Yeast retrotransposon Ty4: the majority of the rare transcripts lack a U3-R sequence

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ABSTRACT

The retrotransposon Ty4 is found in different yeast strains at only one to three copies per haploid genome. In the present study, we aimed at relating the apparent low transpositional activity of Ty4 to transcriptional features of this element. RT–PCR revealed that Ty4 is transcribed at a very low level, being comparable with that of GAL4. Contrary to other Ty elements, the transcriptional rate of Ty4 is not affected in a sin4 background nor by treatment of cells with α factor. From experiments measuring the expression levels in lacZ fusion constructs, we conclude that Ty4 transcription is repressed by a negative regulating element residing within the LTR, whereas positive cis-acting elements, like those that have been found to mediate expression of Ty1/2 and Ty3, are absent from Ty4. Analysing Ty4 transcript termini by the RACE–PCR method, we found several distinct transcriptional initiation sites. But surprisingly, the majority of the polyadenylated Ty4 transcripts terminate shortly upstream from the 3′ LTR boundary, so that these transcripts do not contain a U3-R sequence, which is normally required for obligate strand transfer during DNA synthesis. Thus, the extremely low transcription rate of Ty4 and imperfect Ty4 transcripts are the reason for the low transpositional activity of this element.

INTRODUCTION

Yeast transposable elements are a family of genetically mobile, repetitive DNA sequences. Two different classes have been characterized: the copia-like family, including the Ty1, Ty2, Ty4 and Ty5 elements in Saccharomyces cerevisiae, and the gypsy-like family, including Ty3 in S.cerevisiae (1,2) as well as Tt1 and Tt2 in Schizosaccharomyces pombe (3). Ty elements share structural similarities with and resemble the proviral form of retroviruses (4) in that they transpose through an RNA intermediate (5). Whereas Ty1 and Ty2 occur in some 30 copies throughout the yeast genome, Ty3 and Ty4 are low copy number elements (6,7). The different abundances of the elements appear to be due to the fact that their transcriptional and transpositional activities are differently regulated (8). In accordance with the high transcriptional activity of Ty1, Ty1 RNA is found as one of the most abundant poly(A)+ RNAs in many haploid strains of S.cerevisiae (9). By contrast, little Ty3 RNA is found in haploid cells under normal conditions. Transcription of Ty3, however, can be induced in cells by exposure to the mating pheromone α factor (6). Responsible for this effect is the pheromone-responding element (PRE) located within the Ty3 LTR, sigma (10).

Transcription of Ty1 and Ty3 has been shown to initiate within the 5′ LTR and to terminate in the 3′ LTR, resulting in polyadenylated transcripts which are shorter than the proviral DNA. In both cases, the transcripts contain an R-U5 sequence at the 5′-end and an U3-R sequence at the 3′-end (6,11). During reverse transcription, full-length DNA with complete LTRs is regenerated (12).

Ty4 is 6.2 kb in length and is composed of 371 bp terminal repeats (τau), flanking an internal domain of 5.4 kb. Sequence comparisons at the amino acid level show that the domains in Ty4 diverge considerably from those of the other yeast retrotransposons. Although a number of τau elements are found dispersed throughout the yeast genome, indicating that Ty4 is able to transpose, attempts to obtain direct proof for transposition of this element have failed so far (13,14). For example, the analysis of transformants in which an intact Ty4 copy was tagged by the insertion of the neo gene cassette and overexpressed under the control of the inducible GAL1 promoter, analogous to the constructs used to monitor Ty1 transposition (15), did not reveal any novel integration sites of Ty4 (13). These experiments suggested to us that the transcription rate of Ty4 even under these conditions must be very low and, as transposition is intimately correlated to transcription, that the low rate of Ty4 transcription per se might be the cause for the low transposition frequency of Ty4.

