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# Saturation mutagenesis of a polyadenylylation signal reveals a hexanucleotide element essential for mRNA 3′ end formation in *Saccharomyces cerevisiae*

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**ABSTRACT** **The cis-acting signal sequences required for mRNA 3′ end formation are highly conserved and well characterized in higher eukaryotes. However, the situation in the yeast *Saccharomyces cerevisiae* is still unclear. Several sequences have been proposed which share only limited similarities. One difficulty in identifying yeast polyadenylylation signals might be the presence of redundant signal sequences in the 3′ region of yeast genes. To circumvent this problem we have analyzed the heterologous 3′ region from cauliflower mosaic virus which contains a yeast polyadenylylation signal. We have performed a saturation mutagenesis of the key element TAGTATGTA, which is a condensed version of the polyadenylylation signal TAG . . . TATGTA . . . (TTT) which had previously been proposed. Each of the nine nucleotides was replaced by the three other possible nucleotides and all resulting 1-bp mutants were tested for their capacity to specify mRNA 3′ end formation in yeast cells. The first three nucleotides of this condensed sequence are not required, but mutagenesis of the other six nucleotides had distinct effects on mRNA 3′ end formation. All mutants that were significantly functional had the sequence TAYRTA, and the sequence TATATA had the best capacity for mRNA 3′ end formation. The two thymidine residues at the first and fifth positions are the most essential nucleotides in this sequence. Our results suggest that a degenerate hexanucleotide is essential for mRNA 3′ end formation in yeast. This is reminiscent of the conserved polyadenylylation signal in higher eukaryotes, AATAAA.**

The poly(A) tails of mature mRNAs in eukaryotic cells are generally produced by a two-step process: endonucleolytic cleavage of a precursor mRNA at its poly(A) site and subsequent addition of 50–250 adenosine residues to the newly generated mRNA 3′ end (1, 2). In animal cells, cleavage and polyadenylylation are performed by a large complex consisting of multiple factors, including at least two cleavage factors, a cleavage stimulation factor, a specificity factor, and a poly(A) polymerase (2–6). The specificity factor recognizes the polyadenylylation signal AAUAAA, which is located 10–30 nucleotides (nt) upstream of the poly(A) site and initiates complex formation on the precursor transcript (7). In the yeast *Saccharomyces cerevisiae*, at least four components are required for the cleavage and polyadenylylation reactions: one cleavage factor, one poly(A)-addition factor, a specificity factor, and a poly(A) polymerase (8).

Despite the universality of the cleavage and polyadenylylation processes in lower and higher eukaryotic organisms, fundamental differences in the cis-acting polyadenylylation signals are likely to exist. Polyadenylylation signals in yeast have not been clearly characterized, and attempts to identify a highly conserved sequence element have failed.

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The canonical AAUAAA element of higher eukaryotes is nonfunctional in yeast and shows no homology with the yeast signal sequences which have been proposed so far (9, 10). A decade ago, the tripartite sequence motif TAG . . . TA(T) GT(A) . . . TTT was suggested as a polyadenylylation signal, because a deletion of this sequence had resulted in a *cyc1* mutant defective in mRNA 3′ end formation (11). The further analysis of intragenic *CYC1* revertants that had restored proper mRNA 3′ end formation revealed that some of them had acquired similar motifs including the bipartite sequence TAG . . . TATGTA and the novel putative signal TATATA (12). Mutational analysis of the yeast *GAL7* polyadenylylation signal demonstrated that a sequence element of 26 bp containing an alternating TA stretch was essential for 3′ end formation (13). A similar sequence, the octanucleotide TTTTTATA, has also been suggested as a signal sequence (14).

Despite these well-documented examples, the composition of cis-acting polyadenylylation sequences in yeast seems to be more complicated. First, none of these motifs are capable of directing 3′ end formation alone, but only in combination with other sequence elements at or near the poly(A) site (12, 15–17). Second, a typical yeast polyadenylylation signal—e.g., the *CYC1* polyadenylylation signal—consists of redundant elements where the lack of a potential signal sequence might be compensated for by other sequences (18). Third, some but not all polyadenylylation signals are functional in both orientations, suggesting that a symmetrical RNA structure is involved in this class of signals (15, 19).

