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Expression of Ty-*lacZ* fusions in *Saccharomyces cerevisiae*

B.A.Bowen[1], A.M.Fulton, M.F.Tuite[2], S.M.Kingsman and A.J.Kingsman*

Department of Biochemistry, South Parks Road, Oxford OX1 3QU, UK

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ABSTRACT

We have determined the nucleotide sequence of about 520 bp spanning the 5′ delta regions (Figure 1) of two Tyl elements. There is an open reading frame running out of the deltas for at least 180 nucleotides into the internal region of each element. The functional significance of these open reading frames has been tested by fusing them to a defective E.coli lacZ gene. Expression of B-galactosidase in yeast transformants containing these fusions shows that Tyl elements contain functional translation signals.

INTRODUCTION

The yeast transposable element, Ty, is a member of an ever widening family of genetic elements that includes bacterial transposons (1), the transposable elements of Drosophila (2) and the proviral forms of retroviruses (3). All of these elements are a few kilobases (kb) long, they are flanked by either direct or inverted terminal repeats and they generate short duplications in target DNA as a consequence of integration (e.g.4,5,6). Some of the eukaryotic transposons and retroviral proviruses have further similarities. They are bounded by the same TG-CA nucleotides, they possess sites that could bind a tRNA molecule at one of their terminal repeat/internal sequence junctions and they can alter the expression of neighbouring genes (7,8,9). Furthermore, retrovirus-like particles that contain RNA homologous to the transposable element copia have recently been found in Drosophila (10).

Very little is known about the internal organisation of eukaryotic transposons. Two large transcripts homologous to Ty sequences have been identified (11,12), one of these representing almost a full length transcript. Protein and RNA products encoded by the Drosophila elements, copia and 412, have also been observed (10,13,14).

We have started an analysis of functional regions within the yeast Ty

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element, a study that is complicated by the heterogeneity in the 30-35 copies of Ty in typical laboratory strains of Saccharomyces cerevisiae (15,16). This heterogeneity means that it is very difficult to generate hybridisation probes homologous to specific Ty sequences. As a consequence expression of individual elements cannot be characterised (Kingsman et al, unpublished data). To solve some of these problems we have identified potential Ty coding regions by fusing them to a variety of heterologous genes which lack signals required for either transcription or translation or both. Individual elements are thereby marked with a unique sequence which can be probed for functional expression. In this paper we report the identification of an open reading frame at the 5′ ends of two Ty elements and the fusion of this open reading frame to a defective E.coli lacZ gene.

MATERIALS AND METHODS

a) Strains

E.coli strain AKEC28 = C600 thrC leuB6 thyA trpC1117 hsdRk hsdMk. S.cerevisiae strain MD40-4c = ura2 trp1 leu2-3 leu2-112 his3-11 his3-15. S.cerevisiae strain AH22 = a leu2-3 leu2-112 his4-519 can1.

b) Plasmids

Plasmid pMA40 was constructed by inserting the 3.25 kb double Eco RI fragment from pJDB219 (17) into the single Eco RI site of pAT153 (18). This plasmid therefore carries the yeast LEU2 gene and the 2u plasmid origin of replication, as well as the E.coli ampicillin and tetracycline resistance genes and the Col E1 origin of replication. Yeast transformants (10^5 per ug DNA) are stable and contain about 100 copies of the plasmid per cell (Bowen et al, unpublished data). Plasmid pMC1871 was constructed by Dr. Malcolm Casadaban and contains the E.coli lacZ gene on a 3.1 kb fragment flanked by poly-restriction site linkers. The lacZ gene can be excised on a Bam HI fragment such that it lacks the first 7 codons of the coding sequence (19) (Figure 6).

c) Media

E.coli growth media were prepared according to Miller (20). Yeast growth media were prepared according to Hawthorne and Mortimer (21).

d) Enzymes

Restriction endonucleases, T4 DNA ligase, DNA polymerase, DNA polymerase (Klenow fragment) and BAL31 were purchased from BRL and used according to the manufacturers instructions.