In this study, we carried out experiments to determine the level of Ty4 transcription and to characterize the nature of Ty4 transcripts. By using RT–PCR technology, we show that Ty4 is expressed at a very low level and comparable with that of GAL4. Contrary to the expression of Ty1 or GALA, Ty4 expression is not repressed in a sin4− background. Measurement of β-galactosidase activity from a variety of Ty4–lacZ fusion constructs revealed that the expression of Ty4 at a low level might be due to the presence of negative regulatory elements within the leftmost LTR and the absence of activating cis-regulatory elements within the translated region. Furthermore, an analysis of Ty4 transcript initiation and termination sites using RACE–PCR methodology

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indicates that Ty4 transcripts initiate at different sites within the left LTR, but that the majority of the transcripts terminate shortly upstream from the rightward LTR. Thus these Ty4 transcripts lack the U3-R sequence generally required for strand transfer during DNA synthesis in retroelements.

**MATERIALS AND METHODS**

**Strains and plasmids**

Plasmids were cultured in *Escherichia coli* strain 490A (rks, m kon, met, thr, leu, recA), except pCR-Script SK(+) plasmids (Stratagene, Heidelberg, Germany), which were grown in *E.coli* strain XL1-Blue MRF' Kan (Stratagene). The yeast shuttle plasmids YEp353–358 were cultivated in yeast strain YM4127 (MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-903 leu2-3 leu2-112 tyr1-501) (M. Johnston).

Yeast strain MC45-5/A (MATa, trp1-259, leu3-3, 117, ura3-52, suc2) (M. Ciriacy) and YM4127 were the source of poly(A)* RNA. The wild-type strain YS18 (MATa his3-11 his3-15 leu2-3 leu2-112 can1 ura3 Δ5) (16) and the sin4 mutant strain YS84 (MATa his3-11 his3-15 leu2-3 leu2-112 can1 ura3 Δ5 SIN4:URA3) (D. J. Stillman) was used in the analysis of Ty4 transcript levels. Yeast cells were cultivated as described by Sherman et al. (17).

All constructs used in the β-galactosidase assays are based on the multi-copy *E.coli*-yeast shuttle vectors YEp353–358 carrying a truncated lacZ gene and URA3 as a marker (18). To construct pTy1–PvuII, the entire Ty1–gag region was amplified using primers annealing to the LTR start site (Ty1-1-HindIII) and to the region of the frameshift at position 1600 (Ty1-1600-BglII), thereby introducing artificial HindIII and BglII restriction sites (cf. Table 1).

The Ty1 fragment was subcloned into YEp356, cut out with HindIII and PvuII (position 475) and ligated into the YEp357/Smal site of YEp357. pTy4–EcoRV was constructed by inserting a 1200 bp HindIII–EcoRV fragment containing the Ty4 element (positions 1–907, flanked by genomic sequence) into YEp358.

pTy4-BglII was constructed by amplifying the entire Ty4–gag region using primers annealing to the LTR start site (Ty1-1-HindIII) and to the region of the frameshift at position 1428 (Ty4-1428-BglII), thereby introducing HindIII and BglII restriction sites. The PCR product was cleaved with HindIII and BglII and ligated into YEp356.

Ty1/Ty4 chimeric plasmids were designed using the PCR technique. To construct pTy1-Ty4-lacZ, the delta region and the Ty4 internal region were amplified separately, using the primer pairs Ty1-1-HindIII/Ty1-LTR-Ty4 and Ty4-ATG-Ty1//Ty4-925-EcoRI respectively (the backward primer Ty1-LTR-Ty4 contains extra nucleotides overlapping Ty4; the forward primer Ty4-ATG-Ty1 contains extra nucleotides overlapping Ty4). The resulting products were subjected to PCR for five cycles without primers, followed by 25 cycles with the addition of the ‘outer’ primers Ty1-1-HindIII and Ty4-925-EcoRI. The final PCR product was cleaved with HindIII and EcoRI and ligated into YEp358. pTy4-Ty1-lacZ was constructed in a similar manner except that Ty4-LTR-Ty4 and Ty1-ATG-Ty4 were used as ‘overlapping’ and Ty1-1-HindIII and Ty1-850-EcoRI as outer primers. The resulting PCR product was subcloned in YEp358.

pTy1-Ty4-EcoRV was constructed by replacing the 5′-part of `tau` up to the HpaII site with the corresponding delta region of Ty1. Ty4-230-XhoI and Ty4-910-EcoRV were used in a PCR reaction. The resulting product was subcloned in the SrfI-digested pCR-Script(+) vector, which allows selection for recombinants by including SrfI in the ligation reaction. A clone containing the insert’s XhoI site next to the vector’s HindIII site was selected, digested by HindIII/SacI and the resulting fragment recloned in pUC18. Finally, the 260 bp Ty1 HindIII–XhoI fragment was inserted into this intermediate and the chimeric Ty1/Ty4 HindIII–EcoRV fragment transferred into YEp358. The absence of PCR artefacts was verified by sequencing all constructs between lacZ and the respective LTR.

