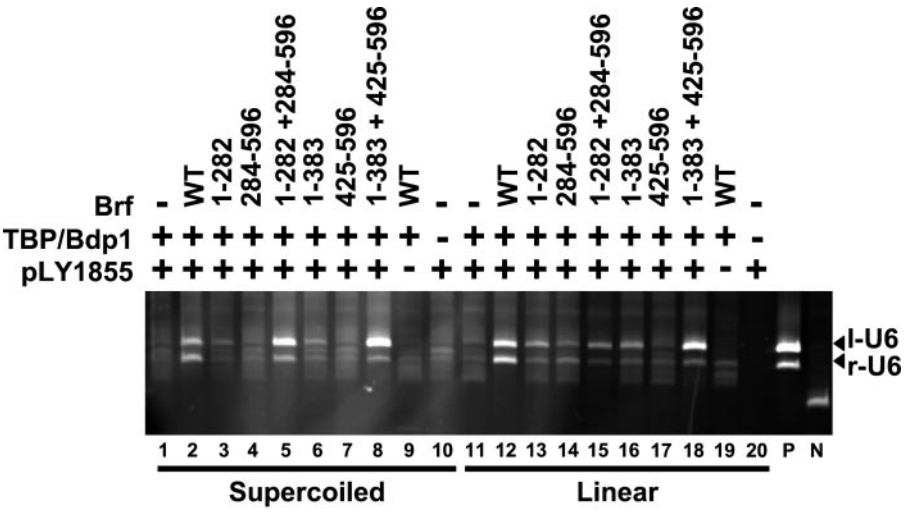

FIG. 1. **The amino-terminal half of Brf1 contains important determinants for Ty3 specific integration into supercoiled and linearized target DNA.** Integration reactions were performed with full-length Brf1 (wild type (*WT*)) or with split Brf1(1–282, 284–596, 1–383, and 425–596) into supercoiled (*lanes 1–10*) and linearized (*lanes 11–20*) *SNR6* target pLY1855 as indicated *above* each *lane*. The presence of wild type TBP, Bdp1, and DNA is also indicated. PCR-amplified integration products separated on a nondenaturing polyacrylamide gel and stained with ethidium bromide are shown. l-U6 and r-U6 integration-templated PCR fragments are labeled on the *right* with *arrowheads*. *Lane P*, products of a positive control PCR reaction using a mixture of plasmids containing Ty3 insertions at l-U6 and r-U6; *lane N*, negative control containing target plasmid pLY1855 alone.

taining Bdp1 half proteins were performed with 150 fmol of Bdp1, 200 fmol of TBP, and 1 pmol of wild type Brf1 in a 25-μl reaction. For Bdp1 internal deletion proteins and Bdp1(224–487), 25 or 50 fmol of mutant protein were used, as indicated for specific experiments, in combination with 50 fmol of TBP and 180 fmol of Brf1 in a 25-μl reaction.

Integration Ladder—In vitro integration events into a *SNR6* target plasmid were amplified as described above using primers 242 and 411. PCR reaction product DNA was digested with restriction enzymes *Xho*I and *Nru*I for 1 h at 37 °C. *Xho*I cleaves within Ty3, 19 bp upstream of the site of integration on the non-transcribed strand; *Nru*I cleaves within *SNR6*, 5 bp downstream of the start site of transcription. Integration downstream of bp +3 on the non-transcribed strand would not be monitored in this assay. Following digestion, reaction mixtures were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. DNA pellets were resuspended in buffered formamide containing tracking dyes. One-third and two-thirds of the sample were used to examine the l-U6 and the r-U6 integration target sites, respectively.

A sequencing ladder was generated using the method of Sanger *et al.* (42) with 5′-^{32}P-end-labeled primer 242 and pLY1855 or pU6LboxB template. Digested DNA from PCR reactions and the sequence ladder fragments were resolved on an 8% 8 M urea sequencing gel. Regions of the gel containing the digested integration products were transferred to nitrocellulose membrane using a semi-dry transfer apparatus and UV cross-linked, and the PCR products were visualized by hybridization with 5′ end-labeled oligonucleotide 451, which is complementary to the plus strand at the downstream (U5) end of the Ty3 element and exposed to a phosphorimaging screen. The length of the hybridized fragment estimated from the sequencing ladder allowed inference of the distance of the Ty3 strand transfer position on the non-transcribed strand from the transcription initiation site.

RESULTS

The Amino-terminal Half of Brf1 Contains Primary Determinants of Ty3 Integration into the SNR6 Gene—B′, comprised of the TFIIIB subunits Brf1 and TBP, was previously shown to be sufficient to mediate a low level of specific Ty3 integration. Of these two subunits, only Brf1 is specific to the pol III transcription initiation complex, suggesting that it may contribute directly to Ty3 targeting. In the case of pol III transcription on the *SNR6* template pU6LboxB, it has been shown that the amino-terminal half of Brf1(1–282) supports transcription in the context of TFIIIB on supercoiled but not linear templates (37). In contrast, the carboxyl-terminal half of Brf1 forms stable TFIIIB-DNA complexes but is transcriptionally nearly inactive on supercoiled DNA (41). The amino-terminal and carboxyl-terminal Brf1 half proteins together reconstitute transcription of linear and supercoiled DNA. To better define the domains of Brf1 involved in Ty3 targeting, integration reactions were performed using half Brf1 proteins in which the TFIIB-like and conserved carboxyl-terminal domains could be evaluated separately. Complete recombinant Brf1 and combinations of proteins representing the amino-terminal segments of Brf1(1–282 or 1–383) and carboxyl-terminal segments (284–596 or 425–596) were used alone or as combined amino- and carboxyl-terminal parts. Supercoiled and linear target DNAs were evaluated. Incubation of supercoiled target DNA with TFIIIB for 60 min at 23 °C followed by addition of virus-like particles and 10 min of incubation at 15 °C left 50% of the DNA supercoiled DNA (data not shown). Because Ty3 integration in the absence of Bdp1 is significantly less efficient, these reactions were performed in the presence of Bdp1. Accordingly, these experiments address the relative contributions of different Brf1 domains but not the minimum requirements for Ty3 integration.