e) Yeast transformation

The method described by Hinnen *et al* (22) was used.

f) DNA isolation

Quick *E.coli* plasmid DNA preparations were performed according to Holmes and Quigley (23) and pure plasmid DNA preparations were prepared by a scaled up version of this procedure followed by banding on a cesium chloride/ethidium bromide gradient. Total yeast DNA was isolated according to Nasmyth and Reed (24) and restriction fragment profiles of these preparations were scanned with a Joyce-Loebl Chromoscan 3 densitometer provided by B.S.Cox, Botany School, Oxford.

g) Sequencing

The dideoxy chain termination method of Sanger *et al* (25) was used in conjunction with the M13mp8 and M13mp9 vectors (26) and the 15 bp primer supplied by New England Biolabs. All reagents for the sequencing reactions were purchased from BRL.

h) B-galactosidase assay

Yeast transformants were tested for B-galactosidase activity on plates according to Rose *et al* (27). Yeast cell extracts were prepared following growth in SC-Leucine medium. Cells were harvested in mid logarithmic growth and then disrupted as described in Rose *et al* (27). Protein was measured using the method of Bradford (28) and reagents were supplied by Biorad. B-galactosidase was measured by standard procedures (20).

i) RNA methods

Isolation of RNA from yeast transformants has been described (29). Dot blot and filter hybridisation conditions were according to Kafatos *et al* (30) and Thomas (31). Purified fragments to be used as hybridisation probes were prepared as described in Dobson *et al* (29).

RESULTS

a) Plasmid constructions:

We have used two cloned Ty family members that represent the two major classes of Ty element observed to date. The first, Ty1-15, is most like the classical Ty1 described by Cameron *et al* (32) and is borne on a 9.6 kb Hind III fragment from plasmid pYe83D4 (15) (Figure 1). The second, Ty1-17, differs from Ty1-15 at a number of restriction sites and by two substitutions (Figure 1). Ty917 (35) and the ADR3-3c insertion (16) are similar to Ty1-17. Ty1-17 is borne on a 7.1 kb Eco RI fragment from phage

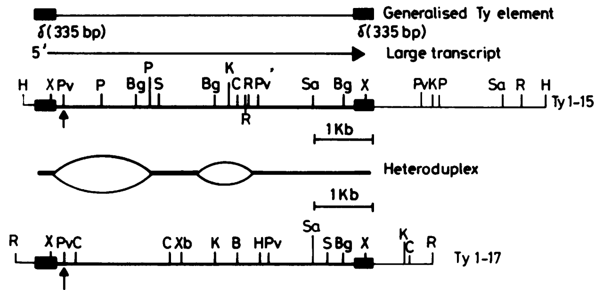

Figure 1. Restriction enzyme cleavage and heteroduplex maps of fragments containing Tyl-15 and Tyl-17. The thick boxes represent delta sequences. The thick line represents the epsilon region of the Tyl elements. The thin lines represent sequences flanking Tyl. The structure marked 'heteroduplex' is a schematic representation of the heteroduplex formed between Tyl-15 and Tyl-17 (15). The large arrow marks the direction of the full length Tyl transcript. The small arrows mark the Pvu II sites referred to in the text; H = Hind III; X = Xho I; Pv = Pvu II; P = Pst I; Bg = Bgl II; S = Sal I; K = Kpn I; C = Cla I; R = Eco RI; Sa = Sac I; Xb = Xba I; B = Bam HI.

