

RNA polymerase III interferes with Ty3 integration

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Abstract Ty3, a gypsylike retrotransposon of budding yeast, integrates at the transcription initiation site of genes transcribed by RNA polymerase III (pol III). It was previously shown that integration in vitro requires intact promoter elements and the pol III transcription factors TFIIB and TFIIC. In order to test the effect of pol III on integration, increasing amounts of a pol III-containing fraction were added to Ty3 in vitro integration reactions. The pol III-containing fraction was inhibitory to integration. These results are consistent with a model where the Ty3 integration complex and pol III recognize similar features of the stable transcription complex and compete with each other for access to the transcription initiation site.

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Key words: Integration; Retrovirus; RNA polymerase III; Transposable element; tRNA gene; Ty3

1. Introduction

A subset of retroelements have been identified which have patterns of integration that reflect dramatic insertion bias for tRNA genes. These include DRE I [1], a nonLTR retrotransposon from *Dictyostelium discoideum*, and the Ty1 and Ty2 copia-like and Ty3 gypsylike LTR retrotransposons from *Saccharomyces cerevisiae* (reviewed in [2]). DRE I elements are close to position –50 nt relative to tRNA gene sequence. Ty1 and Ty2 genomic elements occur predominantly within a region of about 700 nt upstream of tRNA genes [3–5]. Ty3 is even more closely associated with tRNA genes than the other elements [6–8]. Of the two staggered nicks introduced by Ty3 strand transfer into the target, one occurs within 1–2 nt of the transcription initiation site and the other 5 nt distal. The target region spanned by the staggered cleavage therefore overlaps the region contained in the open complex bubble formed upon RNA polymerase III (pol III) transcription initiation [9]. In the case of the *Saccharomyces cerevisiae* elements Ty1 and Ty3, tRNA gene targeting requires intact promoter elements and other genes transcribed by pol III such as 5S and U6 genes are also targets [8,10]. Thus, the processes of transcription and integration are superficially linked, if not actually interdependent.

The Ty3 element is composed of *GAG3* and *POL3* genes which encode proteins with similar sequence and function to those encoded by the retroviral *gag* and *pol* genes [11]. Ty3 proteins condense around the genomic RNA to form a nucleoprotein complex referred to as the viruslike particle (VLP), which is analogous to the retroviral core particle. After re-

verse transcription of Ty3 RNA in association with the VLP, the DNA copy is integrated into the host genome in a process mediated by Ty3 integrase (IN) [12]. Ty3 IN is a homologue of retroviral IN. Recombinant retroviral IN has been demonstrated to have in vitro 3'-end processing and strand-transfer activities (reviewed in [13]). Although Ty3 IN has been demonstrated to be required in vivo for 3'-end processing and integration of Ty3 DNA, integration activity has only been observed in vitro in association with a VLP fraction [14].

Despite the many similarities between retroviruses and Ty3 elements, neither retrovirus integration adjacent to tRNA gene targets nor a bias for tRNA gene-containing regions has been reported. Although, retrovirus insertion has been linked in past studies to genomic DNA which is transcriptionally active, more recent studies are consistent with widespread distribution of integration sites, including regions occupied by nucleosomes [15,16]. Features which target retroviral integration have now been studied in vitro. These studies may also provide further insight into the preferences of retroviruslike elements. Retrovirus IN strand transfer is enhanced by static and induced bends [17–19]. Bent regions within nucleosomes and DNA which is bent by specific DNA binding proteins are favored integration sites. Not all DNA bending proteins are equally active in creating preferred sites, however, as DNA bending proteins which occupy the inside of the bend have been shown to be effective in targeting integration, while those which occupy the outer, distorted side of the bend can actually inhibit integration [20].

In vitro integration of the Ty3 element requires VLPs, and the pol III transcription factors TFIIC and TFIIB, in addition to a DNA target [21]. TFIIC is comprised of six subunits and binds to the two internal promoter elements, box A and box B, of the tRNA gene (reviewed in [22,23]). This factor in turn mediates binding of TFIIB, the pol III initiation factor. TFIIB is composed of three subunits, a 70 kDa subunit with similarity to the pol II transcription factor TFIIB; a 90 kDa subunit, and the TATA binding protein. Although TFIIC is required for binding of TFIIB, the TFIIB-DNA complex is quite stable and can mediate multiple rounds of pol III transcription in the absence of TFIIC [24]. Whether TFIIC is associated with the transcribing tRNA gene has not been directly tested, although genomic footprints of tRNA genes suggest that only TFIIB may be bound [25]. Features which potentially contribute to the targeting of Ty3 to the site of tRNA gene transcription initiation therefore include TFIIC and TFIIB, as well as topographical features of the tRNA gene, such as novel chromatin structure or the bend associated with the transcription initiation site [26]. A previous study showed that pol III did not appear to be essential in vitro for integration to occur [21]. However, it was possible that low levels of pol III present in IIB and IIC fractions were sufficient for transposition. In such a scenario, open complex formation in that event could participate

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in display of the target site to the integration complex. An alternative possibility is that the features of the stable complex could function alternatively to target pol III or the integration complex. These models make distinct predictions regarding the effect of pol III on integration. The former model would predict that transposition is dependent on pol III, while the latter would suggest that transposition might be inhibited by pol III. The current study was undertaken to determine the effect of pol III on *in vitro* integration.