**DNA and RNA methods**

Standard PCR was carried out by the use of a GenAmp PCR System 2400 thermocycler (Perkin Elmer, Weiterstadt, Germany) in a total volume of 50 µl [10 mM Tris–HCl, 1 mM MgCl2, 50 mM KCl, pH 8.3, 200 µM each dNTP, 1 µM each primer and 2.5 U Taq DNA polymerase (Boehringer, Mannheim, Germany)] using thin-walled 200 µl tubes without mineral oil overlay. Cycle conditions were: hold (94°C/5 min), three-step cycle (96°C/1 min,
55°C/40 s, 72°C/1.5 min, 25–35 cycles), hold (72°C/5 min, then 4°C).

PCR products were isolated from agarose gels by the glassmilk method or directly after PCR using QIAquick spin columns (Qiagen, Hilden, Germany). Purified PCR fragments were cloned into the pCR-Script SK(+) vector (Stratagene) according to the manufacturer’s protocol.

Poly(A)+ RNA samples were separated in 0.8% agarose gels (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 5.5–7.9, and 2% formaldehyde) and transferred directly to PALL B filters (Pall, Dreieich, Germany).

Standard procedures were used for labelling DNA probes and in Southern or Northern hybridizations (19).

Isolation of mRNA and RT–PCR

Total RNA was isolated from yeast cells as described (20). After treatment with DNase I (20 U/ml DNase I, 25 mM Tris–HCl, pH 7.5, 5 mM MgCl2, 160 U/ml RNasin; 37°C/60 min), poly(A)+ RNA was enriched using the Oligotex-dT poly(A)+ RNA Mini Kit (Qiagen). mRNA (100 ng) was retrotranscribed in a total volume of 50 µl using oligo(dT) primers and RNase H– reverse transcriptase as recommended by the manufacturer (StrataScript; Stratagene). Aliquots of 2–8 µl of the reaction mixture were used for RT–PCR. The reaction was carried out in a total volume of 50 µl (10 mM Tris–HCl, pH 8.3, 10 mM MgCl2, 50 mM KCl, 200 µM each dNTP, 1 µM each primer and 2.5 U Taq polymerase (Boehringer). Cycle conditions were: hold (94°C/5 min), three-step cycle (94°C/60 s, 55°C/40 s, 72°C/90 s, 25 cycles), hold (72°C/5 min, then 4°C).

RACE

The 5’ RACE protocol of Frohmann et al. (21) was used with minor modifications, poly(A)+ RNA (YS4127) (2.4 µg) was retrotranscribed as above except using a Ty4-specific primer (Ty4–480). The RT product was passed through a QIAquick spin column and polyadenylated by the use of terminal deoxynucleotidyl transferase (TdT) (Boehringer). Excess dA TP was removed by the use of QIAquick spin columns. For amplification, 1–5 µl from the tailing reaction, 0.05 µM RACE-N ('hook') primer, 1 µM Ty4–480 primer and 1 µM RACE-O primer in a 50 µl PCR reaction mixture [1× Taq polymerase buffer (Boehringer), 200 µM each of the four dNTPs] were denatured (95°C/5 min) and cooled to 72°C. Taq polymerase (2.5 U) was added and the mixture incubated for 5 min at 95°C, 5 min at 42°C and 30 min at 62°C in a thermocycler (GeneAmp PCR System 2400; Perkin Elmer). A second round of amplification was carried out using a step program (96°C/1 min, 55°C/40 s, 72°C/90 min, 25 cycles) followed by a 7 min final extension at 72°C, without adding fresh Taq polymerase. Then a nested PCR reaction was carried out using RACE-I and Ty4–428 primers. RACE–PCR products >100 bp were isolated from a 2% agarose gel and cloned. The bacterial colonies were screened by hybridization with a Ty4–gag probe.

