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A single point mutation in the yeast *TRP4* gene affects efficiency of mRNA 3′ end processing and alters selection of the poly(A) site

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ABSTRACT

The yeast *TRP4* mRNA 3′ end formation element is a bidirectional element which functions in both orientations in an artificial *in vivo* test system. In this study, the role of 3′ end formation was analysed in the context of the entire *TRP4* gene. The 3′ untranslated region (3′UTR) of *TRP4* was altered and changes were analysed for their influence on *TRP4* gene expression. The 3′UTR in reverse orientation was fully functional and did not affect *TRP4* gene expression. Exchanging the *TRP4* 3′UTR by the bidirectional *ARO4* or the unidirectional *GCN4* 3′ end formation element allowed efficient gene expression. Deletion of the entire *TRP4* 3′UTR resulted in 70% reduction of *TRP4* mRNA and 50% reduced specific Trp4 enzyme activity in comparison to wild-type. A single point mutation within the *TRP4* 3′UTR caused the same effect on gene expression. This point mutation did not only affect the efficiency of 3′ end formation, but also produced new poly(A) sites which were situated upstream of the wild-type poly(A) sites. Therefore this sequence motif in the *TRP4* 3′UTR acts simultaneously as both an efficiency and positioning element.

INTRODUCTION

3′ end processing of mRNA involves two steps, namely cleavage of the pre-mRNA followed by the addition of adenosine residues at the cleavage site. The signal sequences directing this process are less conserved in yeast than in higher eukaryotes. In higher eukaryotes a highly conserved AAUAAA sequence motif upstream of the poly(A) site and a GU-rich region downstream of the poly(A) site are involved in mRNA 3′ end processing (reviewed in 1). In yeast, all sequence motifs required for this process are normally located upstream of the poly(A) site. Investigations of 3′ untranslated regions (3′UTRs) of different yeast genes revealed a variety of sequence motifs (reviewed in 2). A synthetic signal sequence which was functional in yeast demonstrated the sequence requirements for efficient 3′ end processing of the yeast gene *CYC1*. This signal sequence consisted of three motifs in a defined distance to each other: (i) a far upstream motif which was essential for efficient 3′ end processing, (ii) a near upstream motif necessary for the positioning of the poly(A) site where cleavage occurs and (iii) the poly(A) site itself (3).

Efficiency motifs are usually located upstream of the positioning motif. An exception is the efficiency motif of the yeast *FBP1* 3′ end formation element which is located downstream of a poly(A) site selection motif (4). Sequence motifs which direct efficiency of 3′ end formation vary considerably in different yeast genes. First investigations of the *CYC1* gene revealed a tripartite sequence TAG..TATGT...TTT (5,6). Variations of this motif have also been found in the *TRP1*, *ARO4* and *TRP4* genes (7). A 3′ end formation signal of the Cauliflower mosaic virus (CaMV) was functional in yeast because of a condensed version of the tripartite sequence motif (8). Saturation mutagenesis of the CaMV sequence revealed different functional motifs of which TATATA was the most efficient (9). In some other genes a variation of TATATA, a TTTTTAT stretch, has been found to function as an efficiency motif (10). Among others, the *GCN4* and the *PHO5* 3′ end formation elements include such motifs (7). The *GCN4* 3′ end formation element carries two TTTTTAT stretches. Deletions of either one or both sequences resulted in 30 and 60% decreased processing activity, respectively, in an artificial *in vivo* test system (11). This suggests an accumulative effect of both signals in mRNA 3′ end formation. In most genes, 3′UTRs comprise redundant degenerate efficiency motifs (12). The *ADH2* and the *TRP4* 3′ end formation elements are the only described cases where a single point mutation in one of these motifs led to a total abolishment of 3′ end processing in a highly transcribed test gene (13,14).

A saturated mutagenesis of sequence motifs involved in the positioning of the poly(A) site of the *CYC1* gene revealed AAUAA as the preferred motif (15). This sequence or variations like AAGAA or AAAAA can be found in many, but not all, yeast genes. Deletions of these motifs normally have no effect on the efficiency of 3′ end formation (16). An exception is the *GAL7* transcript which is not processed when a crude deletion of 14 bp, including an AAUAA motif, is located in the 3′UTR of the gene (17). It has been found that cleavage occurs preferentially at PyA sequences and that poly(A) sites can be scattered over a region of 200 bp. Mutagenesis of the adenosine residues resulted in alterations of poly(A) site selection (18).

Testing different 3′ end formation elements in an *in vivo* test system resulted in additional classifications (7). First, the

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efficiency of 3′ end processing varies for different 3′UTRs. Weak elements were not able to process all transcripts that have been initiated at the strong *ACT1* promoter used in the test system. Second, some 3′ end processing elements could function in both orientations (bidirectional) and others only in their natural orientation (unidirectional). The *TRP4* and the *ARO4* 3′ end formation elements used in this study were classified as weak elements which function bidirectionally in an *in vivo* test system while the *GCN4* element represents a strong unidirectional element (7).