It is likely that different types of cis-acting polyadenylylation signals exist in yeast and that these signals are degenerate and redundant. The obvious complexity of yeast polyadenylylation signals makes the analysis of the elements specifying mRNA 3′ end formation rather difficult.

A possible solution to this problem is the use of non-yeast sequences that artificially specify mRNA 3′ end formation. We have recently observed that a plant polyadenylylation signal originating from cauliflower mosaic virus (CaMV) was specifically functional in *S. cerevisiae in vivo* (16). The mRNA 3′ ends were at identical positions in yeast and plant cells. Deletion analysis revealed that three sequence elements were required for proper function of this polyadenylylation signal in yeast: a far and a near upstream element as well as sequences close to the poly(A) site (16). The only key element which is absolutely necessary for proper function was shown to be the near upstream element: a 10-bp deletion of this region abolished mRNA 3′ end formation (16). This element contains the motif TAGTATGTA, which is a condensed version of the bipartite sequence motif of Zaret and

Abbreviation: CaMV, cauliflower mosaic virus.

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Sherman (11). Since mutations in the CaMV polyadenylylation key element were not compensated for by redundant signals (16), this test system is suitable for investigating the sequence requirement of a key polyadenylylation signal in a defined context in more detail. Therefore, we performed saturation mutagenesis of the TAGTATGTA motif and tested which nucleotides within the key element of the CaMV signal are essential and which altered sequences are also able to direct mRNA 3′ end formation in a yeast cell.

## MATERIALS AND METHODS

**Yeast Strains and Methods.** The yeast strain used throughout this study was RH1376, which has the genotype of *MAT***a** *ura3*-Δ. Plasmid-carrying RH1376 derivatives were cultivated in minimal vitamin medium (0.145% yeast nitrogen base/0.52% ammonium sulfate/2% glucose/1% succinate, pH 4.0) at 30°C. Yeast cells were transformed by the lithium acetate procedure (20).

**Construction of the Mutant Plasmids.** Plasmid pME730 is a derivative of pME729 (16). It is a high-copy *Escherichia coli*/*S. cerevisiae* shuttle vector carrying the *URA3* gene for selection in yeast and contains the CaMV polyadenylylation signal as a 0.25-kb DNA fragment. This short sequence was inserted between the actin promoter and the *ADH1* polyadenylylation signal (Fig. 1). The mutant plasmids carrying single point mutations in the sequence TAGTATGTA were constructed by a two-step mutagenesis method (21) using the polymerase chain reaction (PCR). In the first step, nine PCRs were performed, with each using an oligonucleotide derived from the noncoding DNA strand of the *ADH1* sequence and one out of nine degenerate oligonucleotides carrying a mixture of three nucleotides at one position of the TAGTATGTA motif. For all nine reactions, plasmid pME730 was used as the DNA template. The resulting 0.2-kb products were isolated from a low-melting-point agarose gel and applied as primers for a second PCR in combination with an additional oligonucleotide derived from the actin sequence. The template for these nine PCRs was again plasmid pME730. After the isolation of the resulting 0.6-kb products from a low-melting-point agarose gel and after digestion with the restriction enzymes *Pst* I and *Hin*dIII, the resulting 0.25-kb fragments were substituted for the wild-type sequences, resulting in the 27 single-base-pair mutations described in Fig. 1 (nos. 1–27). More than 95% of the resulting clones carried a mutated CaMV insert. The search for the three mutations in each PCR mutagenesis reaction was performed by DNA sequencing.

The same procedure, with oligonucleotides carrying the corresponding mutations, was used to create two plasmids with insertions within the TAGTATGTA motif (Fig. 1, nos. 28 and 29) and one plasmid where the last 8 nt of this motif were replaced by the sequence TTTTTATA (no. 30). All mutations were identified by DNA sequencing with the chain-termination method (22). Sequencing was used simultaneously to verify that no additional mutations were created during the PCRs.