3’-End amplification of Ty4 cDNA (3’ RACE) was performed as described above except omitting the tailing reaction. RACE-N primer was used for reverse transcription; RACE-O, RACE-I and Ty4-specific primers were used in the subsequent amplification reaction.

RESULTS

Ty4 is transcribed at a very low level

In order to investigate Ty4 transcription, poly(A)+ RNA was isolated from MC45-5/A (MATα type) or YM4127 (MATa type) cells, fractionated by electrophoresis in formaldehyde–agarose gels and transferred to nylon membranes. The filter-bound RNA was hybridized to a Ty4–gag–specific probe and a Ty1–gag–specific probe for comparison. An actin gene-specific probe was used for internal standardization. Even at prolonged exposure of the autoradiograms, no Ty4–specific signals could be detected (Fig. 1A). In contrast, major Ty1 transcripts (5.7, 5.0 and 2.2 kb (23)) were visualized by autoradiography after 24 h (Fig. 1B). Therefore, we employed the highly sensitive RT–PCR method (24) to detect Ty4 transcripts. In brief, total yeast RNA was exhaustively digested with RNase-free DNase and enriched for poly(A)+ RNA. After reverse transcription using oligo(dT) primers, a subsequent PCR reaction was carried out using Ty4-specific primers.
Due to the sensitivity of the RT–PCR method, the removal of any genomic DNA from the mRNA preparation is a critical step. The absence of genomic DNA can easily be monitored for mRNAs derived from intron-containing genes. In this case, RT–PCR primers are selected which are separated in the genome by at least one intron: PCR products amplified from the reverse transcribed template are smaller than those that would be derived from the genomic DNA template. For a gene of interest not transcribed template are smaller than those that would be derived by at least one intron: PCR products amplified from the reverse transcribed template are smaller than those that would be derived from the genomic DNA template. For a gene of interest not containing an intervening sequence, completion of the DNase treatment of the RNA preparation can be verified by using primers for an intron-containing gene as an internal control. In our approach, we choose to compare the PCR products obtained from total yeast RNA without DNase treatment and an RNA preparation subjected to exhaustive DNase treatment by using actin gene primers in a control reaction. Employing total untreated RNA as a template, PCR amplified a fragment (Fig. 2, lane 7) of the same length as amplified from a genomic template (lane 12). Such a fragment is no longer generated after DNase treatment of the RNA preparation (lanes 8 and 17). RT of the DNase-treated RNA template followed by PCR amplified a fragment of smaller size (lanes 10 and 18), clearly indicating that all contaminating genomic DNA had been removed completely by DNase treatment. In parallel experiments, the RT templates obtained from the same DNase-treated RNA preparations were used together with appropriate primers for Ty4 amplification from the two yeast strains MC45-5/A and YM4127, carrying two or three copies of Ty4 respectively. GAL4-specific primers served to monitor the sensitivity of the method. As it is technically difficult to retrotranscribe a mRNA >5000 nt, we used primers annealing to the Ty4 3′-region (Ty4-4981 and Ty4-6060, Table 1) in the PCR following reverse transcription. PCR without RT yielded no product in both yeast strains (lanes 2 and 13), whereas PCR of the RT template yielded a product of the expected size in both strains (lanes 4 and 14), confirming the presence of intact Ty4 mRNA. In YM4127, the amount of PCR product was slightly higher than in MC45-5/A, which might reflect the difference in Ty4 copy number. As seen from Figure 2 (lanes 14 and 22), the amount of PCR product from GAL4 is comparable with that obtained from Ty4. This is interpreted to mean that the expression of Ty4 occurs at a level comparable with that of GAL4, which has been shown to be very scarce.

**Ty4 transcription is not influenced in sin4 mutants**

Studies with yeast mutants have shown that the expression of several genes, including Ty1 elements, requires pleiotropic factors of the Swi/Snf class for transcriptional activation. Recent evidence suggests that the SWI/SNF genes, together with the SPT/SIN genes, mediate transcriptional control via effects on chromatin (26,27). Jiang and Stillman (28) found that sin4 mutants display defects both in negative and positive regulation of transcription: the expression of the Ty1 delta element is repressed in these mutants.