Most of the information about 3′ end processing elements was obtained from artificial *in vivo* test systems. Here, we report the influence of the 3′ end formation element on transcription and enzyme activity of the authentic *TRP4* gene. We tested the *TRP4* 3′UTR in both orientations and exchanged it by the bidirectional *ARO4* and the unidirectional *GCN4* 3′ end formation element. These constructs allowed efficient *TRP4* gene expression. A single point mutation in the *TRP4* 3′UTR resulted in the same decrease of *TRP4* gene expression as a deletion of the entire *TRP4* 3′UTR. Additionally, this point mutation resulted in alterations of poly(A) site selection. This is the first example of a single point mutation affecting both efficiency and poly(A) site positioning of mRNA 3′ end formation in the budding yeast *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Yeast strains

The yeast strain RH2063 (*MAT*a, *trp4*::*URA3*, *gcn4*-101, *leu2*) is a derivative of the *S.cerevisiae* laboratory standard strain X2180-1A (*MAT*a *gal2 SUC2 mal CUP1*). The *TRP4* open reading frame (ORF) and the 3′UTR up to the *Eco*RV site have been replaced by the *URA3* gene. Yeast strains were cultivated on YEPD medium or on SD minimal medium with appropriate supplements at 30°C (19).

Plasmid construction and cloning

A 3.0 kb *Sal*I fragment containing the *TRP4* gene was cloned into the *Sal*I site of pSP65 (Promega). The resulting plasmid pME982 was the basis for the construction of all *TRP4* genes carrying different 3′ end formation elements. Deletion of the 3′UTR was achieved by *Nde*I/*Eco*RV digestion, making blunt ends followed by religation. For the single point mutation the *Eco*RV/*Nde*I fragment of pME901 (14) was cloned into the *Nde*I/*Eco*RV sites of pME982. Reverse orientation of the *TRP4* 3′ end formation element was achieved by cloning the *Nde*I/*Eco*RV fragment of pME982 in reverse orientation after blunt-ending. The *TRP4* 3′UTR was replaced by the 325 bp fragment of the *ARO4* 3′UTR by *Hpa*I/*Eco*RV restriction of pME638 (20), making blunt ends and ligation into the *Eco*RV/*Nde*I blunted sites. The 205 bp fragment of the *GCN4* 3′UTR cloned into pME806 (11) was amplified by PCR using oligonucleotides OLCE4 (5′-CGCGGGTACCCGCTGATTTCATTTACC-3′) and OLCE6 (5′-GCGCAGATCTCCCCCATCGTGAGTG-3′) (Gibco BRL), restricted with *Asp*18/*Bgl*II, blunt-ended and cloned into the *Eco*RV/*Nde*I blunted side. The restriction sites were provided with the primers. The orientation of these constructs was determined by PCR and restriction analysis. All constructs were cloned as 3.0 kb *Sma*I/*Pst*I fragments, containing the full *TRP4* gene with its different 3′ end formation elements, into the *Sma*I and *Pst*I sites of pRS315 (21).

RNA analysis

Total RNA from *S.cerevisiae* was isolated according to Cross and Tinkelenberg (22). For northern analysis, 20 µg of total RNA were separated on a formaldehyde agarose gel, transferred to a positively charged nylon membrane (Biodyne B, PALL) by electroblotting and hybridised with ^{32}P-labelled DNA fragments as previously described (11). The DNA fragments were randomly radiolabelled using the HexaLabel DNA Labelling Kit from MBI Fermentas. Band intensities were visualised by autoradiography and quantified using a BAS-1500 Phospho-imaging scanner (Fuji).

Anthranilate phosphoribosyl transferase assay

All enzyme activities were determined *in situ* after permeabilisation with Triton X-100 (23). Anthranilate phosphoribosyl transferase (PRtransferase, E.C. 2.4.2.18) activity was determined as described before (24) by measuring fluorometrically the decrease of anthranilate in the presence of *N*-(5′-phosphoriosyl-1-) anthranilate isomerase. The intrinsic PRtransferase activity of the helper strain RH218 (*TRP1*/*Eco*RI circle) was always <10% of the activity of the cell extract which was assayed, and was deduced from respective values in the assays. Protein determination of whole Triton X-100-permeabilised cells was done as previously described (25).

Reverse transcriptase (RT)–PCR

Reverse transcription of 3′ ends of *TRP4* mRNA was performed using the Superscript™ reverse transcriptase (Gibco BRL). 100 ng of total RNA of the different *S.cerevisiae* strains were reversely transcribed with a poly-18 dT primer. In a following PCR reaction, 3′ ends of the different *TRP4* transcripts were amplified together with the *TRP4*-specific primer TRP-RTF (5′-ATTATC-TAGACCGGCAAGTACCACCTTGGC-3′). DNA fragments were isolated and cloned into pBluescript SK$^+$ (Stratagene). The poly(A) site was determined by sequencing using the T7 Sequencing Kit (Pharmacia).