λgtKG17 (15) (Figure 1). The entire Hind III fragment containing Tyl-15 was inserted into a 2u based vector (pMA40; see Materials and Methods) to produce plasmid pKT40b (Figure 2). The lefthand (Figure 1) Eco RI-Hind

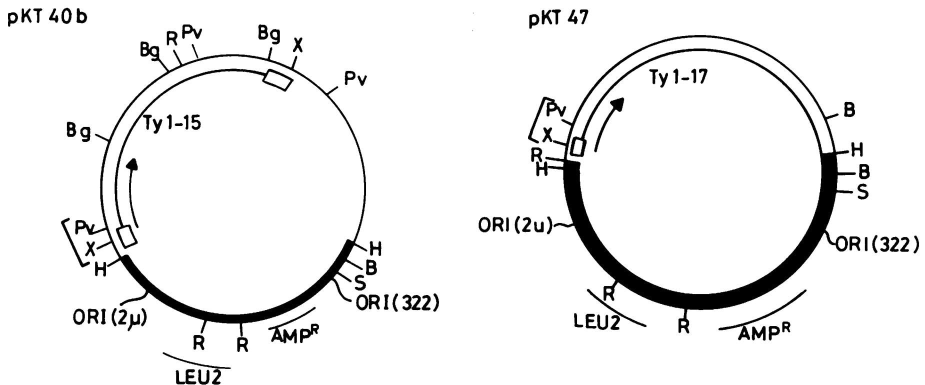

Figure 2. The structures of pKT40b and pKT47. The thick lines represent the 2u:LEU2:pAT153 recombinant plasmid (pMA40) described in the text. The thin lines represent the DNA fragments inserted into pMA40 which contain all of Tyl-15 and part of Tyl-17. The positions of the Tyl sequences are marked on the figure. The open boxes represent the delta sequences and the arrow the direction of Tyl transcription. The square brackets mark the area of interest in this paper. H = Hind III; X = Xho I; Pv = Pvu II; Bg = Bgl II; R = Eco RI; B = Bam HI; S = Sal I.

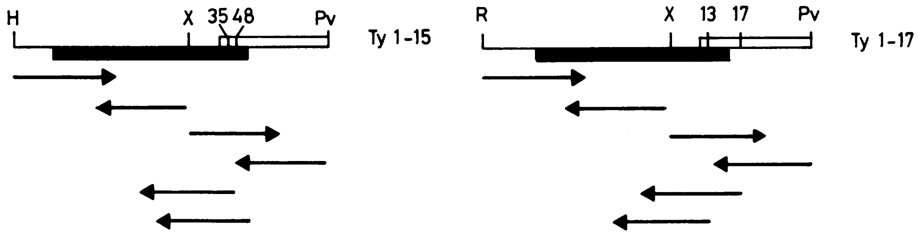

Figure 3. Sequencing strategy. The thick lines show the position of the delta sequences and the open boxes mark the position of the open reading frame discussed in the text. The arrows mark the direction and extent of the sequences determined. The numbers mark the positions of Bal 31 generated deletion end points which were used to produce B-galactosidase fusion proteins. The numbers are arbitrary deletion designations. H = Hind III; X = Xho I; Pv = Pvu II; R = Eco RI.

III fragment from the 7.2 kb Eco RI fragment containing Tyl-17 was also inserted into pMA40 to produce pKT47 (Figure 2).

b) Sequence analysis:

We have sequenced the region in Tyl-15 and Tyl-17 that spans the 5′ delta up to the Pvu II site marked with an arrow in Figure 1. This was achieved by cloning the Hind III-Xho I and Xho I-Pvu II fragments in the vicinity of the 5′ delta of pKT40b into M13mp8 and M13mp9 which allowed sequencing of both fragments in either direction. Similarly the Eco RI-Xho I and Xho I-Pvu II fragments from pKT47 were used. Also, plasmids pKT40b and pKT47 were cleaved with Pvu II and then digested with nuclease BAL 31 to remove about 150 bp. These pools of deleted plasmids were ligated in the presence of an excess of Bam HI linkers (CCGGATCCGG) and then used to transform E.coli AKEC28 to Leu^+ Amp^R. Colonies were screened for plasmids containing a Bam HI site at the deletion endpoints in the two molecules. The resulting Hind III-Bam HI fragments from pKT40b deletion derivatives and Eco RI-Bam HI fragments from pKT47 deletion derivatives were inserted into M13mp9 and M13mp8 respectively and the deletion endpoints determined by sequencing. A summary of the strategy and the sequences found are shown in Figures 3,4 and 5. It can be seen that an open reading frame (ORF) runs out of the two deltas to the Pvu II sites.