To investigate whether Ty4 expression is similarly affected in sin4 mutants, we compared the Ty4 transcript level in a wild-type (YS18) to that in a sin4 mutant (YS84) yeast strain using competitive RT–PCR (Fig. 3). As above, actin gene primers and GAL4-specific primers were used for comparison. Since our PCR conditions were chosen such that no plateau effect occurs, it was also feasible to compare the amount of products in both strains. An equal amount of the competitor Mucomp was added to each of the respective Ty4 RT samples as an internal standard (Fig. 3, lanes 4 and 5 and 21 and 22 respectively). The PCR products of co-amplification were digested with either HpaII (lanes 4 and 21) or MluI (lanes 5 and 22) before loading the samples onto the gel. Direct PCR without RT yielded no product in both strains. PCR after RT resulted in equal amounts of product of the expected size in both strains (Fig. 3, compare lanes 3–5 with lanes 20–22), confirming that the RT–PCR products were derived from RNA. However, no differences between the two strains were observed. Thus, our conclusion is that Ty4 is not repressed in sin4 mutants. In contrast, the sin4 mutation leads to an increase in GAL4 transcription (compare lane 32 with lane 15); the larger bands seen in lanes 25 and 30 in strain YS84 most probably represent products derived from non-specific priming.
Expression of Ty1/2 and Ty3 is regulated by a variety of cellular factors and Ty-specific cis-acting elements (1). The principal regulatory elements of Ty1 are located within the internal translated region rather than upstream of the transcription start site in the leftmost LTR (29–31). Regulation of Ty3 transcription differs in several respects from Ty1 and Ty2 regulation and appears to be largely independent of activation by the internal domain (8).

To address the question whether Ty4 transcription is regulated via potential cis-acting elements, we used the following approach. Two Ty4–lacZ fusions containing different 5′-portions of the element were constructed, transformed into YM4127 (MATa) and β-galactosidase activity was determined in cellular extracts. Ty1–lacZ fusions were employed for comparison. As seen in Figure 4, 216 U β-galactosidase activity was obtained from a construct (pTy1-PvuII, no. 1) containing the 5′-portion of Ty1 including the internal domain up to the ‘critical’ PvuII site (cf. 31), whereas in accordance with earlier observations (31) β-galactosidase activity from a construct in which lacZ was fused to the delta sequence alone was near background. Similarly, pTy4-tau by itself fused to lacZ did not reveal any β-galactosidase activity (not shown). However, β-galactosidase activities from pTy4-lacZ fusion constructs (pTy4-EcoRV and pTy4-BglII, nos 2a and 2b) equivalent to construct pTy1-PvuII were also below the detection level. In view of the low transcriptional activity of Ty4 that could only be monitored by RT–PCR it is not so surprising that the expression of intact Ty4 is too low to be detected by measuring β-galactosidase activity from pTy4-lacZ fusion constructs. The low expression level of Ty4 may be explained by either the absence of activating elements or the presence of negative regulatory elements within the element. To exploit these possibilities, chimeric lacZ fusion constructs were made by combining relevant portions of Ty1 and Ty4, pTy4-Ty1-lacZ (no. 3), in which the region downstream from the Ty4 translational start site was replaced by a Ty1 portion containing the Ty1 translational start site and sequences up to position 850, exhibited weak β-galactosidase activity (9 U) compared with pTy1-PvuII. This result shows that the internal activating elements located within the Ty1–gag region are still capable of stimulating expression in the presence of the upstream Ty4 promoter region, albeit to a much lower degree than in the cognate situation. Normally, arbitrary sequences replacing Ty1 sequences up to the internal activation region do not interfere with Ty1 transcription (30). Thus a possible explanation for our finding is that a negative regulatory element is located within the 5′-portion of the Ty4 LTR. This interpretation is further substantiated by measuring Ty4 expression from construct pTy1-Ty4-EcoRV (no. 4), in which only the 5′-part of tau up to position 238 had been replaced by a 5′-portion of Ty1 delta; in this case, potential Ty4 transcription initiation sites were included. The level of β-galactosidase activity produced from this fusion amounted to 13 U, suggesting that a putative negative regulatory element located within the Ty4 LTR, positions 1–238, had been removed. Finally, the comparably low level of β-galactosidase activity (5 U) obtained from construct pTy1-Ty4-lacZ (no. 5), containing the transcriptional start site of Ty1 but the translational start site of Ty4, suggests that sequences between the transcriptional and translational start sites of Ty4 are required for expression. Furthermore, internal Ty4 elements necessary to initiate transcription might exist: otherwise no expression should be detectable with constructs nos 4 and 5, as it is anticipated that the contribution of the Ty1 LTR portions is nil.