Reaction mixtures contained recombinant TBP, Bdp1, mutant Brf1, and plasmid pLY1855. The pLY1855 plasmid is a derivative of pU6LboxB (23) that contains a modified *SNR6* gene with altered flanking sequence and a gene-internal boxB promoter element optimally placed for TFIIIC binding. In this construct the TATA box is inverted. Although both the l-U6 and r-U6 initiation sites are used, TBP is preferentially bound in the orientation that supports leftward transcription (22). After completion of the incubation, the reaction samples were extracted and processed for PCR, using primers to amplify target DNA containing Ty3 insertions. The products of the PCR reaction were fractionated by gel electrophoresis in nondenaturing polyacrylamide gels, stained with ethidium, and photographed (Fig. 1). As noted previously (23), two TFIIIB-dependent PCR products were generated on this template (Fig. 1, compare *lanes 2* and *12* with lanes lacking one or more components necessary for *de novo* integration (*lanes 1*, *9–11*, *19*, and *20*)). The *upper* and *lower bands* represent integration into the l-U6 and r-U6 initiation sites, respectively. Although the relative yield of PCR product between reactions of a given experiment was reproducible, this assay should be considered semi-quantitative, because it was not possible to accurately estimate the relative specific activities of the mutant proteins. In the absence of either Brf1 or TFIIIB, a somewhat random and dispersed background of integration events was observed (Fig. 1, *lanes 1*, *10*, and *11*). Brf1(1–282) and Brf1(1–383) supported specific integration on supercoiled DNA and a greater amount of specific integration on linear DNA targets (Fig. 1, *lanes 3*, *6*, *13*, and *16*; this is most readily apparent at the l-U6 initiation site, which is less obscured by the TFIIIB-independent background). The Brf1(284–596) and Brf1(425–596) carboxyl-terminal segments failed to support specific integration on supercoiled targets (Fig. 1, *lanes 4* and *7*) at a level significantly above background, but they greatly enhanced integration when

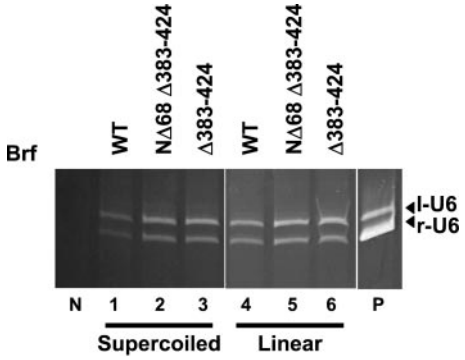

FIG. 2. **The amino-terminal 68 amino acids of Brf1 containing a putative zinc ribbon domain are dispensable for specific integration.** Integration was performed with wild type (*WT*) Bdp1 or TBP, and Brf1Δ383–424, or Brf1 NΔ68 Δ383–424 as indicated *above* each *lane*. The amplified products of specific integration are marked on the *right* with *arrowheads*. *Lanes N* and *P* are as described in the Fig. 1 legend.

combined with Brf1(1–282) and Brf1(1–383), respectively (Fig. 1, *lanes 5* and *8*). Brf1(425–596) likewise failed to support significant specific integration on a linear target (*lane 17*), but integration above background was detected with Brf1(284–596) (*lane 14*). Thus, the portion of Brf1 containing the TFIIB-related putative zinc ribbon, two TFIIB-like repeats, and the primary pol III interaction domain bears a major determinant for position-specific integration in a reaction also containing TBP, Bdp1, and DNA. The Brf1 segment from amino acids 284 to 424, which contains fungal homology region l may also contain a determinant for specific integration.

The Zinc Ribbon Domain of Brf1 Is Not Required for Ty3 Integration into SNR6—Two motifs in the amino-terminal domain of Brf1 contribute to initiation of transcription: a putative amino-terminal zinc ribbon domain and the two TFIIB-related imperfect repeats. Disruption or removal of the zinc ribbon domain of Brf1 generates TFIIIB-DNA complexes that recruit pol III to relaxed DNA templates but display a severe defect in open complex formation. Combination with promoter opening-defective Bdp1 deletions eliminates transcription on supercoiled DNA templates as well (5, 43, 44). The amino-terminal zinc ribbon also appears to be essential for transcription in the minimal pol III transcription system consisting of pol III, Brf1, TBP, and a "preopened" promoter template (36). The Δ383–424 deletion, which removes sequence that is not present among fungal homologues, improves the transcriptional activity of Brf1 *in vitro* (36). To determine whether Ty3 integration is sensitive to the function provided by the zinc ribbon, recombinant Brf1 lacking the amino-terminal 68 amino acids containing the zinc ribbon and amino acids 383–424 (*NΔ68,Δ383–424*) and Brf1 lacking only the internal domain (*Δ383–424*) were tested for the ability to support integration (Fig. 2). Integration was supported by both of these deletion proteins to comparable extents on supercoiled and linear DNA (Fig. 2, compare *lanes 2* and *5* with *lanes 3* and *6*).

