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Convergent Transcription Units and Their Promoters at Both Ends of *Pot2*, an Inverted Repeat Transposon from the Rice Blast Fungus[1]

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The rice blast fungus *Pot2* is an inverted repeat transposon which shares structural features with the Tc*1-mariner* superfamily. In a search for the transposition regulatory mechanism of presently quiescent *Pot2*, we found truncated transcripts for the sense and antisense strands of the transposase (TPase) gene. Reverse transcription-PCR analysis identified the convergent overlapping transcription units that contain the entire coding region of the TPase gene. DNA fragments carrying presumed TATA box motifs at both ends of *Pot2* proved to be functional as promoters in transient expression assays. Using these promoters on transformation vectors, stable transformants of the fungus were obtained at a frequency comparable to that with the established system. Furthermore, the antisense promoter transcribed a marker gene from one of the original sites on the chromosome. The above results indicate that the antisense RNA is transcribed by the antisense promoter at the 3′ end of the element, which has implications for a possible regulatory mechanism. This is the first example of the identification of an antisense promoter within a class II transposon from a eukaryote.

Key words: antisense regulation, blasticidin S resistance, class II transposon, fungal promoter, *Pyricularia oryzae* (*Magnaporthe grisea*).

Transposable elements have been shown to cause chromosomal aberrations, such as deletions and inversions, and are thought to be important determinants of the genetic variation in eukaryotes (*1, 2*). In the transpositioning of class II (DNA-mediated) transposons flanked by terminal inverted repeats (TIRs), the binding of the encoded protein, known collectively as transposase (TPase), leads to insertion into new sites. Its expression level is one of the important factors that affect the frequency of transposition (*3, 4*), and should be tightly regulated by some mechanisms to control the dynamics of the element. So far, several transcription regulatory mechanisms have been proposed for each class II transposon from eukaryotes: *e.g.*, methylation of CpG motifs within the elements (*5, 6*), binding of the encoded regulator protein to the promoter region (*7*), or activation by a cell-type specific enhancer at an adjacent region on the chromosome (*4*). However, the mechanisms regulating transposition are more complex and show greater variety, and remain to be elucidated.

In an effort to explain the pathogenic variability of the rice blast fungus, *Pyricularia oryzae* (perfect stage: *Magnaporthe grisea*), Kachroo *et al.* (*8*) identified a putative transposable element, *Pot2* (*P. oryzae* transposon), that is dispersed on the genome of the fungus. *Pot2* belongs to the *Fot1* family of fungal transposons (*9*), and shares structural features with the Tc*1-mariner* superfamily (*10*); *i.e.*, *Pot2* is a small (1,857 bp) element flanked by short TIRs of 43 bp and also duplicates the dinucleotide TA at the target site. Although *Pot2* proved to code for a TPase like protein (*8*), there has been no direct evidence of its expression in the fungus. Besides, transpositioning of this element was not observed on a laboratory time scale, and thus it has not been used as a tool for tagging genes of interest. As a first step to elucidate the regulatory mechanisms of transposition, we examined whether or not the transposon has the activity to promote the transcription of its own sequence.

Using RNA obtained from growing mycelia of *P. oryzae* isolate P2, we examined RNA of the fungus by Northern analysis (Fig. 1B). In lane 1, the blot was hybridized with an RNA probe of *sdh1* (*11*), the scytalone dehydratase gene, to confirm the good quality of RNA used in the study. When the blot was hybridized with a probe that corresponded to the antisense strand of the TPase gene, a smear band of small RNA fragments was detected (lane 2). Interestingly, truncated transcripts of variable size (0.4-0.8 kb) were also observed on the blot hybridized with the sense strand of the TPase gene (lane 3). This raises the possibility that *Pot2* expression is regulated through the formation of convergent truncated transcripts from both ends of the transposable element. In fact, an additional short reading frame (ORF71) and canonical TATA box motifs, 5′-TATAAA-3′

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Abbreviations: GUS, β-glucuronidase gene; TIR, terminal inverted repeat; TPase, transposase; RT, reverse transcription.