It has been shown by van Arsdell et al. (32), that the Ty3 LTR contains a PRE located within the 5′ LTR, rendering Ty3 susceptible to α factor induction. Two PRE consensus sequences are also present in Ty4. Both of these sequences are located within the gag coding region, downstream of the translational start site, at positions 391 and 1258. To determine whether these elements are functional, all fusion plasmids as described above were introduced into strain YS4127 (MATa) and transformants treated with α factor.
**Figure 4.** β-Galactosidase activity of Ty1–, Ty4– and Ty3– lacZ fusion constructs. Extracts from haploid cell cultures (YM4127) and cells treated with α factor were used. The lacZ portion was fused in-frame to the respective Ty coding region. β-Galactosidase units are reported as µmol o-nitrophenylgalactoside cleaved/min/10^6 unit cells. Extracts made from five independent transformants were assayed in duplicate. The average β-galactosidase activity was calculated; +/- indicates the standard deviation. Ty1 delta regions are shown in grey, Ty1 coding regions as open boxes. Ty4 tau regions are shown as hatched boxes, Ty4 coding regions are black. The Ty3 sigma region is shown as a dotted box. Nucleotide positions, restriction sites and translation products pertinent to Ty1 (or Ty3) are shown underneath the constructs, those pertinent to Ty4 above the constructs.

prior to β-galactosidase activity measurements. Compared with plasmid pTy3-XmnI (Fig. 4, no. 6), used as a control, none of the fusion constructs responded to α factor treatment. Thus, the PRE sequences appear to be non-functional in Ty4.

**Termini of the Ty4 transcripts**

5'-Termini. In order to characterize the Ty4 promoter in more detail, we determined transcription initiation sites by the RACE–PCR protocol (21). The first strand Ty4 cDNA was made using a 21mer reverse transcription primer (Ty4-480) complementary to positions 461–480 in Ty4. After tailing, the 48mer RACE-N primer was annealed and a PCR reaction carried out using the forward primer RACE-O together with the backward primer Ty4-428; a nested PCR followed (RACE-I and Ty4-428). Amplimers were cloned and nine of these clones subjected to sequence analysis. Four different Ty4 transcript 5'-termini were monitored by this approach and are shown in Figure 5. In contrast to the primer extension mapping method, the RACE technique does not allow quantification, so that we cannot specify a major transcriptional start site. To prove the reliability of the RACE method, we used Ty3 in control experiments and found virtually the same three start sites as determined by Clark et al. (6) using reverse transcription extension (data not shown).