2. Materials and methods

2.1. Strains and culture conditions

Construction, maintenance, and propagation of plasmids in *E. coli* were performed according to standard methods [27]. Plasmid manipulations in *E. coli* were carried out using strain HB101 (F⁻ *hdsS20* [$\text{r}_B^- \text{m}_B^-$] *recA13 leuB6 ara-14 proA2 lacY1 galK2 rpsL20* [Sm^r] *xyl-5 mtl-1 supE44*). Transformation and culturing of *S. cerevisiae* strains were according to standard methods [28]. The *S. cerevisiae* strains used in this study were AGY9 (*MATa ura3-52 his4-539 lys2-801 trp1- Δ 63 leu2 Δ 1 spt3*) and yTM442 (*MATa trp1-H3 ura3-52 his3- Δ 200 ade2-101 lys2-1 leu2- Δ 1 can1-100*) (unpublished data, T. Menees and S.B. Sandmeyer). Plasmids were transformed into yeast by the method of Ito [29]. Synthetic medium used for plasmid selection was made according to standard protocols.

2.2. Plasmid construction

Plasmids were constructed by standard techniques. The construction of the plasmid pDLC374 has been described previously [8] (see also Fig. 1). It carries the 2 μ origin of replication for maintenance in yeast, and the *HIS3* gene for selection. It also carries a modified *SUP2* tRNA^{Tyr} gene. The positive control plasmid pTM842 was derived from pDLC374 via an *in vivo* integration of Ty3 at position -1, relative to the nt of tRNA gene transcription initiation in an orientation such that transcription from the tRNA gene and from the Ty3 promoter were divergent [30]. The plasmid pEGTy3-1 has been described previously [31]. It carries the yeast 2 μ origin of replication and the *URA3* gene. Ty3 was under the transcriptional control of the *GALI-10* UAS to confer galactose inducibility upon Ty3. The plasmid pTZ1 carried the *SUP4* tRNA^{Tyr} with a G62C mutation that increased promoter efficiency [32].

2.3. Preparation and fractionation of RNA polymerase III extract

A yeast extract which supported transcription by pol III was generated according to the method of Klekamp and Weil [33], with modifications introduced by Kassavetis et al. [32]. Yeast strain yTM442 was grown to OD₆₀₀ of 3–5 in YPD (2% yeast extract, 1% peptone, 2% dextrose). The cells were harvested and washed once with water. The cell pellet was resuspended in 2 ml solubilization buffer (200 mM Tris-HCl pH 8.1, 10 mM MgCl₂, 1 mM EDTA, 10 mM 2-mercaptoethanol [BME], 1 mM phenylmethylsulfonyl fluoride [PMSF], 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A) per gram of cell pellet. Typically, 12 l of yeast were used per preparation, giving approximately 100–120 g of cell pellet. All subsequent manipulations were done either at 4°C or on ice. The slurry was lysed using a Bead-Beater (BioSpec Products) and 0.5 mm glass beads. Briefly, the cell slurry was combined with 0.5 volumes chilled glass beads, and treated in the bead beater for 30 s, followed by 2.5 min cooling in an ice/NaCl slurry at -17°C. This was repeated 14 times. An S100 fraction was generated by centrifuging the lysate for 1 h at 42000 rpm in a Beckman Ti60 rotor at 4°C. This S100 was subjected to ammonium sulfate precipitation, and the material which was soluble between 35 and 70% saturation at 0°C was applied to a 2.5 \times 20 cm Bio-Rex 70 cation exchange column (BioRad) at a flow rate of 1 ml/min. The column was washed with one volume buffer D (10% glycerol, 20 mM HEPES pH 7.8, 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A) containing 250 mM KCl. A fraction containing TFIIB, TFIIC, and pol III was eluted with buffer D containing 500 mM KCl. The peak protein fractions were pooled and dialyzed against buffer C (20% glycerol, 100 mM NaCl, 20 mM HEPES, pH 7.8, 1 mM EDTA, 1 mM PMSF, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A) and stored at -70°C. This was termed the BR500. The protein concentration was measured by

the method of Bradford [34]; the concentration of protein in the pooled fraction was typically 2–5 mg/ml.