c) The open reading frames are functional:

The presence of an open reading frame does not necessarily mean that it can be translated into a protein. To test this we fused the reading frames in Tyl-15 and Tyl-17 to a lacZ fragment lacking the first seven

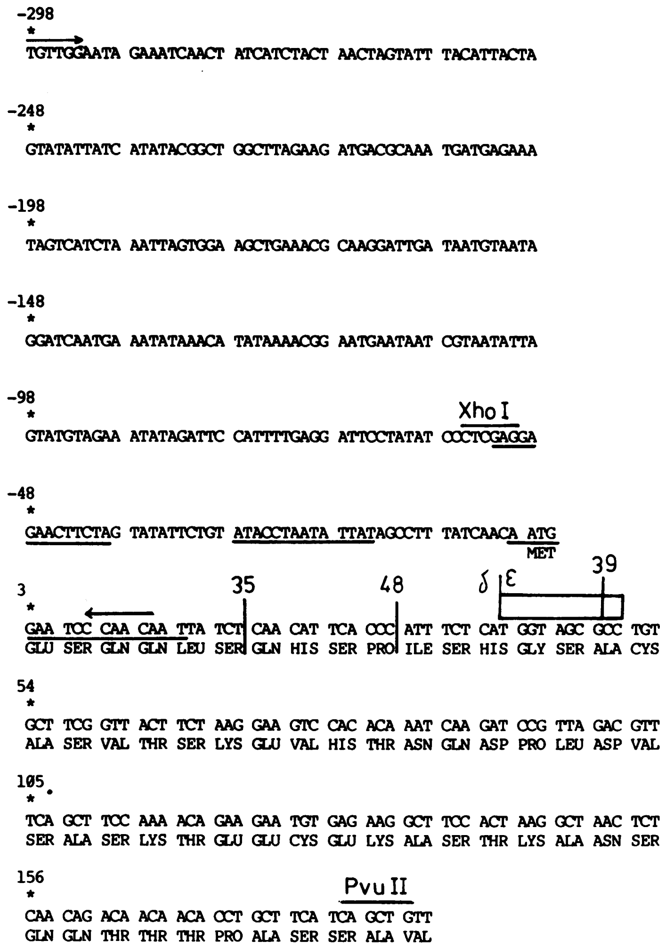

Figure 4. The nucleotide sequence of the 5′ region of Ty1-15. The positions of the end points of deletions 35, 48 and 39 are shown. Fusion of B-galactosidase at deletion 39 results in the B-galactosidase coding region being in frame with the ATG at position +41. The region homologous to met-tRNA is marked with an open box and a 6 bp inverted repeat is marked with arrows. Sequences underlined are those described in reference 16 as being conserved in all delta sequences. The delta/epsilon junction is marked.

codons of the lacZ gene, which, as a consequence requires functional transcription and translation signals for expression (19). The lacZ fragment is available as a Bam HI fragment in pMC1871 (see Materials and Methods), which is suitable for insertion at the Bam HI sites in our deleted derivatives of pKT40b and pKT47. The deletions marked in Figures 4