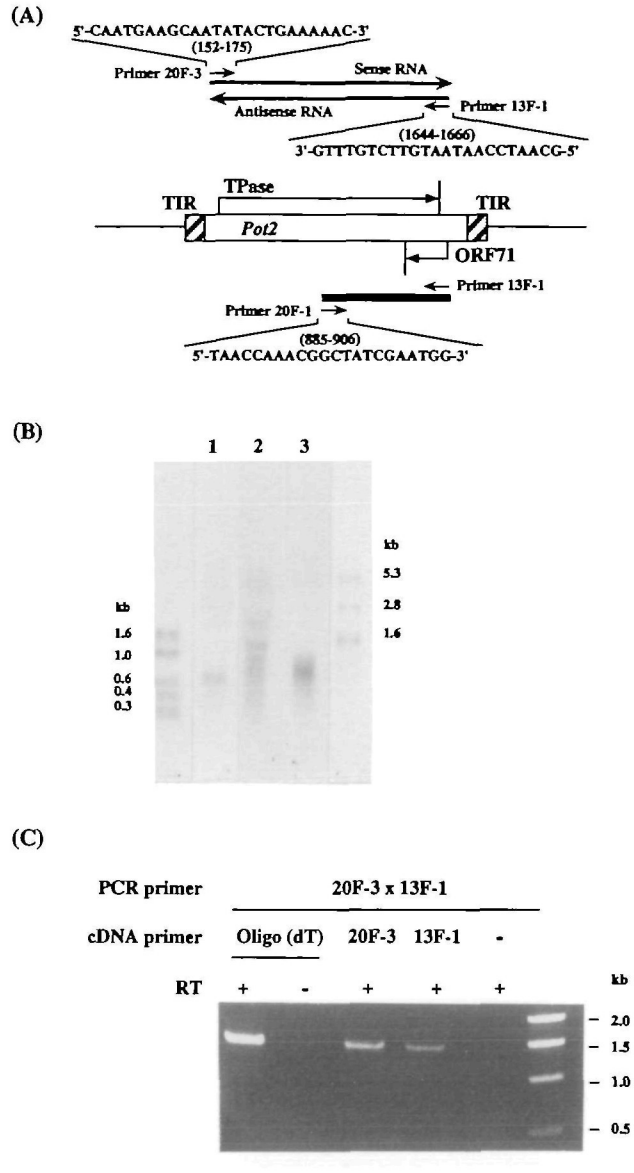

Fig. 1. **(A) Schematic representation of *Pot2*, and the primers used for preparation of RNA probes and for analysis by RT-PCR.** A part of the *Pot2* coding region (shown by a solid box) was amplified by PCR with primers 20F-1 and 13F-1, cloned into pGEM-TEasy (Promega), and used for preparation of the run-off probes for Northern analysis. The RNA probes, transcribed from the T7 promoter (for the antisense strand) and the SP6 promoter (for the sense strand), were prepared with a DIG RNA labeling kit (Boehringer). Primers 20F-3 and 13F-1, used for RT-PCR analysis, correspond to the regions that include the initiation codons of the TPase gene and ORF71 (unidentified), respectively. **(B) Expression of the sense and antisense RNA for the TPase gene.** For preparation of the RNA probe of *sdh1*, the DNA fragment amplified with EX2 and EX3 (see Ref. *11* for the primer sequences) was cloned into pGEM-TEasy and then transcribed from the T7 promoter (for the antisense strand). Total RNA was isolated using a S.N.A.P. total RNA isolation kit (Invitrogen). Ten microgram portions of total RNA were subjected to electrophoresis on a formamide agarose gel and then transferred to a Nytran N membrane (Schleicher & Schuell, Dassel). The membrane was cut into five strips and each strip (except the RNA markers) was hybridized to a different digoxigenin-labeled RNA probe; hybridization was performed with probes that correspond to the antisense strand of *sdh1* (lane 1), the antisense strand of *Pot2* (lane 2), and the sense strand of *Pot2* (lane 3). Digoxigenin-labeled RNA markers (Boehringer) are shown on both sides of the panel. **(C) RT-PCR analysis of *Pot2*.** The RNA samples were treated with RNase-free DNase I and then purified again on the S.N.A.P. column according to the manufacturer's instructions. The first-strand cDNA was primed with either the oligo (dT)-adaptor primer (Takara), or primer 20F-3 or 13F-1 (see panel A). The RT reaction (50°C for 1 h) was carried out on 1 μg of RNA using an RNA PCR kit (Takara Biomedicals). Subsequent PCR with the 5′- and 3′-specific primers generated the corresponding products for *Pot2*, which were cloned into the pGEM-TEasy vector and confirmed by partial nucleotide sequencing. Each primer indicated at the top of the gel was used for the cDNA synthesis, and subsequently subjected to PCR with primers 20F-3 and 13F-1. As controls, RT and the cDNA synthesis primer were omitted from the RT reaction, as shown in lanes 2 and 5, respectively.