Bdp1 Halves Are Redundant for Enhancement of Ty3 Integration into a TFIIIB-DNA Target—The role of Bdp1 in transcription complex formation appears to include a scaffolding function that locks the complex together (4, 33). Binding of Bdp1 to the B′-DNA complex is accompanied by an upstream extension of the DNase I footprint, the introduction of an additional bend between the TATA box and the initiation site, and stabilization of the protein-DNA complex. Nevertheless, deletion analysis has so far failed to identify any specific portion of the protein that is essential for the extended footprint (33). Although B′ alone is sufficient to support Ty3 integration at *SNR6* at a low level, the addition of Bdp1 increases integration, suggesting that Bdp1 introduces additional contacts for the Ty3 preintegration complex, stabilizes the target complex, or changes its conformation (23).

To more specifically define the requirement for Bdp1, recombinant Bdp1(1–352) and Bdp1(352–594) were tested together and separately for activity in Ty3 integration. These proteins support transcription of supercoiled *SNR6* templates together and separately.[2] Bdp1(1–352) and Bdp1(352–594) both supported integration into supercoiled DNA (Fig. 3, compare *lanes 3* and *4* with *lane 1*). In addition, Bdp1 split proteins were tested in reactions with linear DNA. These reactions showed that on linear DNA the half and combined proteins also performed in a manner comparable with that of the wild type Bdp1 (Fig. 3, compare *lanes 10* and *11* with *lane 9*). These results suggest that Ty3 integration, similar to pol III transcription, is not dependent upon Bdp1 for a single contact. Either Bdp1 must have a structural role that does not involve specific contacts, or each part of Bdp1 individually provides a contact that makes the other part nonessential.

TFIIIC Interacts with B′ and Bdp1 to Influence Ty3 Integration Site Selection—TFIIIC is required for *SNR6* transcription and Ty3 integration *in vivo* (2, 19, 46). Although TFIIIC is dispensable for *SNR6* transcription *in vitro* with purified components, TFIIIC-mediated assembly of TFIIIB onto the *SNR6* TATA box specifies a single orientation of TFIIIB for transcription (22). Analysis of Bdp1 function *in vitro* has shown that TFIIIC-dependent transcription exhibits greater dependence upon functions in Bdp1 not required for TFIIIC-independent transcription (33). In particular, certain Bdp1 deletion mutants that are permissive for TFIIIC-independent transcription of supercoiled DNA assemble aberrant TFIIIB-TFIIIC-DNA complexes on TFIIIC-dependent promoters that are transcriptionally deficient or fail (entirely) to assemble these complexes. The experiments that are described next used supercoiled and linear DNA to explore the effect of TFIIIC on Ty3 integration at *SNR6* directed by B′ and also by TFIIIB constituted with wild type Bdp1 or internal deletion mutants of Bdp1.

The effect of TFIIIC on Ty3 integration directed by B′ and TFIIIB was examined first (Fig. 4). There was no major difference in the distribution of integration sites between supercoiled and linear templates (Fig. 4*A*, compare *lanes 1–4* with *lanes 5–8*). As previously observed (23), integration in the presence of B′ alone was primarily into the l-U6 initiation site (Fig. 4*A*, *lanes 1* and *5*), whereas integration in the presence of TFIIIB was more evenly distributed between the l-U6 and r-U6 sites (Fig. 4*A*, *lanes 3* and *7*). This difference has been ascribed to weaker DNA binding of the B′ complex, which allows equilibration toward the optimum orientation of the B′ complex at the TATA box, whereas entry of Bdp1 into the B′ complex prevents dissociation, trapping the initial orientation of the B′ complex. As previously shown (23), integration in the presence of TFIIIC and TFIIIB showed a dramatic shift to the r-U6 initiation site (Fig. 4*A*, *lanes 4* and 8), consistent with TFIIIC orienting TFIIIB to favor initiation of r-U6 transcription into the U6 gene. In contrast, integration in the presence of TFIIIC and B′ generated a small decrease in l-U6 integration on the supercoiled template and greater decrease in integration on the linear template but did not show the dramatic increase in r-U6 integration shown in the presence of Bdp1 (compare Fig. 4*A*, *lanes 1* and *5* with *lanes 2* and *6*). This result could be interpreted to suggest that TFIIIC does not affect the orientation of B′ in the absence of Bdp1, but because entry of Bdp1 is dependent on the prior formation of the B′-TFIIIC-DNA complex (47), this is unlikely. The presence of significant integration at l-U6