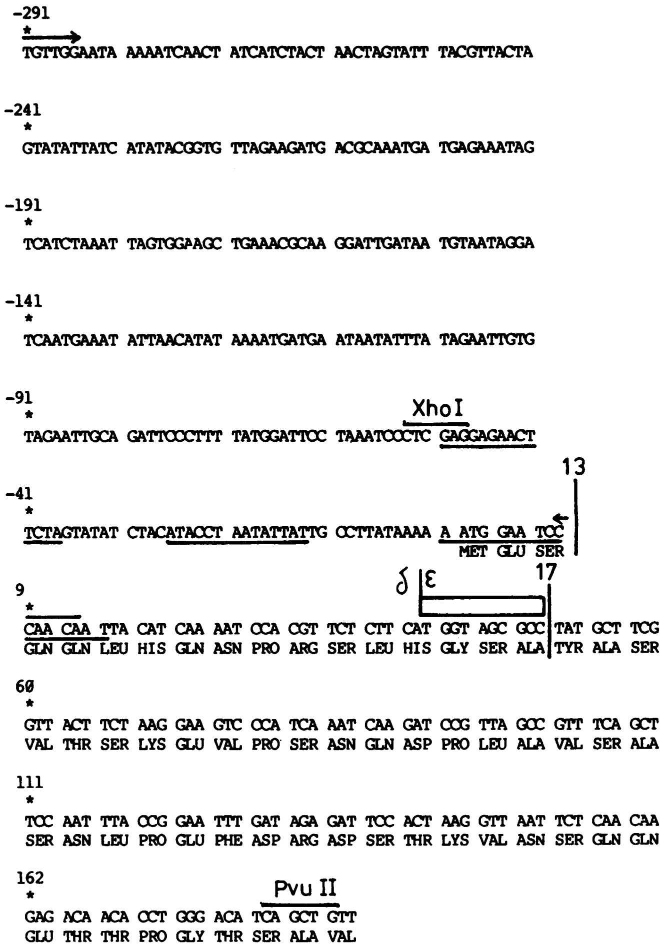

Figure 5. The nucleotide sequence of the 5′ region of Ty1-17. The positions of the end points of deletions 13 and 17 are shown. The region homologous to met-tRNA is marked with an open box and a 6 bp inverted repeat is marked with arrows. Sequences underlined are those described in reference 16 as being conserved in all delta sequences. The delta/epsilon junction is marked.

and 5 (i.e. deletions 35 and 48 for pKT40b and deletions 13 and 17 for pKT47) would be expected to yield fusions with the lacZ which are in frame with the ATG at position +1 (Figure 6).