The BR500 was further fractionated using DEAE-Sephadex. Typically, 1 mg of protein was loaded per ml of column resin. The BR500 was adjusted to 10 mM MgCl₂, and applied to the column. The TFIIB-, TFIIC-, and pol III-containing fractions were eluted with buffer E (buffer D with 10 mM MgCl₂ instead of 5 mM) containing 100, 250, and 500 mM NaCl, respectively. The peak protein fractions were identified and pooled. The fractions were dialyzed against buffer C as above, and stored at -70°C. The protein concentration of pooled fractions was determined by the method of Bradford [34]. The 100 mM NaCl fraction was typically 1–3 mg/ml, the 250 mM NaCl fraction was typically 0.5–1.5 mg/ml, and the 500 mM NaCl fraction was typically 0.2–0.5 mg/ml.

2.4. *In vitro* pol III transcription assays

In vitro transcriptions were performed using the yeast extract generated as above. The reactions contained 110 mM NaCl, 8 mM MgCl₂, 20 mM HEPES pH 7.8, 250 μ M ATP, CTP, UTP, 15 μ M [α -³²P]GTP (16.7 Ci/mmol), 75 fmol template tRNA gene, and 800 ng pIBI20 carrier DNA in a volume of 50 μ l. The reactions were typically incubated for 30 min at 30°C. Some were incubated for 15 min at 15°C, as noted in the text. The reactions were terminated by the addition of 150 μ l of stop buffer (27 mM EDTA, 0.3% SDS, 0.3 μ g/ μ l salmon sperm DNA, and 1.3 M LiCl) [35]. The products were extracted with phenol/chloroform, and precipitated with ethanol in the presence of 2 M NH₄OAc. After a 70% ethanol wash, the products were dried and resuspended in 5 μ l formamide containing 0.1% bromophenol blue, 0.1% xylene cyanol FF. The products were separated by electrophoresis on a denaturing 8% polyacrylamide gel (20:1 acrylamide:bisacrylamide), and visualized by autoradiography. In some cases, the gel was dried, exposed to a Molecular Dynamics phosphorimager screen, and the amount of radioactivity incorporated into the transcription products quantitated using the ImageQuant software. Aliquots of 5 μ l and 9 μ l were used to monitor the activity of the BR500 and DEAE fractions, respectively.

2.5. Viruslike particle preparation

Ty3 VLPs were isolated from yeast strain AGY9 essentially as described [11,36]. Briefly, cells were transformed with pEGTy3-1 and grown to mid-log phase in liquid synthetic medium lacking uracil and containing 2% each raffinose, glycerol, and lactic acid as a carbon source. The cultures were diluted 1:30 into medium containing 2% galactose to induce Ty3 expression from pEGTy3-1. Cells were grown to an OD₆₀₀ of approximately 1.5 and harvested. The cell pellets were resuspended and the cells made into spheroplasts with zymolyase. These were lysed with glass beads and the cell debris removed by centrifugation. The extract was fractionated by velocity sedimentation in a 10%/30%/70% sucrose gradient as described. Fractions containing peak amounts of VLPs were pooled, and the VLPs were concentrated by centrifugation. The pellets were resuspended in 100 μ l buffer B (Mg²⁺) (15 mM KCl, 5 mM MgCl₂, 10 mM HEPES pH 7.8, 10% glycerol) and stored at -70°C. Protein concentration was measured by the method of Bradford [34] and was typically 1–3 mg/ml. *In vitro* integration activity was stable for at least 3 months (C.M. Connolly and S.B. Sandmeyer, unpublished data).

2.6. *In vitro* integration assay

In vitro integration assays were performed as described previously [21] by combining 400 ng target plasmid pDLC374, pol III extract, and 2.5 μ g Ty3 VLPs on ice. Standard integration reactions contained 65 mM NaCl, 5 to 10 mM MgCl₂, 20 mM HEPES pH 7.8, and 1 mM DTT in a 50 μ l volume. Assays were incubated for 15 min at 15°C. The reaction was terminated by the addition of 150 μ l of stop buffer (27 mM EDTA, 0.3% SDS, and 0.3 mg/ml proteinase K) followed by incubation for 30 min at 37°C. The reactions were extracted with phenol/chloroform, and the DNA was precipitated with ethanol in the presence of 2 M NH₄OAc, and collected by centrifugation at 10000 \times g for 30 min at 4°C. The pellets were washed with 70% ethanol, dried, and dissolved in 50 μ l of buffer containing 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA. The products were analyzed by polymerase chain reaction (PCR) [30].

