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Different Classes of Polyadenylation Sites in the Yeast *Saccharomyces cerevisiae*

STEFAN IRNIGER, CHRISTOPH M. EGLI, AND GERHARD H. BRAUS*

Institute of Microbiology, Swiss Federal Institute of Technology (ETH), CH-8092 Zürich, Switzerland

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This report provides an analysis of the function of polyadenylation sites from six different genes of the yeast *Saccharomyces cerevisiae*. These sites were tested for their ability to turn off read-through transcription into the *URA3* gene in vivo when inserted into an *ACT-URA3* fusion gene. The 3′ ends of all polyadenylation sites inserted into the test system in their natural configuration are identical to the 3′ ends of the chromosomal genes. We identified two classes of polyadenylation sites: (i) efficient sites (originating from the genes *GCN4* and *PHO5*) that were functional in a strict orientation-dependent manner and (ii) bidirectional sites (derived from *ARO4*, *TRP1*, and *TRP4*) that had a distinctly reduced efficiency. The *ADH1* polyadenylation site was efficient and bidirectional and was shown to be a combination of two polyadenylation sites of two convergently transcribed genes. Sequence comparison revealed that all efficient unidirectional polyadenylation sites contain the sequence TTTTTAT, whereas all bidirectional sites have the tripartite sequence TAG...TA(T)GT...TTT. Both sequence elements have previously been proposed to be involved in 3′ end formation. Site-directed point mutagenesis of the TTTTTAT sequence had no effect, whereas mutations within the tripartite sequence caused a reduced efficiency for 3′ end formation. The tripartite sequence alone, however, is not sufficient for 3′ end formation, but it might be part of a signal sequence in the bidirectional class of yeast polyadenylation sites. Our findings support the assumption that there are at least two different mechanisms with different sequence elements directing 3′ end formation in yeast.

The mature mRNA in eukaryotic cells is formed by posttranscriptional processing of a longer precursor transcript. In addition to the capping of the 5′ end and the removal of noncoding intron sequences, the formation of a proper 3′ end, including the addition of a poly(A) tail, is a crucial step in mRNA maturation. The processes involved in mRNA 3′ end formation have been well characterized in higher eukaryotic cells where transcription by RNA polymerase II is known to proceed hundreds to thousands of base pairs downstream of the actual mRNA 3′ end. The mature 3′ end is then formed by rapid endonucleolytic cleavage of this precursor transcript, followed by polyadenylation (for a review, see reference 8). The cleavage site is specified by the highly conserved sequence element AAUAAA located 10 to 30 bp upstream of the polyadenylation site (18, 36). Numerous mutagenesis experiments have shown that this hexanucleotide is essential for processing as well as for poly(A) tail addition (43, 46) and that G+T-rich sequences distal to the cleavage site are also required (13, 21, 29).

Most genes of the yeast *Saccharomyces cerevisiae* lack the AAUAAA processing signal. Deletions of different sequences abolished the formation of proper mRNA 3′ ends. Henikoff and Cohen (22, 23) identified the octanucleotide TTTTTATA as being essential for 3′ end formation of a *Drosophila melanogaster* gene expressed in *S. cerevisiae*. Zaret and Sherman found that a *cyc1* mutant defective in normal *CYC1* 3′ end formation lacked a 38-bp DNA region containing the tripartite sequence TAG...TA(T)GT...TTT (45). One or the other of these sequence motifs has been found near the poly(A) addition site of many yeast genes. The well-characterized *CYC1* 3′ end region, as an exception to other yeast genes, contains both of these sequence elements. Deletion analysis of the *CYC1* 3′ end has revealed that either of them can be deleted without distinctly affecting 3′ end formation (35). However, simultaneous deletion of both elements results in a drastic decrease in the formation of normal *CYC1* 3′ ends (45). The absence of a unique processing and polyadenylation signal suggested that there is no single mechanism for 3′ end formation in *S. cerevisiae* (44). The observation that all mRNAs in *S. cerevisiae*, including histone mRNA (15) and aberrant transcripts (45), are polyadenylated indicated that transcription termination and polyadenylation may be directly coupled events. Additionally, several reports pointed out that transcription far downstream of the actual 3′ ends of yeast mRNA is unlikely (34, 35, 39), an observation supported by the fact that the yeast genome has a high density of transcriptional units with only short intergenic regions. This situation would not allow far-extended transcripts, as this might interfere with the transcription of an adjacent transcription unit.