**RNA Analysis.** Total yeast RNA was isolated as described (23). For Northern analysis (24), 5 μg of each RNA sample was electrophoresed in a denaturing 2% agarose gel containing 3% formaldehyde, transferred to Hybond nylon membranes (Amersham), and bound by UV crosslinking. The membranes were prehybridized for 16 hr at 42°C in 50 ml of hybridization solution (50% formamide/50 mM sodium phosphate buffer, pH 6.5/0.8 M NaCl/5× Denhardt's solution/0.5% SDS/1 mM EDTA with denatured calf thymus DNA at 150 μg/ml and torula yeast RNA at 500 μg/ml) and subsequently hybridized at 42°C for 16 hr in 15 ml of the same solution containing randomly labeled DNA fragments. After washing and autoradiographic exposure of the membrane, the radioactive signals were quantitated with a densitometer (OneScanner, Macintosh Apple Computer, Cupertino, CA).

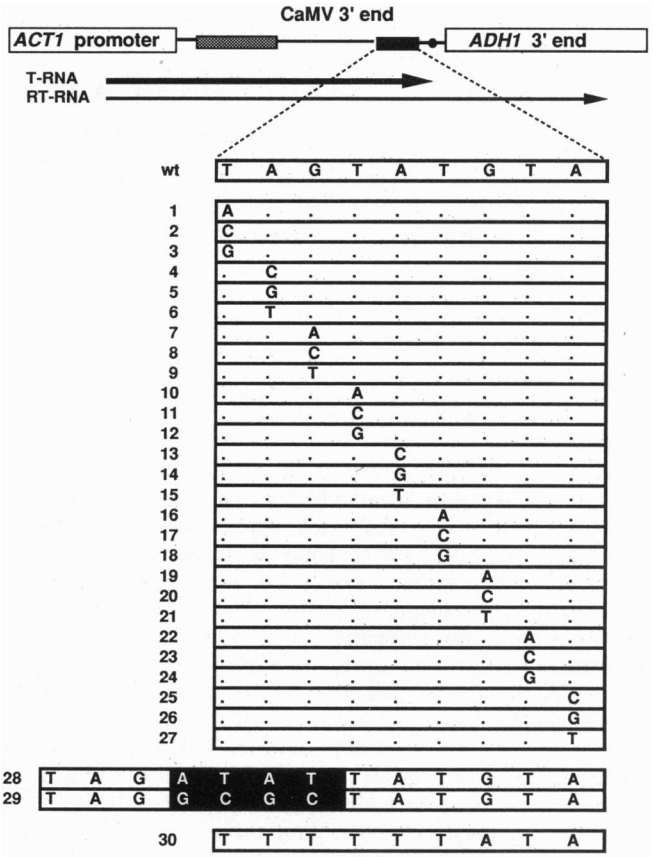



FIG. 1. Test system for wild-type (wt) and mutant polyadenylylation sites in yeast. The test construct contains the 250-bp CaMV polyadenylylation signal, which is inserted between the actin (*ACT1*) promoter and the *ADH1* 3′ end region. The near upstream element (filled box) with the sequence TAGTATGTA is located at positions −43 to −51 relative to the major poly(A) addition site and is the essential element for the function of the CaMV polyadenylylation signal in yeast. Full function requires the additional far upstream element (stippled box, located at positions −150 to −115) and sequences close to the poly(A) site (black dot). The wt sequence causes mRNA 3′ end formation with an efficiency of ≈62% truncated transcript (T-RNA) and 38% readthrough transcript (RT-RNA). The sequences of the various mutant polyadenylylation sites (nos. 1 to 30) are indicated below. All mutations were constructed by using the PCR. For the single point mutations, nos. 1–27, only the changed nucleotide is indicated, and the points represent nucleotides which are identical to the wild type. Constructs 28 and 29 each contain a 4-bp insertion (black box), and construct 30 contains the sequence TTTTTTATA instead of the wt sequence.

**Enzymes and Oligonucleotides.** Restriction enzymes were purchased from Boehringer Mannheim and New England BioLabs. Vent DNA polymerase was purchased from New England BioLabs and T7 DNA polymerase was obtained from Pharmacia. Oligonucleotides were synthesized by Microsynth (Windisch, Switzerland).

## RESULTS

We have used the 3′ end region of CaMV, which contains a yeast polyadenylylation signal (16), as a model system for a yeast polyadenylylation signal that does not contain redundant signals. The key element for recognition of these plant sequences as a polyadenylylation signal in yeast is an upstream element containing the sequence TAGTATGTA. In addition, a far upstream element and sequences close to the poly(A) site are necessary for full function of this polyade-

nylylation site (16). Plasmid pME730 was thereby used as an *in vivo* test plasmid containing the 3′ end region of the CaMV transcript (16). This test plasmid, designed for the *in vivo* analysis of polyadenylylation signals, contained the efficient actin promoter upstream and the efficient *ADH1* polyadenylylation signal downstream of the CaMV insert. The CaMV signal was inserted in a forward direction relative to the direction of transcription starting from the actin promoter (Fig. 1).