RESULTS

Inversion of the *TRP4* 3′ end formation element results in correct *TRP4* gene expression

The *TRP4* 3′ end formation element has been shown to function in both orientations in an *in vivo* test system (14). We were interested whether the inversely orientated element is also functional in the authentic *TRP4* gene and how such a change would affect gene expression. For this purpose, the 147 bp fragment of the *TRP4* 3′UTR was cloned in negative orientation. The *TRP4* 3′UTR contains a TAGT sequence which has been shown to affect 3′ end processing in the *in vivo* test system (14). It is called UTR-E, for 3′UTR-efficiency motif, and is found at position +26 relative to the translational stop codon (Fig. 1). Reverse orientation of the *TRP4* 3′UTR changed the location of UTR-E. Formerly on the antisense strand, it was now located on the sense strand and the first T of UTR-E was situated 138 bp downstream of the translational stop codon. This construct and the others described in the following chapters were cloned into a single copy plasmid and transformed into the yeast strain RH2063 carrying a *TRP4* deletion and a mutated *GCN4* gene. A defective *GCN4* gene prevents an interference of the transcriptional general control system of amino acid biosynthesis in case of starvation for tryptophan. Gene expression was determined by *TRP4* mRNA

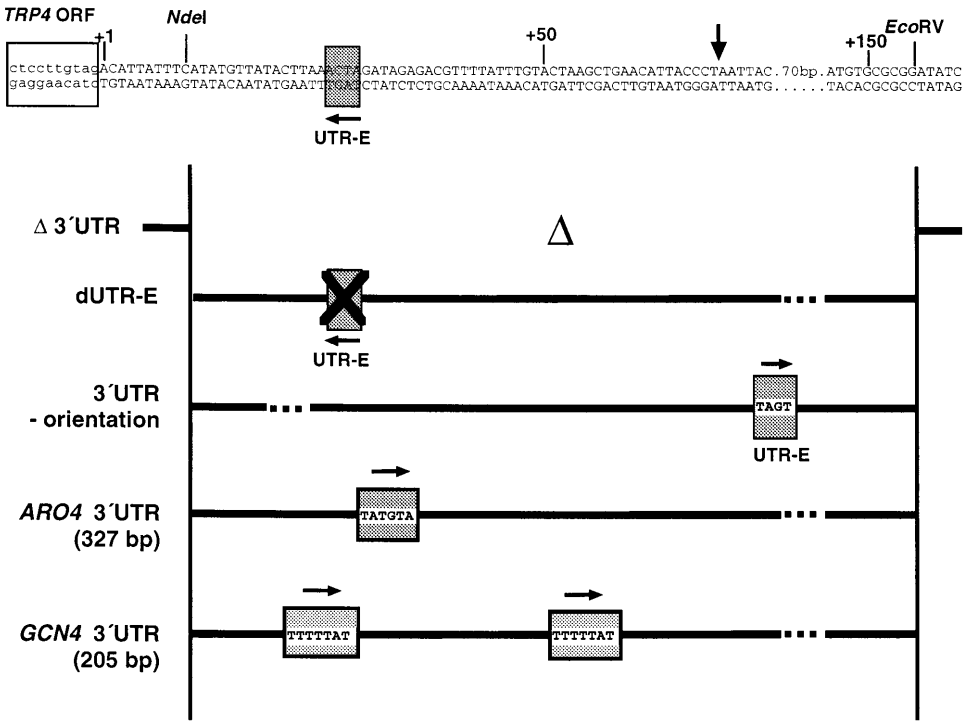

Figure 1. Schematic presentation of the *TRP4* 3′UTR and of constructs with different 3′ end formation elements which have been modified within the *Nde*I/*Eco*RV region of the *TRP4* 3′UTR. The orientation of the 3′ end formation motifs is indicated by horizontal arrows and the wt major poly(A) site by a vertical arrow. Construct Δ3′UTR is a deletion of the *Nde*I/*Eco*RV fragment of the *TRP4* 3′UTR. The following construct dUTR-E carries a point mutation in the sequence motif UTR-E indicated by a cross (for details see text). In the third construct, the *TRP4* 3′UTR has been cloned in negative orientation. The last two constructs represent replacements by *ARO4* and *GCN4* 3′UTRs, respectively. The entirely different *GCN4* signal sequences are underlayed with light grey boxes in contrast to the dark grey boxes of the *ARO4* and *TRP4* motifs.

quantification in northern experiments and measuring *TRP4* encoded specific PRtransferase activity. The data obtained were compared to data from a strain harbouring the wild-type (wt) *TRP4* on the same single copy plasmid.

Growth tests on SD medium with and without supplementation of tryptophan showed no growth phenotype for a strain carrying *TRP4* with negatively orientated 3′UTR. *TRP4* mRNA levels were similar to wt *TRP4* levels (Fig. 2A) and the absence of additional bands implies that 3′ end processing did happen close to wt poly(A) sites. The *TRP4* mRNA with inverted 3′UTR was efficiently translated and resulted in a PRtransferase activity of 130% of wt activity (Fig. 2B). These data demonstrate that the bidirectional *TRP4* 3′ end formation element functions as efficiently in forward as in reverse orientation in the authentic *TRP4* gene.