Integration was detected by PCR amplification of a diagnostic fragment using primers which annealed in Ty3 (primer 278) and upstream of the modified tRNA gene carried on pDLC374 (primer 279) as

described previously [30] and as illustrated in Fig. 1. A 474 bp product is generated in a PCR reaction using the primer set 278/279 from plasmids containing Ty3 insertions at position -1 in the orientation where the direction of Ty3 transcription is divergent from that of the tRNA gene. PCRs contained 4 mM MgCl₂, 500 μM dNTPs, 200 μM primers, 200 ng gelatin (Eastman Organic Chemicals), 27 Ci/mol [³²P]dATP, and 2.5 U Taq polymerase (Perkin Elmer) in a 75 μl volume. Reactions were cycled 27–29 times in a DNA Thermal Cycler 480 (Perkin Elmer). DNA was denatured at 94°C for 60 s, annealed at 50°C for 30 s, and extended at 65°C for 45 s. In reactions to monitor linearity of the assay, increasing amounts of plasmid, pTM842, a target plasmid containing a Ty3 insertion, were combined with the target pDLC374 for a total of 25 ng, the approximate amount of DNA recovered from integration reactions. Products were fractionated by electrophoresis on 8% polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) and stained with ethidium bromide. The bands corresponding to the PCR products were excised, and Cerenkov radiation measured.

In order to normalize for the amount of target plasmid recovered from integration reactions in the 278/279 PCR reactions, PCRs were performed using primers which detect the *HIS3* gene (primers 284 and 285) [30]. This gene is carried on the target plasmid. The PCR reactions were as above, except that reactions contained 2.5 mM MgCl₂ and gelatin was omitted, and primers 284 and 285 were used. The reactions were cycled sixteen times, with a denaturation/annealing/extension profile of 94°C for 45 s, 55°C for 30 s and 72°C for 30 s. The PCR products were treated as described above.

3. Results

In vivo integration of Ty3 upstream of a tRNA gene on a plasmid target showed that Ty3 position specific integration requires a transcriptionally competent tRNA gene. In vitro experiments using pol III, TFIIB and TFIIC containing fractions showed that TFIIB and TFIIC were sufficient for integration, but did not further address the role of pol III. In order to determine whether pol III could further contribute to targeting, a quantitative PCR assay of integration was used to measure the effects of increasing the amount of a pol III-containing fraction on Ty3 integration activity.

As described previously [21], pol III and pol III transcription factors were prepared by passage of a crude extract over Bio-Rex 70 and subsequent fractionation on DEAE Sephadex. The BR500 fraction was eluted from the Bio-Rex 70 column at 500 mM NaCl. The TFIIB-, TFIIC-, and pol III-containing fractions eluted at 100, 250, and 500 mM NaCl, respectively, from DEAE Sephadex. These fractions were assayed using the pTZ1 *SUP4* tRNA^{Tyr} gene template. All three fractions were required for transcription activity, indicating that, by this criterion the factors were present in discrete fractions. As indicated above, single fractions were not sufficient to support integration, but the combination of TFIIC and TFIIB resulted in integration activity.

To investigate the possible effect of pol III on integration, integrations were assayed using a quantitative PCR assay. The inclusion of [³²P]dATP in the assay allowed accurate measurement of the amount of product formed. PCR conditions were determined under which product formation increased linearly with the amount of template. When input integration template pTM842 ranged from 2.5 to 50 amol, the 278/279 PCR products increased linearly, and when target *HIS3*-marked pDLC374 input template ranged from 4.5 to 18 fmol the 284/285 PCR products increased linearly (unpublished data, C.M. Connolly and S.B. Sandmeyer). The amount of product from the 278/279 PCR of experimental samples was normal-