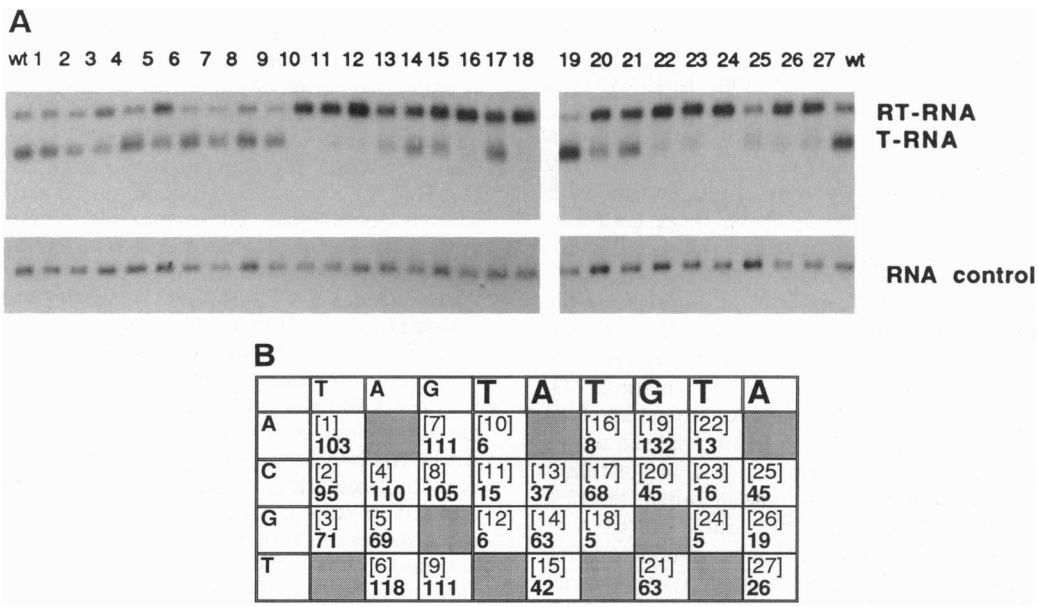
We used this test system to perform a saturation mutagenesis of the motif TAGTATGTA by subsequently replacing each of the nine nucleotides with the three other nucleotides. In addition, we created several additional mutations of this motif. All mutant fragments were created by PCR and inserted in place of the wild-type sequence between the actin promoter and the *ADH1* polyadenylylation signal (Fig. 1).

The mutant plasmids were transformed into the *ura3*-deficient *S. cerevisiae* strain RH1376, and plasmid-carrying cells were selected on minimal medium. Total RNAs isolated from these cells were fractionated and analyzed by Northern hybridization. Functional polyadenylylation signals resulted in the formation of truncated transcripts of ≈500 nt (T-RNA in Fig. 1). Defective signals failed to specify correct 3′ end formation and resulted in the production of 750-nt readthrough transcripts with 3′ ends generated at the *ADH1* polyadenylylation site (RT-RNA). Comparison of the portion of truncated transcripts to the total amount of transcripts initiated at the actin promoter, which are the truncated plus the readthrough transcripts, allowed us to quantitate the efficiency of mRNA 3′ end formation. In the case of the wild-type CaMV sequence, ≈62% of the transcripts were truncated and the remaining 38% were readthrough transcripts (Fig. 2). This value for the truncated transcripts was taken as a standard for all mutations and defined as 100%.

**A Degenerate Sequence, TAYRTA, Is Essential for Function.** Single point mutations of the first three nucleotides of the condensed TAGTATGTA sequence had only minor effects on the activity of the polyadenylylation signal. The activity varied between 69% and 118% when compared with the activity of the wild-type sequence (100%). Only the introduction of G-residues at the first and the second position caused a moderate decrease of truncated RNAs (Fig. 2, mutant 4, 69%; mutant 6, 71%). Some of the mutations even caused a slightly increased activity of the polyadenylylation signal.