In addition, pKT40b and pKT47 were cleaved with Pvu II and then ligated with an excess of Bgl II linkers (CAAAAGATCTTTTG). This yielded plasmids with a Bgl II site in place of the Pvu II sites marked with arrows

```
A)          8   9   10  11
      G GAT CCC GTC GTT TTA ----------
        ASP PRO VAL VAL LEU ----------

B)    ... ... .8   9   10  11
      CCG GAT CCC GTC GTT TTA ----------
      PRO ASP PRO VAL VAL LEU ----------

C)  .. ... ... .8   9   10  11
    CA AAA GAT CCC GTC GTT TTA ----------
       LYS ASP PRO VAL VAL LEU ----------


D)  CCGGATCCGG    Bam HI linker

E) CAAAAGATCTTTTG Bgl II linker
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Figure 6. The structure of the 5′ junctions in the lacZ fusion constructions. a) The structure of the 5′ end of the Bam HI lacZ fragment from pMC1871. b) The structure of the junction between the lacZ Bam HI fragment from pMC1871 and the Bam HI linker inserted at the deletion end points of the Bal 31 generated derivatives of pKT40b and pKT47. c) The structure of the junction between the lacZ fragment from pMC1871 and the Bgl II linker inserted at the left (Figure 1) Pvu II sites in pKT40b (Ty1-15) and pKT47 (Ty1-17). d) The structure of the Bam HI linker. e) The structure of the Bgl II linker. Numbers mark the 'normal' B-galactosidase codons and dots mark the nucleotides arising from the linkers.

in Figure 1. Insertion of the lacZ Bam HI fragment at this Bgl II site should also fuse the B-galactosidase coding sequence in frame with the ORFs in each Ty (Figure 6).

All of these constructions, when introduced into MD40-4c, resulted in expression of B-galactosidase, whereas other constructions, in which the fusions are frame-shifted or which lack the ATG at +1, did not. These results confirm the conclusions drawn from the sequencing data and show for the first time that Ty sequences contain signals which allow both transcription and translation in vivo. The ORFs running out of the 5′ delta of both Ty1-15 and Ty1-17 may, therefore, encode functional products.

d) B-galactosidase activities:

Extracts from transformants containing pKT40b and pKT47 fusion derivatives were assayed for B-galactosidase activity. Bal 31 generated derivatives are given deletion numbers, e.g pKT40bD48, and the derivatives resulting from PvuII cleavage followed by insertion of the BglII linker are designated Pv. A 'Z' is added to the designation if the derivative has the lacZ fragment inserted. For example plasmid pKT47D17Z is a Bal 31 generated derivative of pKT47 with a deletion end point at +51 (Figure 5) at which a Bam HI linker has been inserted, followed by insertion of the lacZ Bam HI fragment at that Bam HI site. Table 1 gives levels of B-galactosidase

Table 1. B-galactosidase activities from log phase cells transformed with Ty-lacZ fusions on 2u based vectors. Values are expressed (with standard error) as nmol o-nitrophenol/min/mg protein as assayed in extracts from cells disrupted by glass beads. Plasmids were selected by omitting leucine from the medium. Plasmid pMA97a contains the same yeast replication and selection system as the Ty fusion plasmids but lacZ is fused to the efficiently expressed PGK gene. Plasmid pMA97b is the same as pMA97a but with the lacZ fragment in the wrong orientation for expression from the PGK promoter.

Plasmid.	Activity.
pKT40bD48Z	285(2%)
pKT40bD35Z	167(1%)
pKT40bPvZ	9597(13%)
pKT47D17Z	35(9%)
pKT47D13Z	20(15%)
pKT47PvZ	3697(12%)
pMA97a	5353(15%)
pMA97b	1(81%)

activity in haploid MD40-4c transformants carrying various pKT40b and pKT47 derivatives.

In similar constructions, Tyl-15 directed expression of B-galactosidase is about 3-10 fold higher than that of Tyl-17 and in both cases expression of the lacZ gene inserted at the Pvu II site is about 50-100 fold greater than that seen with fusions close to or within the delta sequence.

To determine whether these differences in activity are due to variation in the plasmid copy number, total DNA was isolated from each of the transformants assayed, cleaved with Eco RI (pKT47 derivatives) or Eco RI and Hind III (pKT40b derivatives) and the fragments separated on an agarose gel. Plasmid bands were clearly visible after staining with ethidium bromide (data not shown). The copy number was approximated by comparing the intensity of the 2.55 kb band derived from the vector in each preparation with the similarly sized ribosomal DNA fragments produced by these digests. Assuming that each haploid cell contains 110 copies of the ribosomal repeat (36), we calculated that the pKT40b and pKT47 derivatives were present at about 130 and 85 copies per cell respectively.