The *TRP4* 3′ end formation element can be replaced by the bidirectional *ARO4* or the unidirectional *GCN4* 3′ end formation element

3′ end formation elements of yeast genes are different from each other in their sequence motifs involved in 3′ end processing. We were interested whether an exchange of the *TRP4* 3′UTR by 3′ end formation elements of other yeast genes would affect gene expression.

The *ARO4* 3′UTR is an example for a 3′ end formation element with features similar to the *TRP4* element. The *ARO4* 3′UTR functions bidirectionally in the *in vivo* test system and carries two putative efficiency motifs which are located 18 and 98 bp downstream of the translational stop codon. Whereas the proximate seems to be necessary for efficient processing, the more distant motif can be removed without significantly affecting 3′ end processing activity (26). A 327 bp spanning fragment of the *ARO4* 3′UTR was cloned downstream of the *TRP4* ORF to find out whether the *ARO4* 3′ end formation element would allow efficient *TRP4* gene expression (Fig. 1). The fragment started 13 bp from the stop codon within the *ARO4* ORF and included the efficiency motif and the major poly(A) site. Strains harbouring this *TRP4–ARO4* hybrid revealed an amount of *TRP4* mRNA which was similar to wt (Fig. 2A). Surprisingly, the PRtransferase activity was even increased to 178% of the wt activity (Fig. 2B), indicating that the *ARO4* 3′ end formation element can efficiently replace the *TRP4* element with even positive effects on gene expression.

Since it was possible to replace the *TRP4* 3′ end formation element by another bidirectional element, it was of interest whether a 3′ end formation element with different features would also allow efficient *TRP4* gene expression. The *GCN4* 3′ end formation element has been taken as an example for unidirectional elements which function only in the natural orientation in the *in vivo* test system. As efficiency motifs it carries two TTTTTAT sequences which are located 40 and 102 bp downstream of the translational stop codon (11). A 205 bp fragment of the *GCN4* 3′UTR including the two TTTTTAT motifs and the poly(A) site has been cloned downstream of the *TRP4* ORF (Fig. 1). The

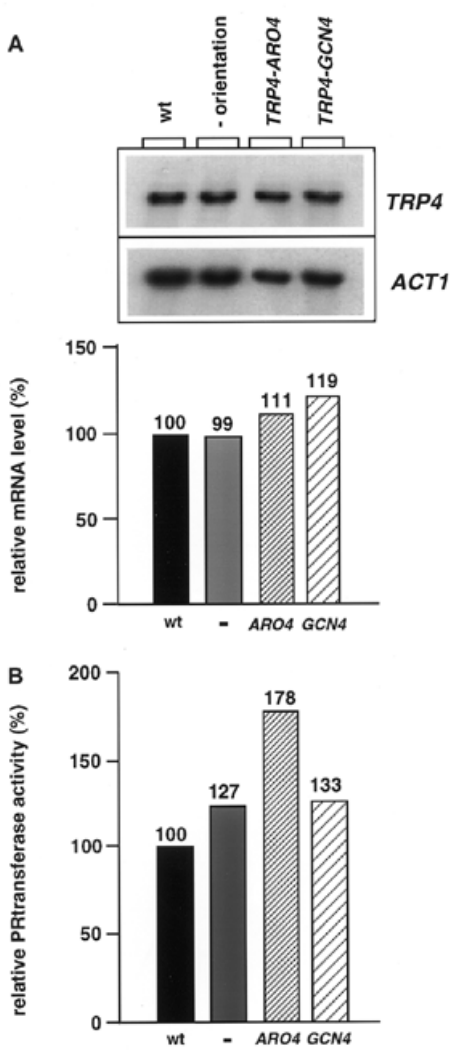

Figure 2. Gene expression of *TRP4* constructs carrying different 3′ end formation elements. (**A**) Determination of mRNA level of *TRP4* constructs carrying the *TRP4* 3′UTR in negative orientation and replacements by the *ARO4* and the *GCN4* 3′UTR, respectively, compared to wt. The *TRP4* transcript was ~1.2 kb in length and was visualised by autoradiography with a radiolabelled 1.5 kb *Mlu*I *TRP4* DNA fragment. As constitutive control a 0.5 kb *Cla*I fragment of the *ACT1* gene was hybridised. Quantification of transcripts by phosphoimager analysis is shown below. Strains carrying the wt *TRP4* 3′ end formation element were set as 100%. The bars represent an average of at least four independent measurements. The standard deviation did not exceed 15%. (**B**) Effects of modified 3′ end formation elements on Trp4 protein have been measured by its PRtransferase activity. In this assay the decrease of anthranilate is measured using fluorescence spectroscopy as previously described (24). One unit of specific enzyme activity is defined as 1 mM anthranilate × min^{-1} × mg $protein^{-1}$. Specific enzyme activity of cells carrying the wt plasmid was set as 100%. This corresponded to 1.0 mM × min^{-1} × mg^{-1}. The bars represent an average of five independent measurements. The standard deviation did not exceed 20%.