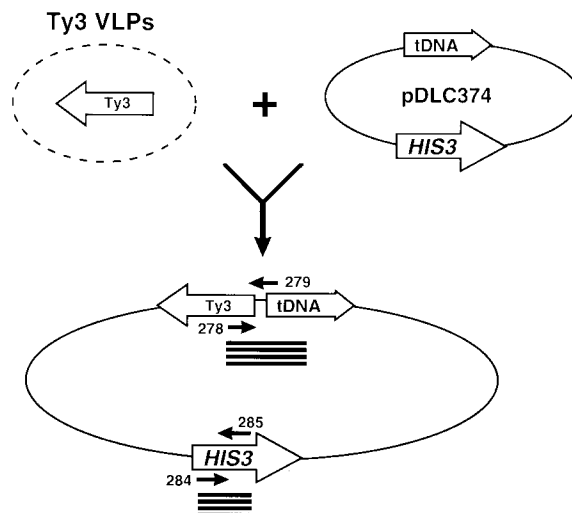


Fig. 1. PCR assay for Ty3 position specific integration [30]. VLPs shown in upper left function as the source of Ty3 DNA and integrase activity. Integration of Ty3 DNA into the target plasmid pDLC374 is monitored by PCR using one primer which anneals within Ty3 (primer 278) and one primer which anneals to the target plasmid (primer 279). A 474 bp diagnostic fragment is generated from products of the integration reaction which contain insertions. This assay detects insertions only in one orientation, but in vivo, insertions occur in both orientations into target plasmids. As a control, levels of a PCR product generated using primers which anneal within the *HIS3* gene on pDLC374 (primers 284 and 285) are also monitored and recovery of Ty3 insertion product is normalized to the amount of *HIS3* product.

ized to the amount of product from the corresponding 284/285 PCR for the same reaction.

The effect of addition of the pol III-containing fraction to in vitro integration reactions was examined. Integrations were performed with a constant amount of TFIIB- and TFIIC-containing fractions (Fig. 2) and variable amounts of pol III. Increasing the amount of the pol III-containing fraction resulted in decreasing levels of Ty3 integration. The inclusion of 15 μl of the pol III-containing fraction resulted in approximately 80% less integration than in the reaction with no pol III added. Although the absolute amount of the active factors in each fraction was not determined, TFIIB is limiting for transcription under these conditions. However, it was possible that another component of the pol III-containing fraction was responsible for the decrease in integration activity. For example, nuclease activity might preferentially degrade the integration products.

In order to determine if contaminating nucleases were responsible for the inhibitory effect of the pol III fraction on integration, aliquots of that fraction were incubated with completed integration reactions (performed in the absence of the pol III fraction) (Fig. 3). In vitro integrations were performed using BR500, and the products were processed and collected. Aliquots of products of the integration reactions were incubated with increasing amounts of the pol III-containing fraction in reactions identical to integration reactions, except that no VLPs or transcription factor fractions were added. The samples were processed as for integrations, and the level of integration products was examined by quantitative PCR assay. The addition of the pol III-containing fraction did not decrease the amount of integration products, indicating that there was no nuclease activity which preferentially af-

fects the amount of integration product in presence of the pol III-containing fraction.

Polyclonal antibodies were used to determine whether pol III was the component of the fraction responsible for the inhibition of integration. Antibodies raised against either pol III or one of two pol III subunits, C34 or C53 [37], were examined for their effect on both *in vitro* tRNA gene transcription and the inhibitory activity found in the pol III-containing fraction. Previous work had shown that these antibodies were inhibitory to pol III transcription. The pol III-containing fraction was preincubated with increasing amounts of either pre-immune or post-immune IgG fraction, and the effects on both transcription and integration were examined. As was previously observed, all three post-immune IgG fractions were found to inhibit pol III transcription, while the pre-immune IgG fraction did not inhibit transcription to an appreciable degree (Fig. 4). This was consistent with results reported previously [37]. Addition of the anti-pol III IgG fraction, together with the pol III fraction, blocked the integration-inhibitory activity seen in the pol III-containing fraction (Fig. 5A, top). Antibodies raised against the C34 subunit showed a moderate blocking of the integration-inhibitory activity (Fig. 5A, middle) but did not completely reverse the effect of pol III, while antibodies raised against the C53 subunit did not block inhibition of integration (Fig. 5A, bottom). The pre-immune IgG fraction did not block integration inhibition by the pol III-containing fraction (Fig. 5B). These results argued that it is pol III which caused inhibition of Ty3 integration.