(complements 1782-1777 and 1771-1766 of Z33638), were found in the complementary strand of the previously identified *Pot2* coding sequence (see Figs. 1A and 2A). This TATA sequence appeared to be responsible for the truncated antisense transcript detected on Northern analysis.

Expression of *Pot2* transcripts was also confirmed by reverse transcription (RT)-PCR. As shown in Fig. 1C, a PCR product of 1,515 bp (the primer pair 20F-3/13F-1, shown in panel A) was obtained from the cDNA primed with the oligo (dT) primer (lane 1). This product was a result of RNA amplification and not contamination by genomic DNA, since we could not detect PCR products in the absence of the RT reaction (lane 2). The transcript of *Pot2* was also detected by PCR of the cDNA primed with primer 13F-1 (lane 4), indicating that the TPase gene is actually transcribed in the fungus. When the cDNA synthesis was primed with primer 20F-3, the same PCR product was obtained (lane 3). Since the *Pot2* transcripts were not detected in the RT reaction without the cDNA synthesis primer (lane 5), RNA-primed (primer 20F-3 independent) synthesis of the cDNA did not occur in the sample under investigation. Therefore, this PCR product can be attributed to the antisense RNA, that overlaps the full-length transcript of the TPase gene. Perhaps the majority of the *Pot2* transcripts were truncated, as shown on Northern analysis, but trace amounts of full-length transcripts might have been detected on sensitive RT-PCR analysis.

The 5′ ends of these convergent overlapping transcripts could not be determined, presumably due to their instability and low abundance (much longer incubation was required to detect these transcripts than that of *sdh1* with Boehringer's digoxigenin detection system on Northern analysis). To demonstrate that the sense and antisense transcripts were generated by the putative promoters within the element (Fig. 2A), we performed transient expression assays with vectors containing *GUS* fused to these promoters (Fig. 2B, left panel): pGR101 contains the *trpC* promoter of *Aspergillus nidulans* (*12*), and pGR102 and pGR103 contain the putative antisense and sense promoters, respectively. Each pGR vector (10 μg) was introduced into 5×10^7 protoplasts of *P. oryzae* by the PEG-$CaCl_2$ method (*13*), followed by incubation in liquid medium for 12 h. As shown in Fig. 2B, GUS activity was detected with pGR102 and pGR103, as well as with pGR101 (4.9-fold over the control level). This result indicated that the putative promoter sequences at both ends of *Pot2* can transiently express the downstream reporter gene when present in multiple copies within the fungal cells.

(A)