Point mutations in the second part of the signal, TATGTA, resulted in more significant effects. The two T residues at the first and the fifth position are the most essential nucleotides of this hexanucleotide core element, and any changes no longer allow proper function. Any replacement of these two nucleotides reduced the function of the CaMV signal to 6–16% of the wild-type activity. The T residue at position 3 did not tolerate changes to A or G but was moderately functional (68%) when mutated to the other pyrimidine, C. Replacement of the two A residues at the second and sixth position caused a significant reduction in mRNA 3′ end formation (19–63% activity in comparison to the wild-type sequence), but the effects were less pronounced than for the T residues. The least effect was that of a transition from A to G at the second position (63%). Finally, mutations of the G residue at the fourth position were tolerated to a certain extent: a T resulted in a moderately functioning polyadenylylation signal (63%), a C further decreased function to 45%. However, an A at the fourth position significantly improved the wild-type sequence to 132% (Fig. 2).

In summary, the saturation mutagenesis experiment demonstrates that the last six nucleotides of the condensed TAGTATGTA sequence are important determinants of the polyadenylylation signal, whereas the first three nucleotides, TAG, seem to be largely dispensable. We cannot rule out the possibility that they might be functionally replaced by upstream TAG sequences. Not all nucleotides of the TATGTA motif have the same degree of conservation. The most active single-point-mutation-generated motifs are the hexanucleotides TATATA, TATGTA, and, with a reduced efficiency, TACGTA. Taken together, these results suggest the consen-




| | T | A | G | T | A | T | G | T | A |
|---|---|---|---|---|---|---|---|---|---|
| A | [1] **103** | | [7] **111** | [10] **6** | | [16] **8** | [19] **132** | [22] **13** | |
| C | [2] **95** | [4] **110** | [8] **105** | [11] **15** | [13] **37** | [17] **68** | [20] **45** | [23] **16** | [25] **45** |
| G | [3] **71** | [5] **69** | | [12] **6** | [14] **63** | [18] **5** | | [24] **5** | [26] **19** |
| T | | [6] **118** | [9] **111** | | [15] **42** | | [21] **63** | | [27] **26** |

FIG. 2. Effects of point mutations on mRNA 3′ end formation in yeast. (*A*) Northern hybridization of total RNA isolated from the wild type and the single-base-pair mutant strains. The truncated RNA (T-RNA, ≈500 nt) and the readthrough RNA (RT-RNA, ≈750 nt) were detected with an actin probe. As control for the loaded RNA (RNA control), the filters were also hybridized to a *URA3* DNA probe. The numbers of the constructs shown above the lanes correspond to the numbers in Fig. 1. (*B*) Quantification of the polyadenylylation function. The films were evaluated with a densitometer. All values (in bold) are an average of evaluations of three Northern blots and represent the ratio between truncated transcripts and the total amount of transcripts initiated at the *ACT1* promoter (total transcript = truncated transcript + readthrough transcript). The polyadenylylation efficiency of the wild-type sequence is taken as a standard, which is defined as 100% and is indicated by stippled boxes. The numbers in brackets correspond to the numbers in Fig. 1. The standard deviations did not exceed 10%.

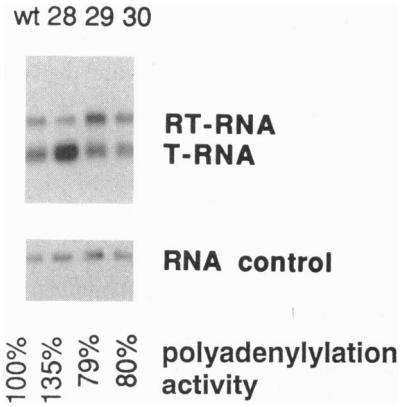



FIG. 3. Northern hybridization of total RNAs isolated from the wild type and mutants 28–30. For nomenclature of mutants see Fig. 1. Quantification of the polyadenylylation efficiency was performed as described in Fig. 2.

sus sequence TAYRTA. A significant activity of >60% was also demonstrated by TATTTA and TGTGTA, suggesting the more degenerate common motif TRYDTA. Two thymidine residues ($T^1$ and $T^5$) are the most essential nucleotides, and interestingly, four of the five functional signals are alternating pyrimidine/purine sequences (Fig. 2).