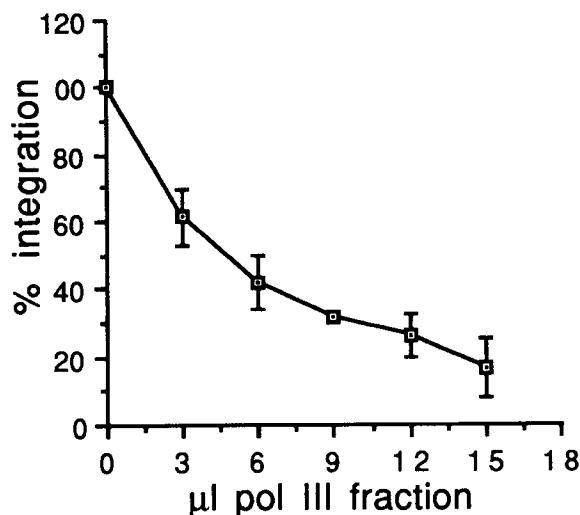


Fig. 2. Titration of the pol III-containing fraction. *In vitro* integration reaction components, except for VLPs, were assembled on ice. Transcription complexes were allowed to form by incubating for 15 min at 15°C. VLPs were added and the reactions were incubated for 15 min at 15°C. The reactions were terminated and processed as described in Section 2. Increasing amounts of the pol III-containing fraction were added to integration reactions containing 15 µl of TFIIB and 5 µl of TFIIC. These reactions were performed in triplicate (except for the 9 µl sample which was performed in duplicate) and the points represent the average of those results. The error bars show the standard deviation. Integration levels are presented as a percentage of integration levels in reactions to which no pol III was added. The reactions were terminated and analyzed using quantitative PCR. Integration levels detected in PCRs performed with the primer set 278/279 were normalized to amounts of target plasmid detected in PCRs using the primer set 284/285.

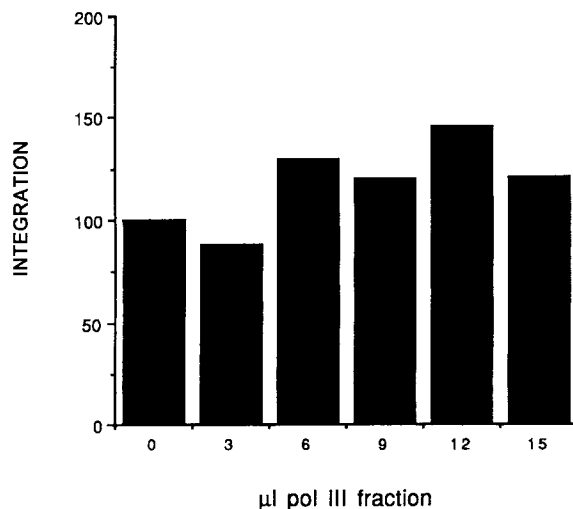


Fig. 3. Examination of nuclease activity in the pol III-containing fraction. *In vitro* integration reactions were performed using BR500 and processed as described in Section 2. The integration products from ten separate reactions were combined. Aliquots were incubated with increasing amounts of the pol III-containing fraction, as noted, for 15 min at 15°C. The reaction conditions were the same as for the titration of the pol III-containing fraction (Fig. 2), except that VLPs and other fractions were absent. The reactions were terminated as for integrations, and analyzed using quantitative PCR. Integration levels detected in PCRs performed with the primer set 278/279 were normalized to amounts of target plasmid detected in PCRs using the primer set 284/285. The data shown are the average of two experiments. Product levels are presented as a percentage of product levels in reactions to which no pol III was added.

4. Discussion

The concentration of a pol III-containing fraction in the Ty3 *in vitro* integration reaction correlated inversely with the amount of integration of Ty3 into the tRNA gene target. These results, derived using a quantitative *in vitro* integration assay and anti-pol III IgG to identify the inhibitory component of the pol III fraction, supported the findings of the previous study that pol III is not required for targeting. Moreover, the findings in this study suggest that the polymerase and integration complex access the target by mutually exclusive contacts.

Despite the proximity of the Ty3 integration and pol III transcription initiation sites, open complex formation appears not to be required for Ty3 targeting. This result extends the conclusions of previous *in vivo* experiments which argued that transcription is not required for Ty3 integration. In one case, the nontemplate strand purines including and surrounding the transcription initiation site were substituted with pyrimidines [8]. As a result of these changes, the major site of transcription initiation moved downstream by several nucleotides, to the nearest available purine, and the level of tRNA expression *in vivo* and *in vitro* was reduced. Several Ty3 integrations into this target were characterized. However, the most frequently used integration site was actually one nucleotide upstream of the most frequently used position of integration in the wt gene. Therefore, in this context, as opposed to the native context, the most common integration site did not coincide with the most common transcription initiation site. If the nucleotide of transcription initiation were mechanistically