The same DNA was used to transform E.coli AKEC28 and several Amp^r Leu^+ transformants in each case were checked for the presence of plasmids with restriction maps identical to the parent (data not shown). No changes in

the restriction patterns of the rescued plasmids could be detected.

In order to gain some insight into the relevance of the differences in B-galactosidase activity in extracts of transformants containing pKT40b and pKT47 derivatives, lacZ homologous RNA levels were determined in dot blot hybridisation experiments (30). Total RNA (20ug) from transformants containing some of the plasmids listed in Table 1 was loaded, in duplicate, onto nitrocellulose filters and then probed with nick-translated, gel-purified restriction fragments. One set of filters was probed with the 2.95 kb Hind III fragment from plasmid pMA3-PGK (41) which contains the yeast PGK gene and the other set was probed with the 3.1 kb Bam HI lacZ fragment from pMC1871. After hybridisation PGK and lacZ homologous RNA was quantitated by counting the filters in a scintilation counter. Specific activities of the two probes were very similar (6.6×10^7 c.p.m./ug for PGK and 7.2×10^7 c.p.m./ug for lacZ) and the ratios of lacZ to PGK homologous RNA from transformants containing pKT40bD48Z, pKT40bPvZ, pKT47D17Z and pKT47PvZ were 1.63, 1.94, 2.76 and 2.48 respectively. No counts above background were obtained with the lacZ probe incubated with RNA from MD40-4c or from a transformant expressing human interferon-alpha from the TRP1 promoter (29). These results suggest that the marked differences in the levels of B-galactosidase seen with the different transformants (Table 1) are not attributable to differences in RNA levels but that either the different messages are translated with different efficiences, or that the hybrid proteins have different stabilities or activities.

DISCUSSION

We have sequenced the region spanning the 5′ delta of Ty1-15 and Ty1-17. The deltas of these two Ty elements are 339 and 332 bp respectively and are 70% A:T. Roeder and Fink (37) have proposed that there are two basic types of delta and that both types can flank Ty elements which differ in the organisation of the central epsilon region. We have compared the homologies between twelve 5′ deltas and a schematic interpretation is shown in Figure 7. All deltas share patches of homology that are highly conserved (>99%), but these are interspersed with regions derived from two parental sequences, typified by TyB10 and Ty917, which are only 55-75% homologous. Different deltas can have different combinations of these sequences, suggesting that variants might arise by gene conversion (16,37). A conserved TATAAA box (38) is positioned as indicated in Figure 7. Ty RNA synthesis initiates approximately 70 bp downstream from this site and

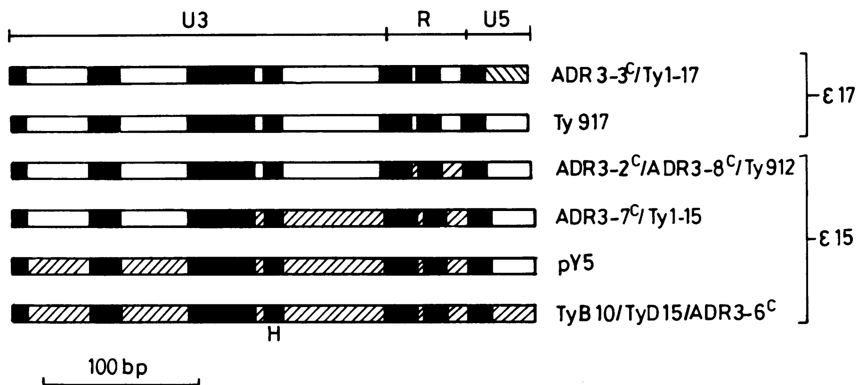

Figure 7. Homologies between 5′ delta sequences. Shaded regions indicate sequences (>10 bp) which are highly conserved (>99%) in all 5′ deltas. Open and hatched regions share at least 95% homology but differ from one another by 25-45%. The U3-R-U5 annotation is described in the text. 'H' denotes the position of a conserved TATAAA box. ε 15 and ε 17 refer to the two types of internal epsilon sequences (Tyl-15-like and Tyl-17-like respectively) to which each delta is linked. The sequences are taken from references 33,34, 35, 36, 16 and this work.

terminates in the 3′ delta, thereby creating a terminal redundancy of approximately 40 bp (12). It is possible, therefore, to subdivide the deltas flanking Ty elements into U3, R and U5 regions by analogy with retroviral proviruses (3) (Figure 7), where the R sequence represents the terminal redundacy of the large Ty transcript (12). In contrast to the LTRs of related proviruses, which have conserved R and U5 sequences but vary in their U3 region (5,39), Ty deltas can vary throughout. However, within the sample shown in Figure 7 there is a correlation between R region type and epsilon region type. It seems unlikely that the different 5′ delta sequences affect Ty transcription since lacZ homologous RNA levels from our Tyl-15 and Tyl-17 fusion constructions are very similar. However the regions upstream of the TATAAA boxes in Tyl-15 and Tyl-17 are almost identical (Figure 7).