**5′ Flanking AT Sequences Increase, Whereas 5′ Flanking GC Sequences Decrease, Activity of the 6-bp Motif.** Since the mutagenesis of the first three nucleotides, the TAG element, showed, in some mutants, a moderate increase or decrease in the activity of mRNA 3′ end formation, and since a bipartite sequence was originally proposed as a putative signal, we tested the influence of adjacent sequences on polyadenylylation by using two additional insertion mutants. In both cases, four nucleotides, TATA (mutant 29) and GCGC (mutant 30), respectively, were inserted between the TAG and TATGTA elements (Fig. 1). Neither mutation abolished polyadenylylation, but they resulted in opposite effects on polyadenylylation. Whereas the insertion of the sequence ATAT upstream of the TATGTA motif improved the activity of the polyadenylylation signal (135%; Fig. 3), the GCGC mutant reduced the activity of the CaMV signal (79%). These data indicate that adjacent A- and T-rich sequences have a stimulating effect, whereas C- and G-rich sequences show inhibitory effects on polyadenylylation. In agreement with the results of the point-mutation analysis, these data also demonstrate that the bipartite character of the signal is not essential.

**The Sequence TTTTTATA Partially Compensates for the Lack of the CaMV Polyadenylylation Sequence.** The octanucleotide sequence TTTTTATA is another motif that has previously been proposed for mRNA 3′ end formation (14). If only the last six nucleotides, TTTATA, are considered, this motif corresponds to a version of the original sequence, TATGTA, which contains one mismatch (a T at position 2 instead of an A, which reduced the activity in mutant 15–42% of the wild type; Fig. 2), and one "up" mutation (an A at position 4, which increased the activity in mutant 19 to 132% of wild type; Fig. 3). To test whether this element could act as a polyadenylylation signal in our system, we replaced the sequence AGTATGTA by this element (Fig. 1, mutant 30). Northern hybridization showed that the $T_5$ATA motif could restore mRNA 3′ end formation with an efficiency that was ≈80% of the wild-type activity (Fig. 3). This value corresponds to a compensation of the down mutation by the up mutation and supports the idea that the polyadenylylation signal is degenerate.

## DISCUSSION

The signals used for polyadenylylation were always described as one of the major differences between mRNA 3′ end formation of mammals and yeast. Whereas animal genes require the conserved motif AATAAA, polyadenylylation signals in yeast are rather complex and show no significant similarities to higher eukaryotic signals. The major finding of this paper is that the key signal element for mRNA 3′ end formation in yeast has the same size as the corresponding element in higher eukaryotes and is a sequence-specific hexanucleotide.

We have chosen the plant CaMV polyadenylylation signal, inserted into a yeast test plasmid, as an artificial model for a yeast polyadenylylation signal. The heterologous CaMV polyadenylylation signal is functional in yeast, and a deletion of its key element, located 43–51 nt upstream of the poly(A) site, resulted in the inactivation of the polyadenylylation signal (16). A special feature of this key element is that it corresponds to the sequence motif, TAGTATGTA, that previously has been proposed to be a signal sequence in yeast (11). The characterization of the short, 9-bp oligonucleotides allowed us to define the features of cis-acting signal sequences that specify RNA 3′ end formation of RNA polymerase II transcripts in *S. cerevisiae* and to reveal the sequence specificity of the element.

The detailed mutational analysis demonstrated that the first three nucleotides of the TAGTATGTA element play only a minor role in this polyadenylylation signal. Our data suggest that a high A and T content in the 5′ flanking region of the actual key element, which comprises the last six nucleotides, has a stimulating effect on its activity. Single point mutations within the TATGTA motif, however, have significant effects on the activity of the polyadenylylation signal. These results confirm the data of Hyman *et al.* (9) that in yeast, as in higher eukaryotes, it is possible in principle to inactivate a polyadenylylation site by a single nucleotide exchange. The most drastic reduction of mRNA 3′ end formation was observed when one of the three T residues of the hexanucleotide TATGTA was mutated. Each mutation decreased the activity of the signal to below 20%, except for the central T, which could be partially replaced by a C residue. Mutations of either one of the two A residues or of the G residue were partially tolerated and reduced mRNA 3′ end formation to 20–60% of wild type. An opposite effect was caused by the replacement of the G by an A residue (TATATA), which resulted in increased activity of the polyadenylylation signal. These data are in agreement with the analysis of intragenic revertants of a *cyc1* mutant incapable of 3′ end formation. Some of these revertants had acquired the sequences TATGTA, TATATA, or TACATA in the 3′ region of the *CYC1* gene (12). That 14 out of the 18 possible single-base pair mutations reduce polyadenylylation by >50% indicates the sequence specificity of the element.