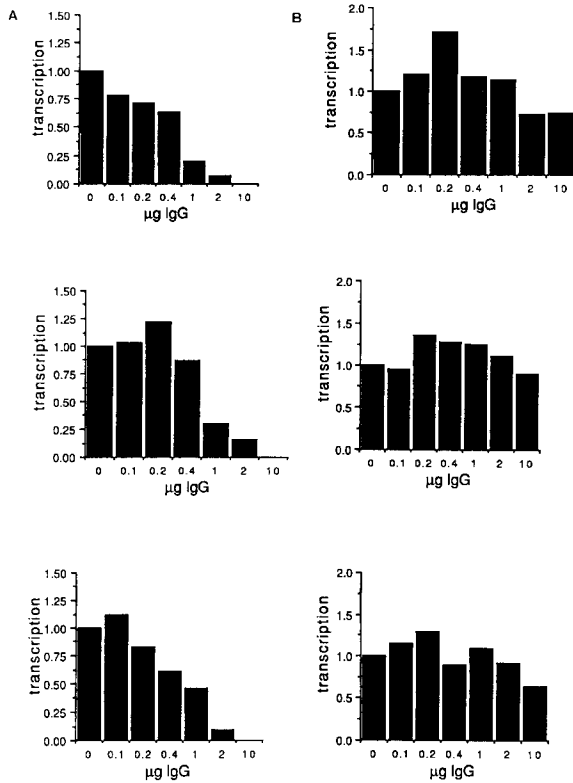


Fig. 4. Transcription inhibition by polyclonal antibodies. The effects on in vitro transcription of antibodies raised against either pol III and one of two subunits of pol III (C34 or C53) were examined. Increasing amounts of antibodies, as noted in the graphs, were preincubated with 15 µl pol III-containing fraction for 1 h at 4°C. These were then used in transcription reactions with 10 µl TFIIC-containing fraction and 7.5 µl TFIIB-containing fraction. The plasmid pTZ1, carrying a *SUP4* tRNA^{Tyr} was used as a template. The reactions were assembled on ice, then incubated for 15 min at 15°C, and processed as described in Section 2. The products were separated by electrophoresis on 8% polyacrylamide gels (20:1 acrylamide:bisacrylamide). The gels were dried, exposed to a phosphorimager screen and the intensity of the bands determined using ImageQuant software. The transcriptions were performed twice, and representative data is shown. The data shown are presented as relative to reactions in which no antibody was present in the preincubation and are from one representative experiment. A: Effect of post-immune IgG fraction on in vitro transcription. Top, anti-pol III IgG; middle, anti-C34 IgG; bottom, anti-C53 IgG. B: Effect of pre-immune IgG fraction on in vitro transcription. Top, anti-pol III pre-immune IgG; middle, anti-C34 pre-immune IgG; bottom, anti-C53 pre-immune.

linked to integration, these sites would be predicted to show such linkage.

The second result that suggested that transcription is not required for integration is that tRNA genes which appear to be transcriptionally less active can be as competent as wt genes as targets for Ty3 integration [8]. For example, the *sup2* tRNA gene modified by substitution of pyrimidines for purines in the vicinity of the initiation site showed levels of transcripts in vivo and in vitro which were about one-twentieth those of the wt gene. When the modified gene was inserted in tandem with a wt *SUP2* tRNA gene target, however, a similar number of insertions was recovered into each gene. Similarly, the level of transcripts produced from a mutant *SUP2* tRNA gene in which the box A region was deleted and a synthetic box A sequence was inserted upstream of

the natural position was reduced compared to wt *SUP2*, but this gene was used at a frequency similar to that of the wt gene when carried on the same target plasmid. Although these two examples did not prove that transcription is not required for targeting, they did suggest that transcription is not limiting for the integration reaction.

Antibodies raised against pol III blocked integration interference by the pol III fraction. The addition of the pol III-containing fraction reduced integration up to 80%. However, preincubation of the pol III-containing fraction with polyclonal antibodies raised against pol III blocked the ability of the fraction to inhibit integration. This was consistent with the interpretation that the component of the pol III transcription