Recent experiments showed that various in vitro synthesized pre-mRNAs can be endonucleolytically cleaved at their poly(A) addition site when incubated in a yeast cell-free extract (1, 10, 11). These findings suggest that, similar to what is found in higher eukaryotes, the processing of longer precursor transcripts in *S. cerevisiae* is involved in mRNA 3′ end formation in vivo. It still remains unclear, however, whether all or only some yeast mRNA 3′ ends are formed by this mechanism.

Since all knowledge concerning 3′ end formation in *S. cerevisiae* derives from a limited number of genes, we have investigated yeast polyadenylation sites from six different genes by using an in vivo test system in order to detect differences in their function. This test system consists of an *ACT-URA3* fusion gene. The DNA fragments containing the various polyadenylation sites were inserted into the intron of the fusion gene, and their function was tested for the ability to turn off read-through transcription into the *URA3* gene. A

* Corresponding author.

similar approach using *HIS4* as a reporter gene was used in earlier studies to analyze the function of the *CYC1* 3′ end region (38). As a result, we find two different classes of polyadenylation sites in *S. cerevisiae*, (i) strong unidirectional and (ii) less efficient bidirectional polyadenylation sites. All strong poly(A) sites contain the sequence TTTT TAT, which deviates slightly from the Henikoff-Cohen sequence (22), whereas all bidirectional sites contain the tripartite sequence TAG...TA(T)GT...TTT. These sequences are not sufficient for directing 3′ end formation. Mutagenesis revealed that mutations in the TTTTTAT motif had no effect on 3′ end formation in the class of unidirectional polyadenylation sites. A mutation of the tripartite sequence, however, reduced the efficiency of 3′ end formation in the class of the bidirectional polyadenylation sites.

MATERIALS AND METHODS

Strains and media. All yeast strains are derivatives of the *S. cerevisiae* laboratory strains X2180-1A (*MAT***a** *gal2 SUC2 mal CUP1*) and X2180-1B (*MATα gal2 SUC2 mal CUP1*). RH1242 (*MAT***a** *aro7 leu2-2*) was used for RNA studies, and RH1367 (*MAT***a** *his3-11 his 3-15 leu2-3 leu2-112 can1 Δura3*) was used for complementation studies. YEPD complete medium and MV minimal media were used for the cultivation of *S. cerevisiae* (32). Yeast transformation (26) and DNA isolation (9) were previously described.

Escherichia coli MC1061 [Δ(*lacIPOZYA*)*X74 galU galK strA hsdR* Δ(*ara leu*)] (12) was used for propagating plasmid DNA and was maintained on Luria broth (31).

Oligonucleotides. Synthetic oligonucleotides were purchased from Microsynth AG, Windisch, Switzerland.

Plasmid constructions. Plasmid pME621 was used for the testing of the polyadenylation sites and was created as follows: the 1.2-kb *Bam*HI fragment on pNKY48 (2) containing an *URA3* cassette (270 bp of the *HIS3* gene fused in frame to the *URA3* gene) was cloned into the *Bgl*II site of the actin gene (*ACT*) on plasmid pYact1 (33). The reading frame from the *ACT* to the *URA3* gene was maintained. The *ACT-URA3* fusion gene was isolated as a 3.4-kb fragment by digestion with *Pst*I and *Kpn*I and placed into the multiple cloning site of YEp351 (24), a 2μm-based *E. coli-S. cerevisiae* shuttle vector carrying the *LEU2* gene for selection in *S. cerevisiae*, resulting in pME621. DNA 3′ end fragments containing the polyadenylation sites of interest were inserted into the single *Xho*I site of this plasmid as follows: the *TRP1* 3′ end fragment and a control fragment from the *TRP1* coding region were isolated as a 291-bp *Alu*I-*Rsa*I fragment and a 232-bp *Eco*RV-*Hin*dIII fragment, respectively, from plasmid pEMBLYr25 (6). The *TRP4* 3′ end was isolated as a 264-bp *Bst*EII-*Eco*RV fragment from plasmid pME511 (19), and the *ADH1* 3′ end was isolated as a 200-bp *Hin*dII-*Hpa*II fragment from plasmid pAAH5 (3). The *GCN4* 3′ end was obtained as a 261-bp *Taq*I-*Taq*I fragment (25). The *PHO5* 3′ end was isolated as a 202-bp *Rsa*I-*Hin*dIII fragment from p31R (5), and the *ARO4* 3′ end was isolated as a 257-bp *Hpa*I-*Bcl*I fragment from plasmid pME638 (35a). Blunt ends of all fragments were produced by filling in with AMV reverse transcriptase (BioRad, Richmond, Calif.), and the fragments were cloned into the filled-in *Xho*I restriction site of vector pME621.