These data and the size of this essential sequence for mRNA 3′ end forming in yeast are reminiscent of the AATAAA polyadenylylation signal in animal genes. Since in yeast only mutations of the T residues of the TATGTA motif resulted in strong effects, and the effects of mutating the other nucleotides seemed to be more variable, the yeast hexanucleotide is certainly less conserved than its counterpart in higher eukaryotes. In higher eukaryotes all single point mutations except one (AATAAA to ATTAAA) caused a loss of activity to below 20% (25). The degenerate character of the yeast sequence is also supported by the observation that in yeast the hexanucleotide TTTATA, which consists of an up (A at position 4) and a down (T at position 2) mutation relative to TATGTA, is functional. Its 3′-end-formation efficiency is 80% in comparison to the wild type, which corresponds to the average value of the two single point

mutations (TTTGTA, 42%; TATATA, 132%). Therefore, we suggest that a hexanucleotide element containing essential T residues with a preference for an alternating pyrimidine and purine stretch is the polyadenylylation signal for yeast.

The sequence specificity of the element suggests that this element interacts with one or several proteins. It seems likely that this protein is an RNA-binding protein, presumably an analogue of the mammalian specificity factor (8). However, a possible role of DNA-binding proteins cannot completely be ruled out. The fact that the TATATA element has been shown to be the most effective polyadenylylation signal is surprising because it is identical to a functional TATA element in the promoter 5′ region of a gene (26). The speculation that the general role of the TFIID transcription factor in the promoters of all RNA polymerases could be extended to the end of a gene is tempting and remains to be elucidated.

Since it has been difficult to abolish the polyadenylylation function in yeast by point mutations or very small deletions (18), it may be that at least some yeast polyadenylylation signals are composed of redundant elements or of several essential but not sufficient elements. The elimination of one element seems to be compensated for by other elements, which complicates the identification of the cis-acting signal sequences by classical point-mutagenesis experiments. The CaMV polyadenylylation signal contains three elements which are necessary for full function in yeast (16). Whereas deletions in two of these elements reduced the efficiency of polyadenylylation but did not abolish it, a deletion of the third element, which corresponds to the TAGTATGTA sequence, resulted in the loss of the polyadenylylation function (16). Therefore, the use of the artificial CaMV polyadenylylation site created a situation in yeast in which full function was dependent on a short, 9-bp sequence with no other absolutely essential elements being present. For example, in the wild-type *CYC1* gene, the key element of the polyadenylylation signal is located within a 38-nt region, but no smaller deletions in this region, not even 20 nt, abolished *CYC1* 3′ end formation (18, 19), indicating that the 38-nt region contains two or more elements that function simultaneously as polyadenylylation signals. This 38-nt region has neither a perfect TATATA nor a TATGTA element but does contain several sequences with great similarity to these elements. It is tempting to speculate that yeast polyadenylylation signals are composed of several partially active signals that, in combination, result in an efficient polyadenylylation signal and are able to substitute for each other. This situation is similar to the initiation of transcription where, as in the yeast *TRP4* promoter, numerous redundant cryptic initiator elements can compensate for a loss of function of a single initiator, without drastic effects on overall transcription initiation (27). Since mRNA 3′ end formation and transcription termination are essential processes with respect to the proper expression of adjacent genes, which often have only short intergenic regions, the redundancy of signals directing these processes might be a safety mechanism. Redundancy would inhibit a spontaneous mutation in the polyadenylylation signal of a gene from inactivating the promoter of an adjacent gene by transcriptional interference. If yeast genes have acquired redundant signals during the evolution of their "high gene density" genome, the conservation of a fully active polyadenylylation signal would not be required and signals with a partial activity would be sufficient if two or more of them were present in the 3′ end region.

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