3'-Termini. To fulfill a basic requirement for a retrotransposition intermediate, the Ty transcript has to begin in the left LTR and to terminate within a redundant sequence, downstream of the homologous position in the right LTR, called the R region (12,33). To see whether the Ty4 transcripts fulfill this requirement, we used the RACE technique to map the 3'-end(s). First strand Ty4 cDNA was synthesized using the 48mer RACE-N primer, followed by PCR using RACE-1 as a backward and Ty4-5035 as a forward primer. Various PCR products were directly cloned and sequenced. Surprisingly, the majority of these transcripts were found to terminate upstream of the Ty4 3' LTR boundary located at position 5743. Twelve out of 17 Ty4 transcripts analysed terminated exactly 97 nt upstream of this boundary. To get an estimate of the relative abundance of the transcripts, the PCR products were size fractionated and hybridized to a Ty4 3'-terminal probe (Fig. 6A, lane 1). The major Ty4-hybridizing bands were (a) 800, (b) 520 and (c) 380 bp in size, of which the most abundant band (b) represents the transcript ending 285 bp upstream of the 3' LTR (Fig. 6B). Minor bands >800 bp indicated,
Figure 6. Ty4 termination sites as determined by 3′ RACE–PCR. (A) Southern analysis of PCR products. Samples were separated on a 2% agarose gel, blotted onto a nylon membrane and hybridized with a 32P-labelled terminal Ty4 probe. Resulting bands are designated a–e. (B) Schematized results from the 3′ RACE–PCR experiments. (Top) 3′-region of Ty4. PCR products visualized in (A) are represented by the bars; the part corresponding to the RACE-I primer is shown in grey. Product f is that derived from secondary PCR reactions.

however, that larger transcripts also appear to exist. To allow mapping in the Ty4 region further downstream, PCR reactions were carried out using Ty4-specific forward primers located closer to the 3′-end of Ty4 (Ty4-5644 and Ty4-5840) in combination with RACE-I as a backward primer, followed by hybridizations as described above (Fig. 6A, lanes 2 and 3 respectively). The result indicated that polyadenylated transcripts terminating within the 3′ LTR do exist, although in minor quantities. This notion was further substantiated by experiments using the above PCR products in a second round of PCR reactions with a forward primer corresponding to the left boundary of the LTR (data not shown). However, using the PCR product of the Ty4-5035/RACE-I primer set (Fig. 6, lane 1) as template together with RACE-I and Ty4-5840 primers, we detected a 460 bp band which represents a polyadenylated transcript terminating beyond the 3′ LTR (marked as f). The outcome of the experiments is schematized in Figure 6B. It might be mentioned that we validated the approach taken for Ty4 by performing parallel experiments with Ty3, confirming the data reported by Clark et al. (6).

DISCUSSION

The low copy number element Ty4 from yeast shares several characteristics with other retrotransposons. Although some 15–25 copies of Ty4 LTRs dispersed throughout the yeast genome (1) indicate that Ty4 is able to transpose, no direct proof of transposition of this element has been obtained (14), possibly due to the fact that Ty4 expression occurs at a low level. In this study, however, we were able to visualize Ty4 transcripts. Using GAL4 as a standard (25), we estimate that Ty4 is transcribed at a comparable level, i.e. Ty4 mRNA represents only 1.6 × 10–5 of total mRNA (∼0.1 molecules mRNA per cell). Care was taken in these experiments to exclude any DNA contamination of the mRNA preparations: to ensure that genomic DNA was not serving as the template in the PCR reaction after RT, the RNA samples from MC45-5/A and YM4127 yeast cells were exhaustively digested with RNase-free DNase. Additionally, we confirmed that the RT–PCR products were derived from RNA by using actin gene primers as an internal control and the accuracy of all PCR products was verified by diagnostic restriction enzyme analysis.

Studies with yeast mutants have shown that the expression of Ty1 is influenced by pleiotropic factors of the Swi/Snf class, which, in conjunction with the SPT/SIN genes, mediate transcriptional control via effects on chromatin (26,27). Especially, sin4 mutants have been shown to repress transcription from a Ty1 delta element (28,34). To investigate whether Ty4 expression is similarly affected in sin4 mutants, we compared the Ty4 transcript level in a wild-type (YS18) to that in a mutant yeast strain (YS84) using competitive RT–PCR. Our results clearly demonstrate that Ty4 expression is not influenced by the pleiotropic factor Sin4p. Similar experiments in other mutant spt or sin backgrounds will be necessary to see any influence of members of the Spt/Sin family on Ty4 expression.