pGR102

```
      PstI
ctgcagtctgcacatagagccaagctccgagttcgggTAACGTTGCGTACCCCCTGTTCGGCACCCCCCCTGTTCGGCACCCTGAAAATC  90
                                  DraI                TPase Stop
TAACAACGAAATGCCTACTTTTATAAACTGATTTAAATATAAAATTATCAACGGACAAAAATACAATCGTTTTCACGCTTCTCCGGTTCA 180
                                                 ClaI
TTAACCTCTTCTTCATCAGCTAAAGGTTCCAAATTTTCTATATCATTTTCCTGCAATCGAT                              241
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pGR103

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     KpnI
ggtaccggttgattaccctgcatcgcccatacgtttgccatttgccacgaaagcgatcccaaagagacaagggtataatataggggcgac  90
      NspV
tctattcgaaggatctaaagctaagtgtaagaaagaaggaaggcacgcagtcccagtcgcgtacacgcgcctaagcgaacaaacaacaaa 180
cgaaccctggcgccttatcacgtggggtggagctggtcacggttagcagtcgtccaggcataccattccgtaacggagggtctgaccaaa 270
gttcaaTAACGTTGCGTACCCCCTGTTCGGCACCCCCCCTGTTCGGCACCCAAAAATAACAACACTTTTATTTTTATCCTCCAACTTCTA 360
                                                                          HindIII
TTATAAATATCTCGATGAATCTGTCACTTTTGGATTTACTTTTTTCTGATTTTAATTCTCCATTTTCGATAAGCTT               436
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(B)

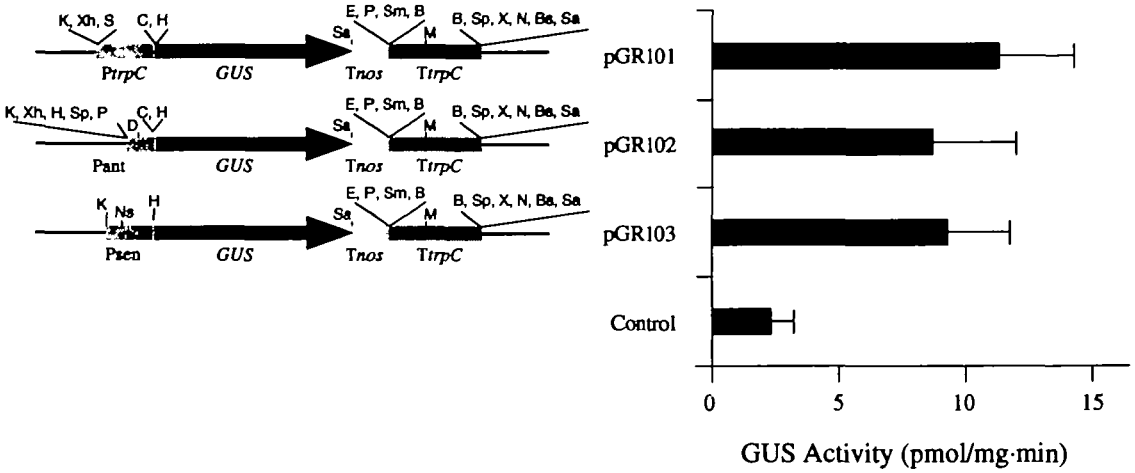

Fig. 2. **(A) The sequences of the promoter fragments of pGR102 and pGR103.** The sequences within the "TA" target sites of *Pot2* are shown in capital letters. The 43 bp inverted terminal repeats are underlined and the 16 bp direct repeats present within the inverted repeats are italicized. The putative TATA box motifs are shown in bold type. Since a C to T transition was found in the *Pot2* sequence (which corresponds to C at bp 1773 of Z33638), a new *Dra*I site was generated in the promoter region of pGR102. **(B) Determination of the promoter activity of *P. oryzae*.** In the left panel, the structures of the vectors (circular) for transient expression of the GUS reporter are shown. Thick lines represent vector pBluescriptSK$^+$. Plasmid pBI221 (Clontech) was digested with *Sma*I and then the *Hin*dIII linker (5′-CCCAAGCTTGGG-3′) was ligated to the blunt end. After double digestion with *Eco*RI and *Hin*dIII, the fragment containing *GUS* was cloned into the corresponding site of pBF101 (*14*). The resulting plasmid, named pGR101, was used as a standard vector to evaluate the strength of the *Pot2* promoters by means of transient expression assays. For the construction of pGR102 and pGR103, DNA fragments containing the *Pot2* promoters were amplified by PCR with primers containing mismatched bases (underlined): pGR102, primers U-I (5′-GTTTTCCCACTCGAGACGTTG-3′) and D-I (5′-GAACATTAATCGATTGCAGG-3′); pGR103, primers U-III (5′-CAAAGGTACCGGTTGATTACCCTGC-3′) and D-III (5′-CATTCGAAAATGGAGAATTAAAATCAG-3′). The PCR products were double digested with *Cla*I-*Xho*I and *Kpn*I-*Nsp*V (partial), for pGR102 and pGR103, respectively, and the resulting promoter fragments were replaced by the *trpC* promoter of pGR101. Abbreviations: P*trpC*, the promoter of *trpC*; Pant, the antisense promoter of *Pot2*; Psen, the sense promoter of *Pot2*; T*trpC*, the terminator of *trpC*; T*nos*, the terminator of the nopaline synthase gene; B, *Bam*HI; Bs, *Bst*XI; C, *Cla*I; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Mlu*I; N, *Not*I; Ns, *Nsp*V; P, *Pst*I; S, *Sal*I; Sa, *Sac*I; Sm, *Sma*I; Sp, *Spe*I; X, *Xba*I; Xh, *Xho*I. In the right panel, the result of the transient assay is shown. Young regenerated mycelia were disrupted in 1 ml of lysis buffer (10 mM EDTA, 0.1% Triton X-100, 0.1% *N*-lauroylsarcosine, 10 mM 2-mercaptoethanol, 50 mM phosphate buffer, pH 7.0) by sonication on ice. The GUS activity of the extract was measured by means of the fluorometric assay using 4-MUG (4-methyl-umbelliferyl-β-D-glucuronide) as a substrate (*28*). Although the level of GUS activity varied, depending on the condition of the protoplasts in an independent experiment, the relative strength of each promoter showed a similar pattern of expression. A control reaction was performed with pBI221, which contains promoterless *GUS* in pUC19.

We next examined whether or not these *Pot2* promoters are also capable of driving the expression of a heterologous gene from a chromosomal locus. For this purpose, we used these promoters to generate drug-resistant transformants and compared their efficiencies with that of the *trpC* promoter. Plasmids pBF102 and pBF103 were constructed by replacing the *GUS* and *nos* terminator cassettes of pGR102 and pGR103 with *BSD*, the blasticidin S (BS) resistance gene from *Aspergillus terreus* (*14*); the 0.6 kb *Cla*I-*Sma*I fragment of pBF101 (*14*) and the 0.5 kb *Hin*dIII-*Pst*I fragment of pUCSV*BSD* (*15*) were cloned into the corresponding sites of pGR102 and pGR103,

respectively. Each of the BS resistant plasmids, pBF101 (with the *trpC* promoter), pBF102, and pBF103, was transformed into protoplasts of *P. oryzae* as described previously (*13*). After 5 days incubation, stable transformants were obtained with substantially equal efficiencies (approx 200 transformants/μg of DNA) with any of these BS resistant plasmids. The efficiencies correlated well with the promoter activities, as measured by means of transient expression assays (Fig. 2B). Therefore, the antisense transcript of *Pot2* would have been generated by the antisense promoter at the 3′ end of the element, not through read-through transcription from the adjacent region.

Nine randomly selected pBF102 transformants (with the antisense promoter) were subjected to Southern analysis. The blot was probed with pBF101, which hybridizes to the vector sequences in these transformants, since approx. 100 copies of *Pot2* are present in the genome (*8*). As shown in Fig. 3A, different patterns of hybridization signals were observed for the genomic blot of each transformant. Interestingly, transformant BRA102-2 appeared to contain one copy of pBF102 integrated in the genome through homologous recombination, as suggested by the banding pattern on Southern blotting. This was further confirmed by sequencing of the expected PCR product (see Fig. 3B), which was amplified with primers M13-M4 and 20F-1 (data not shown). These results indicate that the antisense promoter transcribed the downstream sequence from one of the chromosomal loci where the transposons were originally positioned in the wild type genome.