[2] A. Kumar and G. Kassavetis, unpublished data

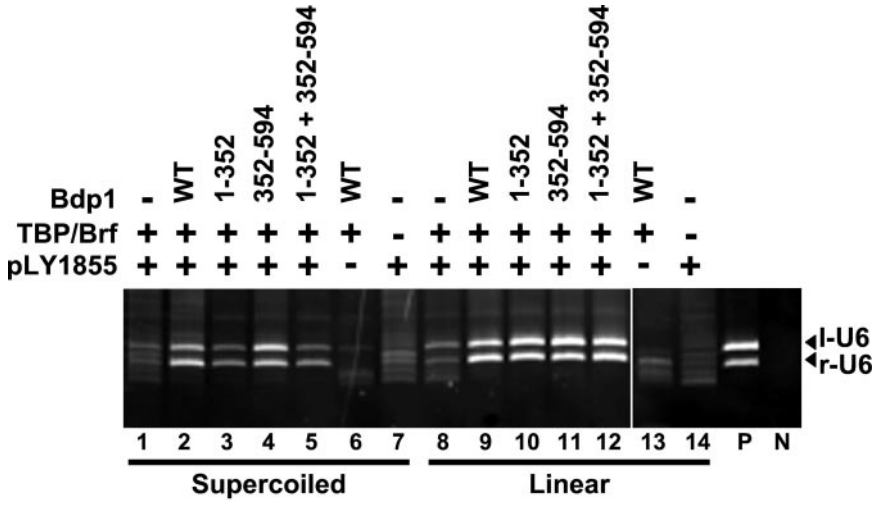

FIG. 3. **Split Bdp1 in Ty3 integration.** Wild type (*WT*) and split Bdp1 proteins (1–352 and 352–594) were used for integration assays with wild type Brf1 and TBP on supercoiled (*lanes 1–7*) and linear plasmid DNA (*lanes 8–14*), as indicated *above* each *lane*. *Lanes N* and *P* correspond to negative and positive controls, as described in the legend to Fig. 1. Specific integration events are indicated on the *right*.

may indicate that not all of the templates contain B′ and TFIIIC. The absence of integration at r-U6 could stem from the fact that DNA surrounding the start site of transcription in B′-TFIIIC-DNA complexes is occluded by TFIIIC from attack by integrase; in the case of transcription, Bdp1 is required to lift TFIIIC from this site for transcription initiation to occur (47). The additional decrease in l-U6 integration observed here in the presence of Bdp1 (Fig. 4*A*, compare *lanes 2* and *6* with *lanes 4* and *8*) could reflect the greater stability of the TFIIIB-TFIIIC complex compared with the B′-TFIIIC complex, trapping more of the target in this form.

The site of Ty3 integration *in vivo* is precisely defined. It was of interest to determine whether the specificity of integration was conserved *in vitro* in the context of minimal integration targets. To gain information concerning the overall distribution of integration sites, PCR products were digested with *Xho*I and *Nru*I to remove both DNA ends, leaving an internal fragment the size of which was proportional to the distance of the integration site from the duplicated *SNR6* transcription initiation sites (see "Materials and Methods"). These fragments were fractionated by electrophoresis on sequencing gels, transferred to nitrocellulose (48), and probed with a ^{32}P-labeled oligonucleotide that anneals to the end of the Ty3 element to visualize only one DNA strand. The PCR products of integration into the l-U6 and r-U6 transcriptional initiation sites separated into more slowly (l-U6) and more quickly (r-U6) migrating sets of fragments. The locations of integration sites were deduced from sequencing ladders and from parallel experiments with positive control plasmids pDLC370 and pLY1842, containing sequenced sites of integration at l-U6 and r-U6 (Fig. 4*B*). The results of this analysis showed that integration in the presence of B′ or TFIIIB occurred at one major site for l-U6 (Fig. 4*C*, *lanes 1–8*) and at two sites for r-U6 (Fig. 4*D*, *lanes 1–3* and *5–7*). (Note that the relative amounts of l-U6 and r-U6 radioactivity do not reflect the distribution of integration events, because different amounts of PCR product were loaded on each gel to obtain nearly equivalent radioactive signals.) Fragments generated in the restriction digestion of the PCR reaction were offset by one nucleotide from the major *in vivo* site of integration on the positive control r-U6 plasmid, but corresponded to sites that are also used *in vivo*. Integration sites were also mapped to identify the effects of TFIIIC on Ty3 integration site usage in the presence of B′ and TFIIIB (Fig. 4, *C* and *D*). There was no redistribution of residual integration sites at l-U6 caused by TFIIIC. TFIIIC also had no effect on the pattern of integration at r-U6 in the presence of B′. However, the pattern of integration at r-U6 in the presence of TFIIIC and TFIIIB was dramatically different from that of TFIIIB alone, with integration sites spread downstream into *SNR6*, from −7/−3 to −1/+4 (non-transcribed/transcribed strands). This pattern contrasted with the positions of sites observed *in vivo* (predominantly at positions −6/−2 and −7/−3).

Mutations in Bdp1 That Affect Open Complex Formation Do Not Affect Ty3 Integration—The roles of specific Bdp1 domains in TFIIIC-dependent and TFIIIC-independent integration can be further defined using Bdp1 internal deletion mutants. Analysis of the effect of a set of such mutants on pol III transcription *in vitro* (4, 33, 44) identified an internal segment defined by mutants Bdp1Δ355–372, Δ372–387, Δ388–409, and Δ409–421, within which deletions do not eliminate the ability of TFIIIB to recruit polymerase but do interfere with formation of the open promoter complex. This domain is thus implicated either in isomerization of the polymerase or in DNA duplex destabilization (37). DNA structure has been found to affect integration activity of retroviral integrases (49–51). Thus, Bdp1 containing a deletion within this defined region offered an interesting *in vitro* test of the potential role of Bdp1 in creating a specific structure required by the Ty3 integrase for activity. Bdp1Δ355–372 was tested on linear and supercoiled *SNR6* targets. It stimulated integration well over the levels observed with B′ alone on both templates, with no significant change in distribution between l-U6 and r-U6 initiation sites (Fig. 5, *lanes 4* and *12* relative to *lanes 1* and *9*).