mRNA level expressed by the *TRP4–GCN4* hybrid was at least as high as the wt *TRP4* level (119%). A slight increase could be observed for the *TRP4–GCN4*-derived PRtransferase activity which was 133% of wt activity (Fig. 2A and B). Consequently, a unidirectional 3′ end formation element like the *GCN4* element can act as an alternative for the *TRP4* element despite of its different features. In summary, *TRP4* gene expression does not depend on the specific *TRP4* 3′ end formation element, since replacements by a similar 3′ end formation element or an entirely different element did not affect gene expression in a negative way.

Deletion of the 3′UTR significantly reduces *TRP4* transcript level and enzyme activity

The maximum effect of *TRP4* 3′ end formation on gene expression was achieved by deleting a 147 bp fragment of the entire 3′UTR containing all essential sequences for 3′ end formation (14) (Fig. 1). This construct is referred to as *TRP4* Δ3′UTR. Growth experiments of a strain carrying *TRP4* Δ3′UTR on SD medium lacking tryptophan revealed growth rates similar to cells carrying wt *TRP4* (data not shown). The *TRP4* mRNA level of the *TRP4* Δ3′UTR construct was reduced to 30% of wt level independently of a supplementation with tryptophan (Fig. 3A). Additional bands resulting from transcripts of significant difference in length could not be detected, suggesting that no stable readthrough transcripts are present in the cell. The PRtransferase activity of strains carrying *TRP4* Δ3′UTR was reduced to 58% of wt activity, implying that the *TRP4* Δ3′UTR transcripts are efficiently translated (Fig. 3B). These results show that an extensive deletion of the entire *TRP4* 3′UTR halves gene expression, but the reduced expression does not result in a growth phenotype.

A single point mutation within the 3′UTR has the same effect on *TRP4* gene expression as the deletion

A specific point mutation in the *TRP4* 3′UTR had been shown to abolish 3′ end processing activity in the *in vivo* test system lacking the *TRP4* ORF (14). This prompted us to analyse the influence of this point mutation on gene expression of the authentic *TRP4* gene. The point mutation results in an exchange from A to G at position +29 relative to the *TRP4* translational stop codon, which is the last base of a TAGT motif located on the antisense strand, named UTR-E. The *TRP4* 3′UTR was replaced by the mutated version and the resulting construct was called dUTR-E (Fig. 1). The data obtained from this construct were compared to strains carrying wt *TRP4* and *TRP4* Δ3′UTR. Like the *TRP4* Δ3′UTR carrying strain, the strains harbouring the point mutation showed no growth phenotype. In northern experiments the dUTR-E-carrying strains showed *TRP4* transcripts of similar sizes to the wt *TRP4* strain. However, the *TRP4* mRNA level was reduced to 32% of wt level which was a similar effect to in the *TRP4* Δ3′UTR strain (Fig. 3A). The same correlation can be observed for the PRtransferase activity, which is reduced in dUTR-E-carrying strains to 55% of wt activity (Fig. 3B). These data demonstrate that the UTR-E motif is crucial for mRNA 3′ end formation efficiency in the *TRP4* 3′UTR of the authentic gene.

The 3′ end formation efficiency motif UTR-E is required for correct poly(A) site selection of the *TRP4* mRNA

RT–PCR was performed to find out whether the identified efficiency motif UTR-E also affects cleavage and maturation of the different pre-mRNAs. The wt *TRP4* revealed two poly(A) sites in RT–PCR experiments at positions +68 and +72 relative to the translational stop codon (Fig. 4). In case of the reverse orientation of the *TRP4* 3′UTR, a new poly(A) site was located