Although the expression levels of the sense and antisense promoters were shown to be similar to that of the strong *trpC* promoter, the amounts of the convergent *Pot2* transcripts appeared to be very small (see Northern analysis). Such a negative effect on transcription was also observed in the *cyc1-512* mutant of *Saccharomyces cerevisiae*; *i.e.*, deletion of the termination signals between *CYC1* and the adjacent gene causes convergent overlapping transcription from opposite strands and has a markedly deleterious effect on the levels of mRNA yielded by both transcription units (*16*). The decreased amounts of transcripts might be

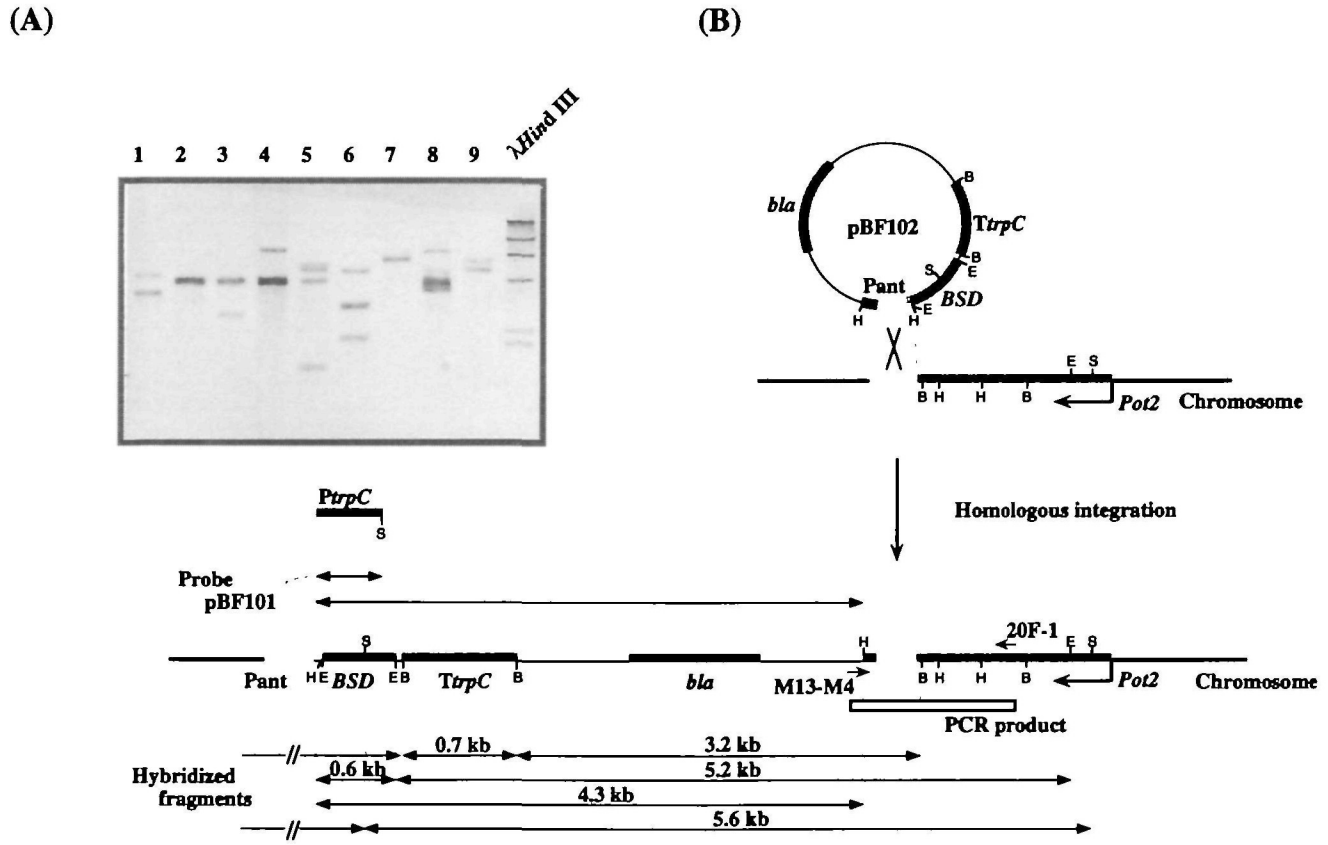

Fig. 3. **(A) Southern analysis of transformants carrying pBF102.** Lanes 1-9, genomic DNA of transformants BRA102-1-BRA102-9 digested with *Hin*dIII. Genomic DNA was isolated from the *P. oryzae* transformants by the miniprep procedure of Sweigard *et al.* (*29*). When the blot was probed with digoxigenin-labeled pBF101, a single band of 4.3 kb was detected for transformant BRA102-2. If a transformant carries a single copy of pBF102 on the genome due to homologous integration between the *Pot2* antisense promoter sequences, such a banding pattern is expected to occur on a Southern blot. **(B) Homologous integration of pBF102 into the genome of *P. oryzae*.** A single cross-over between the anti-sense promoter segment of *Pot2* (201 bp of pBF102) and its homologue in the genome leads to integration of the vector sequences between copies of this 201 bp segment in a direct repeat. The genome of such transformants should contain the universal primer (M13-M4) sequence of the pBluescript vector immediately downstream of *Pot2*. Transformant BRA102-2 appeared to have been generated through such homologous integration, because the predicted fragment was amplified by PCR with primers M13-M4 (5′-GTTTTCCCAGTCACGAC-3′) and 20F-1 (see Fig. 1A). In further support of the above results, Southern analysis (pBF101 as a probe) revealed hybridization signals of 0.7 kb+3.2 kb, 0.6 kb+5.2 kb, and 5.6 kb for the genomic DNA digested with *Bam*HI, *Eco*RI, and *Sal*I, respectively (data not shown). See the legend to Fig. 2B for abbreviations.

attributed to colliding RNA polymerase molecules that cause transcription in both directions to cease, or perhaps to an altered chromatin structure that causes the polymerase to fall off the template when both strands are transcribed.