Two domains of Bdp1 (I and II) are protected from hydroxyl radicals upon entry into theTFIIIB-*SUP4* complex (33). Interactions involving domains I and II are required on an either/or basis for TFIIIC-independent transcription, but both are required for TFIIIC-dependent transcription. Bdp1 deletions in these domains were used to test whether domain I or II was required for the Bdp1 enhancement of specific integration over activity of B′ alone in the absence of TFIIIC. Bdp1Δ272–292 and Bdp1Δ424–438, representing deletions in regions II and I, respectively, were shown to be as active as wild type Bdp1 for TFIIIC-independent integration into linear and supercoiled *SNR6* gene targets (Fig. 5, compare *lanes 3* and 5 with *lane 2* and *lanes 11* and *13* with *lane 10*). The finding that domains I and II of Bdp1 were not individually required for TFIIIC-independent integration is congruent with prior analysis of transcription.

Bdp1Δ424–438 fails in TFIIIC-dependent transcription because it does not assemble into the B′-TFIIIC-DNA complex. Bdp1Δ272–292 assembles into the B′-TFIIIC-DNA complex but fails in TFIIIC-dependent transcription because it does not displace TFIIIC from the initiation site so as to allow pol III access (33). The effects of wild type Bdp1, Bdp1Δ424–438, and Bdp1Δ272–292 on Ty3 integration in the presence of TFIIIC

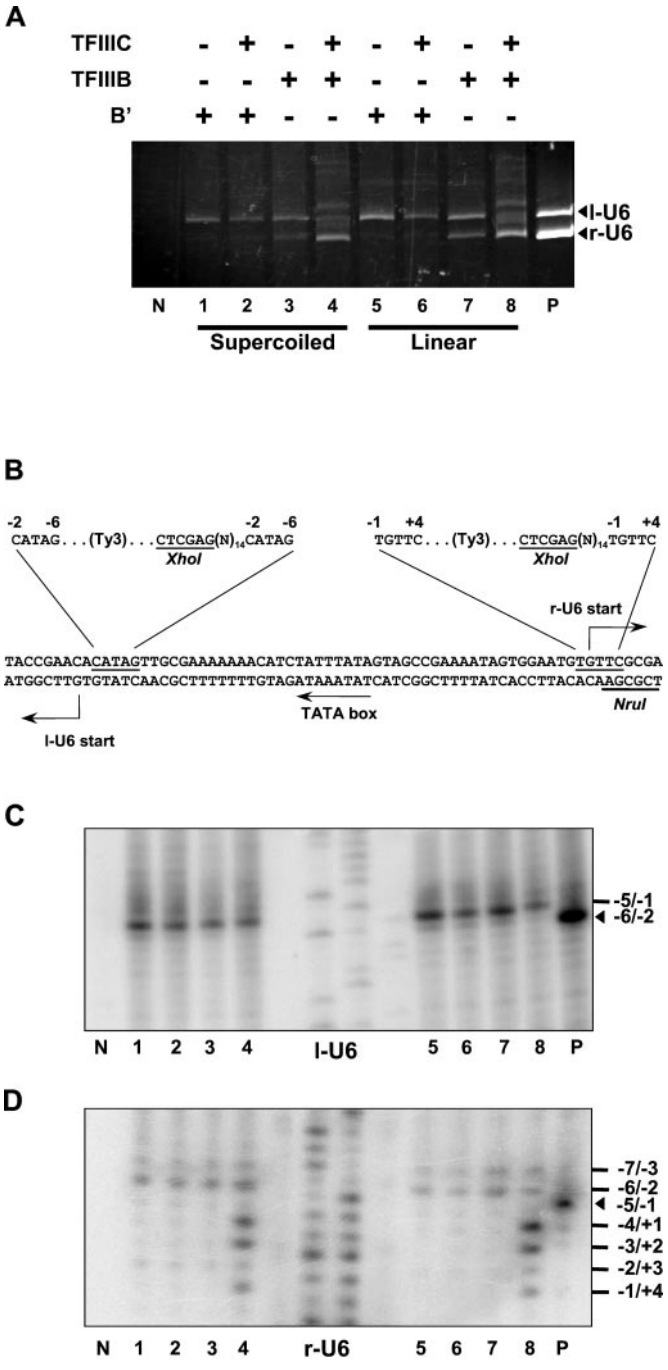

FIG. 4. **TFIIIC shifts the pattern of Ty3 integration.** *A*, integration reactions contained TFIIIC, B′, and TFIIIB, as indicated *above* each *lane*, with supercoiled (*lanes 1–4*) or linearized (*lanes 5–8*) plasmid DNA targets. l-U6 and r-U6 integration products are indicated with *arrowheads*. *B*, the sequence shown represents the integration region at the U6-l and U6-r transcription initiation sites on pLY1855. Integration reaction DNA was amplified by primers in Ty3 and *SNR6* (not shown), cleaved with *Xho*I and *Nru*I, and fractionated together with a sequencing ladder by gel electrophoresis as described under "Materials and Methods." The plus strand (*top*) was visualized by probing with a radioactive minus strand oligonucleotide. The length of the plus strand fragment was determined by comparison with a sequencing ladder and used to infer the positions of integration indicated in *C* and *D*. *C* and *D*, Southern blots of restriction endonuclease-cleaved PCR products corresponding to positions of integration at l-U6 (*C*) and r-U6 (*D*). *Lanes 1–8* and *lanes P* and *N* correspond to the samples analyzed in *A*. Negative (*N*) and positive (*P*) controls are described in the Fig. 1 legend. The correspondence between PCR product size and sites of Ty3 strand transfer is shown on the *right*. The *unlabeled lanes* are sequencing ladders (described under "Materials and Methods") used to determine the sizes of hybridizing fragments.