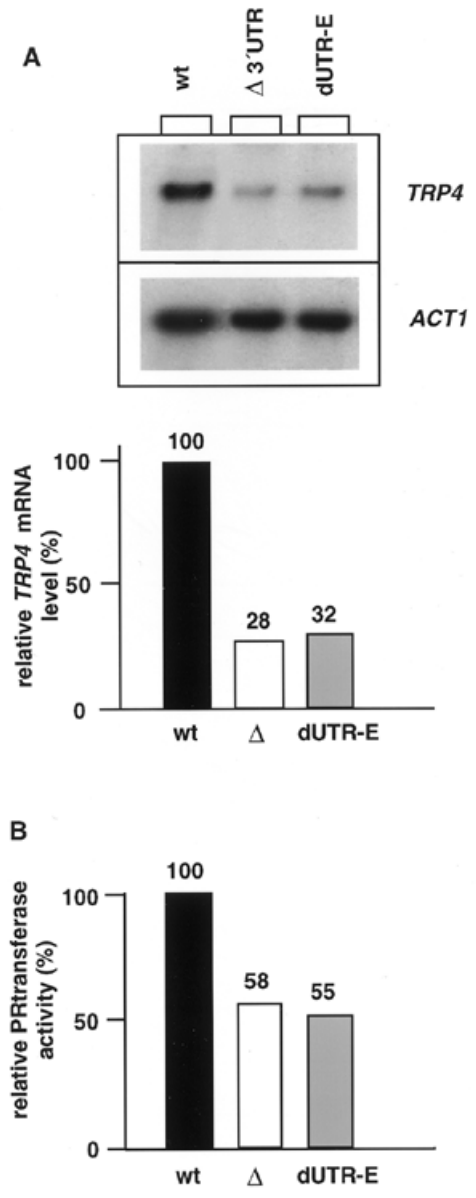

Figure 3. *TRP4* gene expression of alleles carrying a deletion and a specific point mutation in the 3′UTR. (**A**) The steady state *TRP4* mRNA levels were determined by northern hybridisation using total RNA from yeast strains carrying *TRP4* wt, a deletion of the *TRP4* 3′UTR and an allele carrying a specific point mutation in the *TRP4* 3′UTR (dUTR-E). Northern analysis was performed as described in the Materials and Methods and the legend of Figure 2A. *ACT1* RNA served as constitutive control and the quantification by phosphoimager analysis is shown below. Wild-type level was set as 100%. The bars represent an average of four independent measurements and the standard deviation did not exceed 15%. (**B**) Effects on the Trp4p level determined by measuring PRtransferase activity. PRtransferase activity of cells harbouring the different plasmids has been determined as described in the Materials and Methods and Figure 2B. The enzyme activity of cells carrying the wt plasmid was set as 100%. The bars represent an average of five independent measurements. The standard deviation did not exceed 20%.

28 nt downstream of the former *Eco*RV site (position +177 compared to the translational stop codon). The distance between the UTR-E motif and the poly(A) site in wt and reverse orientation is very similar with 43 and 39 nt, respectively (Fig. 4).

In the case of the *TRP4* Δ3′UTR construct, in which all mapped wt poly(A) sites and the UTR-E motif were removed, two new poly(A) sites were located 28 and 37 nt downstream of the UAG stop codon (originally positions +166 and +172). Destruction of the UTR-E motif resulted in poly(A) sites which were located further upstream relative to the translational stop codon. The dUTR-E construct, carrying a single point mutation in UTR-E, identified poly(A) sites at positions +36, +43 and +52. These data show that a single point mutation affects efficiency and poly(A) site selection of the *TRP4* mRNA 3′ end formation process and suggest that UTR-E is a sequence motif involved in both functions. Interestingly, the poly(A) sites of the *TRP4* Δ3′UTR and the dUTR-E construct were located in a distance of ~40–50 bp to another TAGT motif located in the ORF.

DISCUSSION

We have shown here that a single point mutation in the 3′UTR of *TRP4* influences the efficiency of *TRP4* gene expression on mRNA and protein levels and alters the position of poly(A) sites. In addition, we demonstrate that the *TRP4* 3′UTR can be inverted without affecting gene expression. Other 3′UTRs, such as the *ARO4* 3′UTR or the *GCN4* 3′UTR, are capable of replacing the corresponding *TRP4* region without negative effects on *TRP4* transcript or enzyme levels.

The *TRP4* 3′ end formation element has been investigated earlier in an artificial *in vivo* test system which consisted of the strong *ACT1* promoter and the 3′ end formation element of the *ADH1* gene. In between, different modified *TRP4* 3′UTRs have been cloned (14). This resulted in a transcript which could be quantified but had the drawback that no protein was encoded by the transcript. The results obtained from the test system allowed us to select a mutation within the *TRP4* 3′UTR which affected 3′ end processing activity there. The primary aim of this work was to assay if the results with the test system are transferable to the authentic gene. In addition, we wanted to know how we could inactivate the 3′ end formation signal and how inactivation of the 3′ end formation signal affected *TRP4* gene expression. The data we obtained for *TRP4* gene expression verified those obtained from the test gene. Minor differences between the authentic *TRP4* and the test system can be explained by the use of the strong *ACT1* promoter in the test system. Differences between an efficient 3′ end formation element like the *GCN4* and a less efficient element like the *TRP4* element could only be observed in the test system, where the less efficient signal could not process all transcripts initiated from the *ACT1* promoter (11,14). Both classes of 3′ end formation elements are able to direct efficient gene expression on *TRP4* transcript and enzyme level. This might be due to the fact that the *TRP4* promoter is not highly efficient for transcription and it is not, therefore, necessary to supply this gene with a strong 3′ end formation signal.