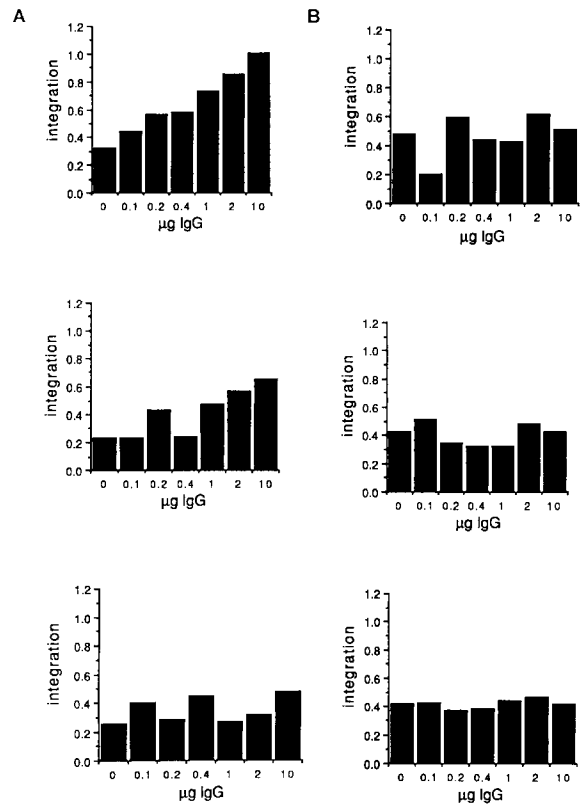


Fig. 5. Effect of polyclonal antibodies on Ty3 integration inhibition by the pol III-containing fraction. Polyclonal antibodies raised against pol III or one of two pol III subunits (C34 or C53) were examined for their effect on the Ty3 integration-inhibitory activity seen in the pol III-containing fraction. Increasing amounts of antibodies were preincubated with 15 µl pol III-containing fraction for 1 h at 4°C. In vitro integration reactions were performed with these together with 10 µl TFIIC-containing fraction and 7.5 µl TFIIB-containing fraction as described in Section 2. The reaction were assembled and incubated for 10 min on ice, then incubated for 15 min at 15°C and processed as described in Section 2. The data shown are normalized to reactions in which no antibody was present in the preincubation. The experiments were performed twice, and representative data is shown. The integration levels were assayed using quantitative PCR. Integration levels detected in PCRs performed with the primer set 278/279 were normalized to amounts of target plasmid detected in PCRs using the primer set 284/285. A: Effect of post-immune IgG fraction on the integration-inhibitory activity present in the pol III-containing fraction. Top, anti-pol III IgG; middle, anti-C34 IgG; bottom, anti-C53 IgG. B: Effect of pre-immune IgG fraction on the integration-inhibitory activity present in the pol III-containing fraction. Top, anti-pol III pre-immune IgG; middle, anti-C34 pre-immune IgG; bottom, anti-C53 pre-immune.

extract which interfered with integration was, in fact, pol III. Blocking of the interference attributed to pol III was also observed for anti-C34 IgG, although to a lesser extent. It was not observed, however, for the anti-C53 IgG. Cross-linking experiments have mapped the positions of the C34 subunit and the C53 subunit proximal and distal to the transcription initiation site, respectively [38]. Thus, one explanation for the difference in effectiveness of blocking between these two antisera is that antibody against the C53 subunit is directed distal to the region accessed by the integration complex, potentially presenting less of an obstacle to pol III binding at the target site. The C53 subunit is reported to be more loosely associated with the pol III complex [39] so it is also possible that it is dislodged from the polymerase by its interaction with the antibody, leaving the remainder of the polymerase available to bind to the target.

Data in the current study are consistent with mutual exclusion or interference of the integration complex and pol III at the target site. However, whether the integration complex and pol III recognize the same features or different target features was not addressed by these experiments. No striking similarity in primary sequence has been observed between known Ty3 components of the integration complex and potential components of the stable transcription complex or subunits of pol III present in the yeast genome data base (unpublished data, C.M. Connolly and S.B. Sandmeyer). Nevertheless, it is possible that some element of the integration complex has structural similarity to transcriptional components. For example, previous experiments have provided support for the existence of a heterodimeric Ty3 reverse transcriptase composed of one reverse transcriptase and one reverse transcriptase-IN fusion protein [11,14] as is the case for RSV (reviewed in [40]). RNA- and DNA-dependent polymerases have some global similarities in their so-called right hand three-dimensional structure. One attractive, but highly speculative, explanation for the observed interference is that the integration complex actually resembles one or more subunits of the stable transcription complex or polymerase III and docks at the target through interactions mediated by that component.

The targeting of several retrotransposons with tRNA genes in different organisms encourages the search for global regulatory significance of the association. In the case of Ty3, integration of the element adjacent to at least one tRNA gene was found not to significantly disrupt expression of the tRNA gene [41]. Nevertheless, disruption of tRNA gene promoters did relieve an apparent repression of Ty3 gene expression suggesting that tRNA genes could potentially limit the activity of retrotransposons. Whether the competition observed here might have any similar physiological outcome was not addressed in this study. In one study a tRNA gene could be footprinted in vivo, suggesting a high percentage of occupancy of the gene by transcription factors [25]. However, the percentage of genes actually occupied by pol III was not estimated. Ty3 is naturally transpositionally active only in cells which have recently mated [42]. If pol III occupancy of tRNA genes or particular sets of tRNA genes were low during this time, the results of this study suggest that it could enhance their use as targets relative to other times in the yeast life cycle. The effect of VLPs on transcription was not measured quantitatively in this study. However, physiological Ty3 transposition does not occur at high frequencies. Therefore, transient blocking of pol III access to tRNA genes by Ty3

integration complexes is unlikely to have major regulatory implications even if it occurs transiently.

The results of this study argue, that although a tRNA gene must be transcriptionally competent, pol III does not contribute directly to Ty3 target establishment. In contrast, the features of the transcription complex which target pol III transcription may resemble those which target integration. Therefore, transcription and integration are mutually exclusive activities.

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