were compared in order to determine whether TFIIIC would prevent integration by virtue of start site occlusion or whether the presence of Bdp1 in the TFIIIB complex and integrase together would suffice for TFIIIC displacement (Fig. 6). Integration in the presence of wild type TFIIIB alone produced more integration into the l-U6 than into the r-U6 initiation site (Fig. 6*A*, *lane 1*). As expected, TFIIIC redistributed this pattern to favor the r-U6 initiation site with new sites of integration downstream (Fig. 6, *A* and *B*, *lanes 2*). Redistribution did not occur in reactions containing Bdp1Δ272–292, which assembles into the B′-TFIIIC-DNA complex (*lanes 3*) or, as expected, in the presence of Bdp1Δ424–438 (*lanes 5*), which does not assemble into a B′-TFIIIC-DNA complex. The core amino acid 224–487 fragment, which retains competence for TFIIIC-dependent transcription (33), yielded less integration but significantly redistributed integration sites in response to TFIIIC (Fig. 6*B*, compare *lanes 1* and *6*).

The observation that the presence of TFIIIC generates unique sites of integration at r-U6 (Fig. 4*D*) clarifies the analysis of TFIIIC effects on integration (Fig. 6*B*), because it substitutes a qualitative effect for a quantitative assessment that is burdened with a substantial background. TFIIIC generated downstream integration events with TFIIIB-DNA complexes containing wild type Bdp1, Bdp1Δ355–372, and Bdp1(224–487) (Fig. 6*B*, compare *lanes 2*, *4*, and *6* with *lane 1*) but not with TFIIIB-DNA complexes containing Bdp1Δ272–292 (*lane 3*) or Bdp1Δ424–438 (*lane 5*). These results imply a requirement for Bdp1-mediated displacement of TFIIIC from the site of Ty3 integration.

DISCUSSION

These experiments define the roles of Bdp1 and B′ domains in position-specific integration of Ty3 and extend the parallels between Ty3 targeting and recruitment to the stable transcription initiation complex. Distinctions are identified between requirements for pol III transcription initiation and Ty3 integration for the first time. Unexpectedly, our findings suggest that *in vitro* interactions between TFIIIB and TFIIIC lead to a characteristic pattern of Ty3 integration extending just downstream of the initiation site. This pattern is not observed *in vivo* or in the absence of TFIIIC *in vitro*. The findings are summarized in Table I, and the implications are discussed below.

Requirements for Ty3 Integration Resemble Those for pol III Recruitment—The location of Ty3 integration sites and the protein requirements of Ty3 targeting resemble those for pol III recruitment to the promoter. Initiation of transcription at position +1 follows the sequential stages of promoter opening that unpair a DNA segment extending from bp −9 to bp +7 (52). Ty3 strand transfer occurs on the transcribed strand between +1 and −1 and on the non-transcribed strand between −5 and −6 (14), within the DNA segment that is eventually unwound by pol III. Previous work identified the B′-DNA complex as the minimal target for Ty3 integration at *SNR6 in vitro*. The observation that pol III is actually inhibitory to Ty3 integration *in vitro* (18) suggested that some of the contacts in Brf1 used in recruitment of pol III might also be used in Ty3 targeting. Thus, it was of interest to investigate the extent to which pol III transcription initiation complex and Ty3 strand transfer share determinants at the resolution level of specific protein domains.

In the work described here, mutant Brf1 and Bdp1 proteins, previously characterized for pol III transcription, and highly purified TFIIIC were used to define protein domains required for Ty3 integration and to better understand the contributions of TFIIIB and TFIIIC to targeting. Analysis of the Ty3 targeting activity at *SNR6* of two pairs of Brf1 amino- and carboxyl-terminal fragments (1–282, 284–596, 1–383, and 425–596) in

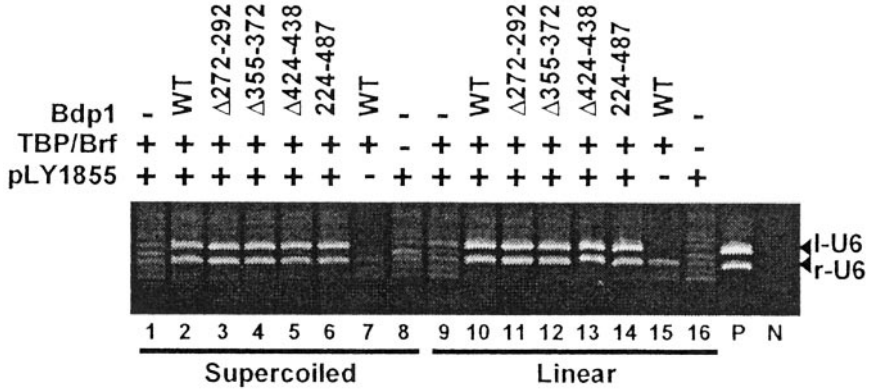

FIG. 5. **Integration reactions mediated by TFIIIB reconstituted with mutant Bdp1 proteins.** The reactions contained wild type (*WT*) Bdp1 (75 fmol), Bdp1Δ272–292 (50 fmol), Bdp1Δ355–372 (25 fmol), Bdp1Δ424–438 (25 fmol), or Bdp1(224–487) (50 fmol) with wild type Brf1 and TBP, as listed *above* each *lane*. Integration into supercoiled (*lanes 1–8*) and linear (*lanes 9–16*) target plasmid pLY1855 was tested. The *arrowheads* indicate integration products at the l-U6 and r-U6 target sites. *Lanes P* and *N* are the positive and negative controls as described in the Fig. 1 legend.