The *TRP4* 3′ end formation element functions bidirectionally in an *in vivo* test system (14) and, as we could show here, also in the authentic *TRP4* gene. For the authentic gene there is no difference in gene expression when the 3′UTR is cloned in forward or in reverse orientation. Data obtained from the test system revealed a 20–40% reduction of the 3′ end processing activity when the element is cloned in negative orientation (14). This difference might also be caused by the promoter strength of the *ACT1* promoter in the test system and might not play a role for less efficiently transcribed genes.

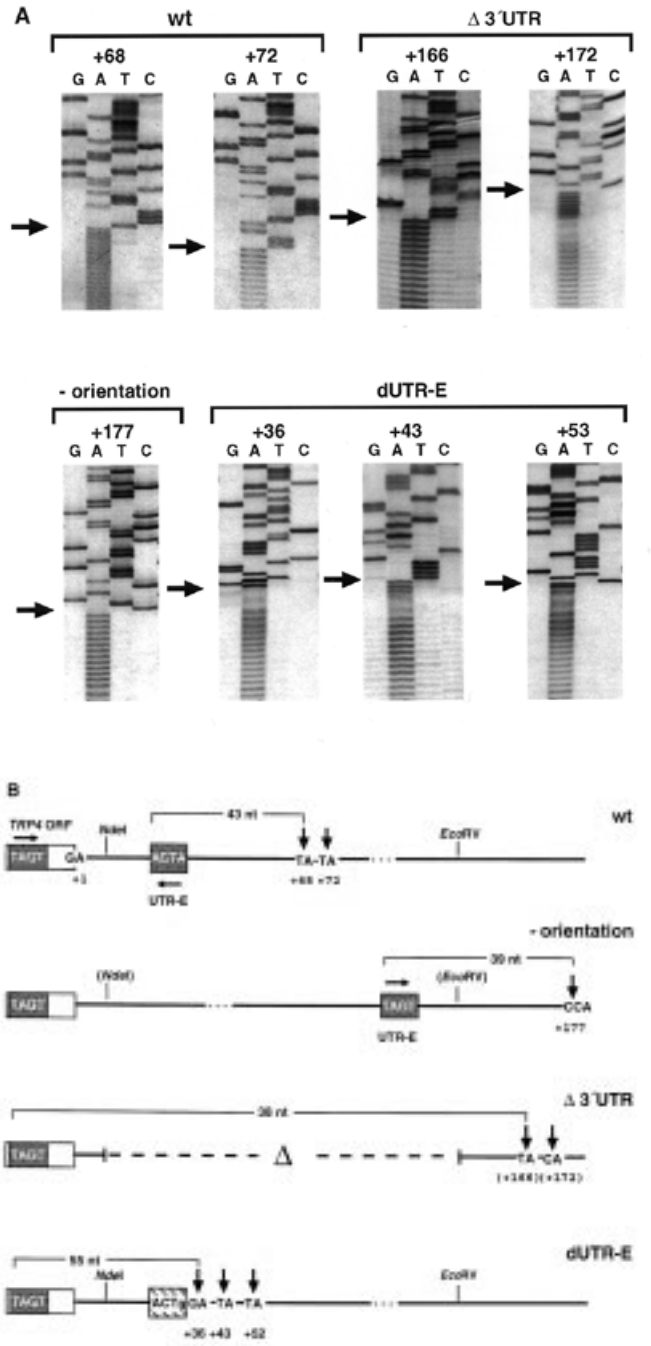

Figure 4. (**A**) Sequence data of RT–PCR. RT–PCR was performed with wt *TRP4* RNA, the deletion construct Δ3′UTR, the specific point mutation dUTR-E and the 3′UTR cloned in negative orientation. Each picture represents sequence reactions of at least three independent clones. The startpoint of the poly(A) tail is indicated by an arrow. (**B**) Schematic presentation of poly(A) sites of constructs carrying the *TRP4* 3′UTR cloned in negative orientation, a deletion of the *TRP4* 3′UTR and the specific point mutation (dUTR-E) compared to wt poly(A) sites. 3′ end processing motifs are represented by dark grey boxes, motifs which carry point mutations (small letters) are outlined by diagonal stripes. The poly(A) sites which are represented by vertical arrows have been determined by RT–PCR and sequencing of the amplified fragments (A). The sequences have only been shown for the 3′ end formation motifs and the dinucleotides forming the poly(A) site. Numbering of the sites is relative to the first nucleotide following the UAG stop codon, designated as +1. The distance between TAGT motifs and the first poly(A) site of each construct includes all nucleotides of the sequence motifs.

The *TRP4* 3′UTR is not essential for viability of yeast cells under standard laboratory conditions on medium lacking tryptophan. Deletion of the *TRP4* 3′UTR led to 30% of wt mRNA and 50% of protein level. A similar effect could be observed for the yeast *ARO4* gene. The *ARO4* encoded DAHP synthase activity was reduced to 41% in a strain carrying a deletion of the *ARO4* 3′UTR (26). In the case of the *CYC1* gene, a deletion within the 3′UTR resulting in deficiency of 3′ end formation decreased the amount of mRNA and protein levels to 10% of wt levels (27). There are obviously differences in the importance of efficient 3′ end formation signals for different genes.