The organisation of Ty elements differs from that of retroviral proviruses in one other respect. Our data demonstrates the presence of an open reading frame (ORF) running out of the 5′ delta of Tyl-15 and Tyl-17 for at least 180 nucleotides. The gag coding region of all known retroviruses starts downstream of the 5′ LTR sequence (3). Preliminary data (not shown) indicates that the Ty ORF may in fact extend for at least 350 nucleotides, and analysis of Ty912 has shown that the analogous ORF in this

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AMINO ACID HOMOLOGY IN TY OPEN READING FRAMES TO PvuII AT +189
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::

         1                                                50
         .                                                 .
         00000-0-00--0000000000-000000000000000--0-00000000
TY1-15   ESQQLSQHSPISHGSACASVTSKEVHTNQDPLDVSASKTEECEKASTKAN
TY1-17   ESQQLHQNPRSLHGSAYASVTSKEVPSNQDPLAVSASNLPEFDRDSTKVN
TYpY5    ESQQLSQHSPISHGSACASVTSFEVHTNQDPLDVSASKINEYDKASTKAN
TY912    ESQQLSQHSPISHGSACASVTSKG
TY917    ESQQLSQNSPNL
TYB10    ESQQLSNYPHISHGSACAS
         *****       **** *****  *  ***** ****   *    *** *

         51         62
         .           .
         000000000000
TY1-15   SQQTTTPASSAV
TY1-17   SQQETTPGTSAV
TYpY5    SQQTTTPASSAV
         *** ***  ***
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Figure 8. The amino acid sequences of putative Tyl encoded proteins. Above the amino acid sequences identity or the nature of a change is marked. A '0' indicates a score of 0 or >0 in a Dayhoff log odds matrix (42), a '-' indicates a score of <0. Where more than one difference is observed a non-conservative change is given preference over a conservative one. Below the amino acid sequences complete identity across all the samples is marked with a '*'. Data is from references 16,33,34,35, 36 and this work.

element is about 1600 bp long (P. Farabaugh, personal communication). Alignment of the Tyl-15 and Tyl-17 sequences suggests that each has suffered several frameshifts, but that closely linked second-site revertants have been selected so that the ORF is preserved. The amino acid sequences encoded by these ORFs are shown in Figure 8 and are compared with data derived from other published sequences. These putative Ty proteins are highly conserved over their N-terminal regions, and differ mainly in a number of conservative amino acid substitutions.

We have also shown that the ORFs in both Tyl-15 and Tyl-17 can be translated in vivo. Three different Ty::lacZ fusions in each case can express B-galactosidase in yeast (Table 1). Different Ty::lacZ fusions yield very different levels of B-galactosidase activity but this cannot be explained by differences in plasmid copy number or integrity or differences in RNA levels. It seems most likely that B-galactosidase specific activity is very sensitive to the precise nature of the fusion protein.

Ligation of the lacZ fragment to a series of deletions running into the 5′ delta of each Ty element results in a Lac^+ phenotype only when the fusion is in frame with the ATG at position +1 (Figures 4 and 5). Fusions that are in frame with, for example, the ATG at position +41 in both

elements do not express B-galactosidase. It is highly unlikely that lacZ expression results from fusion to an ORF initiating upstream since in frame termination codons precede the ATG at +1 in both elements. Furthermore, Sherman and Stewart (40) have noted that translation in yeast is initiated *in vivo* only at the AUG codon nearest the 5′ terminus of the message, a condition which would be satisfied by the ATG at +1 in both Ty1-15 and Ty1-17. The extent of the ORF in these two elements and the nature of their protein products will be the subject of further publications (Fulton *et al.*, in preparation; Dobson *et al.*, in preparation).

The data reported here shows that at least two of the 30-35 members of the Ty family possess signals which allow the expression of protein products in vivo. Given the heterogeneity of Ty sequences, this suggests that different Ty elements may encode different proteins within the cell. It will be interesting to see what function these proteins have and whether this varies according to the individual Ty element from which each is expressed.

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[1]Present address: Department of Applied Genetics, John Innes Institute, Colney Lane, Norwich, UK
[2]Present address: Biological Laboratory, University of Kent, Canterbury, UK

*To whom correspondence should be sent

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