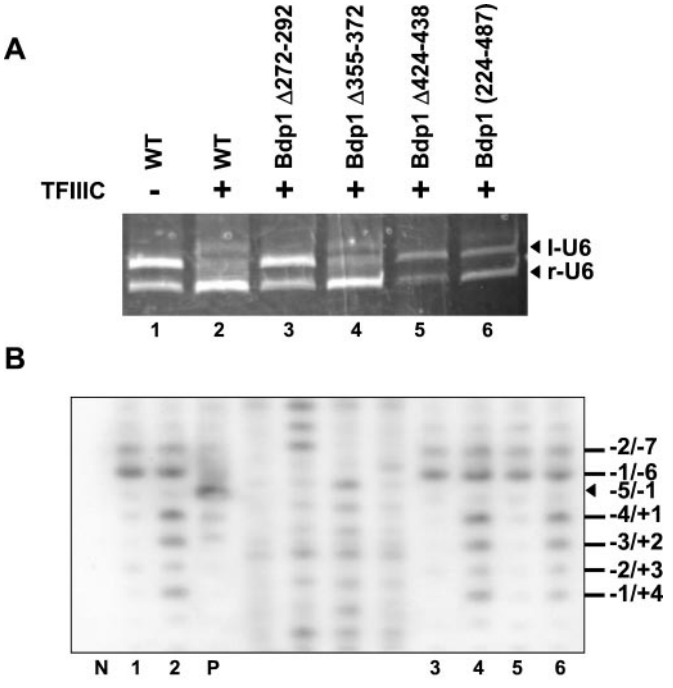

FIG. 6. **TFIIIC-dependent integration into supercoiled pLY1855 in the presence of Bdp1 mutant proteins.** *A*, integration reactions mediated by combinations of mutant Bdp1 and TFIIIC. All reaction mixtures contain wild type (*WT*) TBP and Brf1; Bdp1 mutant proteins are indicated *above* each *lane*. *B*, analysis of restriction enzyme-cleaved PCR products showing the distribution of integration events in the r-U6 target region at the nucleotide level. The *lane numbers* and reactions correspond with those shown in *A*. *Lanes P* and *N* are positive and negative controls, as described in the Fig. 1 legend. The *unlabeled lanes* show the sequencing ladder used to determine the sizes of hybridizing fragments.

TABLE I

TFIIIC-independent transcription from and integration activity at the l-U6 and r-U6 initiation sites on supercoiled and linear DNA templates

Txn, transcription; Int, integration; −, <2% of wild type TFIIIB activity; ND, not determined. The transcription activity was determined previously (Refs. 33, 37, 41, and 52 and G. Kassavetis, A. Kumar, and S. Shah, unpublished data).

	Supercoiled		Linear	
	Txn	Int	Txn	Int
Brf1				
1–596	+	+	+	+
1–282	+	+	−	+
284–596	−	+/−	−	+/−
1–383	+	+	−	+
425–596	−	+/−	−	+/−
NΔ68Δ383–424	+	+	−	+
Δ383–424	+	+	+	++
Bdp1				
1–594	+	+	+	+
1–352	+	+	ND	+
352–594	+	+	ND	+
Δ272–292	+	+	+	+
Δ355–372	+	+	−	+
Δ424–438	+	+	+	+
224–487	+	+	ND	+

the context of TFIIIB showed that the amino-terminal portions of Brf1 supported significant amounts of TFIIIB-dependent integration on the supercoiled target but that the carboxyl-terminal portion activity was difficult to distinguish from background. On the linear target, Brf1(1–282) and Brf1(1–383) amino-terminal domains were clearly more effective for TFIIIB-dependent integration, but the Brf1(284–596) carboxyl-terminal domain also supported detectable levels of TFIIIB-dependent integration. Brf1(425–596), which contains the major sites of interaction with TBP and Bdp1, remained inactive for integration into linear DNA. Removal of the zinc ribbon of Brf1 was also shown not to have any effect on Ty3 integration directed by TFIIIB.

These findings point to similarities in the targeting of pol III and Ty3 integrase to the TFIIIB complex. First, in the context of TFIIIB, there is a redundancy for sites of interaction with pol III and Ty3 integrase. The amino-terminal TFIIB-related half of Brf1 is the major determinant of transcription activity (41) and integration activity (Fig. 1), but weaker transcription activity (41, 52) and integration activity (Fig. 1) is retained with the carboxyl-terminal half of Brf1(284–596). Second, Brf1 and TBP suffice as the minimal target for pol III and integrase (23, 36). Third, the zinc ribbon region is not absolutely essential for transcription or integration. It is apparent, however, that the zinc ribbon region plays a major role in transcription and targeting of pol III but not in integration. Removal of the amino-terminal 68 amino acids of Brf1 greatly destabilizes the pol III-TFIIIB-DNA complex, generates a 2-fold decrease in the transcription of supercoiled DNA templates, and abolishes transcription of linear DNA templates (52). In contrast, the same deletion has no effect on integration on supercoiled or linear plasmid DNA (Fig. 2). The amino-terminal half of Brf1 has been shown to interact with the carboxyl-terminal lobe of TBP, partially overlapping with the domain contacted by TFIIB (27, 41). Brf1 also interacts with the 34- and 17-kDa subunits of pol III (35). Similarities between the primary sequences of these polymerase subunits and the Ty3 integrase are not readily apparent, so it is not yet possible to specify the relationship between the determinants for Brf1 interaction.