Cells carrying a deletion of the *TRP4* 3′UTR were able to grow with a growth rate like wt cells even though inefficient 3′ end processing reduced Trp4p activity to 50%. It had been shown earlier that 25% of wt activity of the *TRP4* encoded PRtransferase were rate limiting in tryptophan biosynthesis and resulted in a growth phenotype (23). Consequently, *TRP4* gene expression produces a surplus of gene product in cells growing under standard laboratory conditions and reduction of gene expression does not necessarily lead to selective pressure.

Reduction of *TRP4* gene expression was achieved by a deletion of the 3′UTR. Interestingly, a single point mutation in the 3′UTR affected *TRP4* gene expression in the same intensity as the entire deletion. In the artificial test system this single point mutation led to a total abolishment of 3′ end processing activity (14). The drastic effect of a single point mutation is surprising since most yeast 3′ end formation elements carry redundant sequence motifs (12). The loss of 3′ end processing could be due to a change in the RNA secondary structure. Computer prediction of RNA secondary structures revealed alterations in case of the UTR-E point mutation (mfold server, http://mfold1.wustl.edu/mfold/rna/form1.cgi).

There are two additional TA(T)GT motifs in the *TRP4* 3′UTR. One of these motifs is located on the sense strand upstream of the UTR-E motif, the other downstream on the antisense strand like the UTR-E motif. These motifs had only minor effects on 3′ end processing in the *in vivo* test system (14) and point mutations within these motifs in addition to the point mutation in the efficiency motif did not further reduce *TRP4* gene expression (data not shown). These findings further corroborated our earlier results obtained with the *in vivo* test gene construct.

Another important finding was that this single point mutation in the *TRP4* 3′UTR did not only influence efficiency of 3′ end processing but also the positioning of the poly(A) site. The current model which is predominantly based on investigations of the *CYC1* 3′ end formation element proposed two different sequence motifs for these functions. The sequence motif which we found to be involved in both processes resembles the core part of a tripartite sequence motif which had been shown to act as efficiency motif in the *CYC1* gene (5). Previously described positioning motifs were composed of entirely different sequences (15) which cannot be found in the *TRP4* 3′UTR.

There is a major difference between positioning motifs previously described and the bifunctional sequence motif in the *TRP4* 3′UTR. Point mutations in positioning motifs generally led to downstream cryptic poly(A) sites (15,16). In case of the *TRP4* point mutation, new poly(A) sites are located upstream of the wt poly(A) site. In wt and in the inversely orientated *TRP4* 3′UTR, poly(A) sites can be found in a minimal distance of 40–50 nt relative to the efficiency motif. Therefore, in both cases, the poly(A) sites are located downstream of the efficiency motif. This suggests that the efficiency motif of the bidirectional *TRP4* 3′ end

formation element functions only in a downstream direction for the positioning of the poly(A) site and is therefore functionally unidirectional. Deletion of the *TRP4* 3′UTR and a point mutation within the efficiency motif resulted in new poly(A) sites upstream of the wt sites. A similar distance of 40–50 nt between a TAGT motif located in the ORF and the cryptic new poly(A) sites can be observed. The TAGT sequence in the ORF might represent a backup system which would direct positioning of the poly(A) site when the 3′UTR efficiency motif is destroyed. It is not known whether the C-terminus of the PRtransferase is necessary for enzyme activity.

Our findings suggest that intact efficiency motifs have an influence on cleavage of pre-mRNA. There are several possibilities how binding of protein factors to such an efficiency motif might influence poly(A) site selection. A bound factor could prevent cleavage factors from cleaving the RNA. Alternatively, a protein factor might bind and act as cleavage (mediating) factor itself catalysing cleavage or the recruitment of cleavage factors in a distance of 40–50 nt. The processing factor Rna15p has been discussed to bind to positioning motifs. SELEX experiments revealed a $UGUGUAU_2$ sequence motif which was bound by Rna15p (28). Similar experiments with the human CstF-64 factor of which the RNA binding domain has high homology to Rna15p resulted in three conserved sequence motifs of at least 15 nt, which also were GU rich (29). Whether Rna15p could recognise the shorter and only slightly resembling UAGU motif in the *TRP4* transcript remains speculative. However, RNA binding activity had also been shown for other components of the 3′ end processing machinery (30).

Yeast protein factors involved in 3′ end processing show significant homologies to the processing machinery of higher eukaryotes (31–34). On the other hand, the signal sequences in higher eukaryotes which direct 3′ end formation are highly conserved in comparison to yeast sequence motifs, and efficiency and positioning functions are strictly separated. In yeast, 3′ end processing does not necessarily need a separation of efficiency motifs and motifs directing the positioning of the poly(A) site as we could show in case of the *TRP4* 3′ end processing signal. Although the yeast processing machinery might be similar to higher eukaryotes, it seems to act much more flexibly in directing processing of the very different 3′ ends of yeast transcripts.

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