To coordinate expression with the yeast life cycle, transcription of Ty1/2 and Ty3 is controlled by a variety of cellular factors in conjunction with specific Ty internal cis-acting elements (1). For example, Yu and Elder (31) showed that a region internal to the Ty1 coding sequence is essential for transcription, while the delta region by itself had little or no ability to promote transcription. In Ty3, major regulatory elements reside within the 5′ LTR that can be viewed as a compact, highly regulated, mobile promoter responsive to cell type and mating (8). In these cases, detailed analyses could largely be based on sequence homology, which revealed the presence of particular elements, and on direct
measurement of RNA levels and/or expression levels of reporter genes in appropriate constructs. In Ty4, these approaches are complicated by two facts: (i) inspection of the Ty4 sequence did not reveal elements similar to those found in the other yeast retrotransposons or yeast gene promoters, except two PRE consensus sequences detected at positions 391 and 1258 respectively; (ii) Northern blot analysis was hampered by the low level of Ty4 transcription. As an alternative, we therefore attempted to determine the influence of particular regions from Ty4 on the expression of lacZ in reporter constructs. These constructs were also tested for their ability to promote expression under α-pheromone induction. In Ty4 constructs (Fig. 4, nos 2a and 2b) that are similar to that of Ty1 (Fig. 4, no. 1), used for comparison, expression is virtually undetectable. Only when sequences from the Ty4 5′ LTR are removed (and replaced by ‘inert’ Ty1 sequences; Fig. 4, nos 4 and 5) does expression occur at a low level. The comparison of constructs nos 4 and 5 suggests that internal Ty4 sequences are necessary for expression, but that a negative regulatory element located within the first 238 bp of Ty4 has been abolished. The existence of such a negative element is also suggested by comparing constructs nos 1 and 3; the internal control region of Ty1 is still capable of initiating expression when fused to the Ty4 5′ LTR, although the expression level is reduced more than 20 times as compared with the cognate Ty1 construct. In accordance with this interpretation is the finding of Bilanchone et al. (10) that a negative regulatory element is present in the Ty3 LTR which shows matches (7 of 9 bp) to sequences in the Ty4 LTR that are located in a similar position with respect to the 5′-end of the LTR. Nevertheless, all of these interpretations have to be taken with precautions, because mutual (positive or negative) interactions conferred by the sequences in the above constructs cannot be excluded and sequence similarities might also be misleading. From our experiments, for example, we have to conclude that despite a reasonable match to the PRE element (35), the PRE consensus sequences present in Ty4 (14) have no relevance for α-pheromone induction. Altogether, more detailed experiments are necessary to clarify this point.

A requirement for active transposition of retrotransposons is the synthesis of RNA intermediates that are capable of serving as templates to regenerate full-length DNA with complete LTRs. As a basic requirement for a retrotranspostion intermediate (33), that in conjunction with an appropriate termination site would be homologous to the transcriptional start site and the site of poly(A) addition respectively, as found for Ty1 and Ty3 (6,11,37). Furthermore, a putative ‘efficiency element’ (TACATATA) found in some yeast genes (38,39) is present at a canonical distance (∼100 bp) upstream of this polyadenation site in Ty4. However, no ‘positioning element’ (38) is present in the interspace. It should be noted that inspection of the Ty4 LTR sequence has not revealed any further reasonable array of the three polyadenylation signals as described for yeast genes (38,40,41) nor any one of the signal signatures described for Ty1 or Ty3 (6,42). Because of these notions, we have thus far discounted position 154 (Fig. 5) as a major initiation site. Of course, we have to consider the possibility that yet unknown signals operate in Ty4 giving rise to appropriate ‘full-length’ transcripts. In any case, however, we can rule out that these are major Ty4 transcripts.

From the above results we arrive at a two-fold explanation for the anticipated low transpositional activity of the Ty4 element. Firstly, we have shown that transcription of this element occurs at an extremely low level. From the experiments measuring the expression levels in lacZ fusion constructs, we favour the idea that active repression of Ty4 transcription is the major reason for this effect. This is supported by preliminary experiments in our laboratory indicating that a protein(s) specifically binds to the 5′-part of the tau element, which presumably contains a repressor binding element. It will be of interest to identify this protein(s) which might influence Ty4 transcription. Secondly, the generation of truncated Ty4 transcripts over ‘full-length’ transcripts significantly reduces the predisposition for transpositional activity. Altogether, these findings suggest that Ty4 constitutes a type of retroelement whose behaviour drastically deviates from that of other retrotransposons or retroviruses described thus far.

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