In addition to *Pot2*, convergent overlapping transcription units have been found in four transposable elements; bacterial IS*10* (*17*), fly *micropia* (*18*), maize *MuDR* (*19*), and *TOC1* of green alga (*20*). Although the antisense RNAs of *MuDR* and *TOC1* were not the result of transcription by specific promoters within the elements, the other two transposons proved to carry the related antisense promoters: the antisense transcript derived from the pOUT promoter of IS*10* bound to the sense TPase RNA and blocked ribosome binding; and the *micropia* retrotransposon produced antisense RNA complementary to the RT and RNase H coding regions from the testis-specific antisense promoter. However, these antisense RNAs are not transcribed from the 3′ end of the element and overlap by a small part of the entire sense transcription unit. In these respects, the transcription of *Pot2* is unique relative to that of other characterized prokaryotic and eukaryotic transposons.

At both the nucleotide and peptide sequence levels, *Pot2* shows significantly high similarity to *Flipper* from *Botrytis cinerea* (*21*) and *Fot1* from *Fusarium oxysporum* (*9*). At the 3′ ends of these transposons, TATA-like motifs for the convergent direction were found; *i.e.*, 5′-TATATA-3′ and 5′-TATAT-3′ (complements 1787-1782 and 1779-1775 of U74294, respectively) for *Flipper*; and 5′-TATTTA-3′ (complement 1857-1852 of X64799) for *Fot1*. Comparison of these antisense sequences suggests that ORF71 of *Pot2* might not be translated to produce a functional protein involved in transposition, since the antisense RNAs of these closely related transposons do not contain any ORFs similar to ORF71. It remains to be determined whether or not these TATA-like motifs also produce the antisense RNA for the TPase gene.

In the transpositioning of the Tc*1-mariner* superfamily element, the purified TPase is sufficient to mediate the phosphoryl transfer reaction *in vitro*, and no species-specific host factors are necessary for the transpositioning (*22-24*). Considering the similar structural characteristics of *Pot2* and its insertion sites, expression of the encoded TPase might induce transpositioning. If this is the case, one possible explanation for the lack of transpositioning might be some mutations that abolish the function of the encoded TPase. However, the presence of both sense and antisense RNAs in presently quiescent *Pot2* leads to the more appealing hypothesis that expression of the TPase gene could effectively be repressed by the function of the strong antisense promoter; *i.e.*, antisense RNAs that are retained in the nucleus bind to the target sense transcripts, inhibit the transport from the nucleus to the cytoplasm (*25*), and lead to degradation (*26*). The regulatory mechanism of transpositioning could be analyzed by means of transient expression excision assays (*27*) using a series of *Pot2* plasmids each carrying a mutation or deletion in the antisense promoter sequence. Further investigations will lead to elucidation of the regulatory mechanism of *Pot2*.

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