Despite the ability of B′ to support minimal levels of Ty3 integration and pol III transcription on specialized templates (36), Bdp1 enhances both processes. In the case of transcription, Bdp1 has roles in both recruitment and pol III isomerization. On fully duplex DNA the presence of Bdp1 is essential for

bringing pol III to the start site of transcription; even transient assembly of pol III (as measured by protein-DNA photochemical cross-linking) could not be detected in the absence of Bdp1 (37). This suggests that Bdp1 either contributes directly to binding pol III or to displaying essential pol III interaction sites on Brf1. In addition, two observations suggest that Bdp1 plays a post-recruitment role in the initiation of transcription by pol III; first, transcription can be made Bdp1-independent through the introduction of heteroduplex bubbles at the site of open complex formation (36), and second, Bdp1 mutants with short deletions within the region of the amino acid 355–421 segment of the wild type protein bind pol III but do not allow promoter opening (37).

Comparison of the Bdp1 domain requirements for pol III transcription and Ty3 integration provides additional insight into what constitutes the Ty3 target. In particular, it was of interest to consider separately how domains implicated in the structural and implied DNA-flexing functions of Bdp1 related to the Bdp1 domain requirements for integration. It was previously shown that the positive effect of Bdp1 on Ty3 integration is consistent with a model involving stabilization of B′ binding (23). In this work, it has been shown that the amino- and carboxyl-terminal halves of Bdp1 separately support equivalent, elevated levels of integration into *SNR6* templates. Therefore, no single region of Bdp1 is absolutely required for Bdp1 enhancement of integration, just as no single region of Bdp1 is absolutely required for *SNR6* transcription. This is similar to what has been interpreted as the scaffolding role of Bdp1 in pol III recruitment.

A Bdp1 internal deletion mutant, Bdp1(Δ355–372) was used to test whether the post-recruitment function of Bdp1 contributes to Ty3 integration. This assay showed that a mutant capable of supporting pol III open complex formation on a supercoiled but not a linear template (37) was equivalently active for integration into both types of DNA. If the role of Bdp1 in promoter opening that is lost in Bdp1Δ355–372 involves DNA flexure or altering the path of DNA so as to facilitate strand opening by pol III, this function is not required for Bdp1-enhanced integration. We suggest instead that Bdp1 plays a relatively nonspecific scaffolding role in Ty3 integration, acting primarily to stabilize the B′ complex on the DNA rather than providing specific structures at the initiation site.

Although TFIIIC is not essential for *in vitro* integration at *SNR6*, it directs the orientation of TFIIIB and therefore the choice of transcription initiation sites used by Ty3. In this study, in which the pattern of Ty3 strand transfers at a particular site was mapped, a more striking TFIIIC effect has surfaced. In the absence of TFIIIC, the predominant sites of strand transfer of the Ty3 3′ ends to the target DNA at r-U6 were flanking positions −7/−3 and −6/−2, similar to what is observed *in vivo* (primarily −5/−1). In the presence of TFIIIC, the sites used extended from −7/−3 to −1/+4. This pattern of integration *in vitro* in the presence of TFIIIB and TFIIIC is not as precise as that observed *in vivo*, suggesting that the integration site is differently exposed *in vivo*, perhaps because TFIIIC is not present at the time of integration. Because pol III competes with Ty3 in the *in vitro* integration reaction, it is assumed that it also does not occupy the initiation site during integration. Genomic footprinting of the *SUP53* tRNA and *SNR6* genes indicates that occupancy of the boxA and boxB promoter elements, presumably by TFIIIC, is considerably lower than occupancy of upstream DNA, presumably by TFIIIB (21, 53). However, a recently identified mutant with a truncated TFIIIC 95-kDa subunit displays only a subtle effect on transcription, but a relatively dramatic effect on Ty3 integration at a tRNA gene target (54). The phenotype of this mutant may support the alternative interpretation that TFIIIC is present when integration occurs but that there are differences between target presentation *in vitro* and *in vivo*.

TBP can bind to the nearly symmetric *SNR6* TATA box in either orientation both in the presence and in the absence of Brf1. In the presence of TFIIIC a single orientation of B′ over the TATA box is obtained because of the interaction of the TFIIIC τ120 subunit with Brf1. Paradoxically, TFIIIC has relatively little effect on B′-dependent integration site selection in the presence of B′ alone (Fig. 4). Bdp1Δ272–292 is competent for stimulating integration and is competent to enter the B′-TFIIIC-DNA complex; yet there is no effect of TFIIIC on the orientation of TFIIIB assembled with Bdp1Δ272–292, as assayed by Ty3 integration. Amino acids 272–292 of Bdp1 lie in very close proximity to DNA upstream of the TATA box (45), and this interaction appears to be required in order for Bdp1 to lift TFIIIC away from the start site of transcription (33). This suggests a simple (and plausible) explanation for the paradox; B′-TFIIIC-DNA complexes are not a substrate for Ty3 integration because of start site occlusion. Integration is only observed in those B′-DNA complexes that have not been assembled by TFIIIC.

The experiments presented here extend the parallels between the requirements of pol III and Ty3 preintegration complexes for target gene docking. Those results underscore the specific role of the TFIIB-like domain of Brf1 and the apparent structural role of Bdp1 in both processes. Although TFIIIC is known to contact Brf1, the current study showed that Bdp1 is required to produce the TFIIIC-dependent pattern of Ty3 integration. Finally, the current results argue that despite the congruence of the region of DNA melting in the pol III transcription initiation open complex and the positions of Ty3 strand transfer, Ty3 integration does not depend upon a Bdp1-imposed structure at the initiation site. Overall, these results support in some detail the model that the Ty3 preintegration complex mimics pol III in the mechanism of recruitment to its target.

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