Mutagenesis. Point mutations were generated by using the Amersham oligonucleotide-directed in vitro mutagenesis system (Amersham, Amersham, United Kingdom), based on the method of Taylor et al. (41). The mutant oligonucleotide primer was hybridized to single-stranded DNA, and the complementary DNA strand was synthesized in the presence of thionucleotides by the action of Klenow polymerase and DNA-ligase. The resulting double-stranded plasmid was cut with *Nci*I. This enzyme is unable to digest the thionucleotide-containing strand, thus leaving the mutated strand intact. The nicked nonmutated strand was degraded with exonuclease III, and a new complementary strand was synthesized by DNA polymerase I, generating a double-stranded mutated plasmid. Mutated plasmids were identified and confirmed by DNA sequencing.

Isolation of RNA and Northern blotting. Total RNA was isolated according to the method of Zitomer and Hall (47) using glass beads to disrupt the yeast cells. Poly(A)$^+$ RNA was selected by oligo(dT) cellulose (4). For Northern (RNA) hybridizations, 30 μg of total RNA or 5 μg of poly(A)$^+$-enriched RNA was separated on a denaturing formaldehyde gel according to the method of Rave et al. (37). After being transferred to Hybond nylon membranes (Amersham), the bound RNA was hybridized at 42°C with randomly labeled DNA fragments (17) or at 60°C with in vitro synthesized labeled RNAs (30).

Nuclease S1 mapping. For the mapping of 3′ ends of transcripts, DNA fragments were labeled with α-^{32}P nucleotides and AMV reverse transcriptase. Radiolabeled fragments were annealed to 10 μg of poly(A)$^+$ RNA in 30 μl of 80% formamide–0.04 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–0.4 M NaCl–1 mM EDTA by denaturing them for 15 min at 85°C followed by hybridization at 42 to 50°C (16). A total of 300 μl of ice-cold S1 buffer (0.28 M NaCl–0.05 M sodium acetate [pH 4.6]–4.5 mM $ZnSO_4$–20 mg of denatured sonicated calf thymus DNA per ml) and 100 U of nuclease S1 (Boehringer, Mannheim, Federal Republic of Germany) were added and incubated for 30 min at 37°C. The reaction was stopped by the addition of 75 μl of 2.5 M ammonium sulfate–50 mM EDTA. The nucleic acids were then ethanol precipitated and fractionated on denaturing polyacrylamide gels containing 7 M urea.

Primer extension. Primer extension analysis was performed according to the method of Kassavetis and Geiduschek (27) by using 20 μg of poly(A)$^+$ RNA and 5×10^6 cpm of oligonucleotide primer labeled at the 5′ end with polynucleotide kinase (Pharmacia, Uppsala, Sweden) and [γ-^{32}P]ATP.

RESULTS

Test system for the analysis of polyadenylation sites. We created a test system to analyze and compare the functions of different polyadenylation sites in the yeast *S. cerevisiae*. We constructed the 2μm-derived plasmid pME621 containing part of the yeast actin gene (*ACT*), including its promoter, exon 1, intron, and a portion of exon 2, fused to an *URA3* cassette (2) in such a way that the reading frame was maintained (Fig. 1A). DNA fragments from the 3′ end of six different genes of *S. cerevisiae* were cloned in both orientations into the *Xho*I restriction site in the intron of the fusion gene. All inserted fragments spanned a 200- to 300-bp DNA region from the end of the coding region to at least 18 bp downstream of the mapped poly(A) addition site (Fig. 1B). The influence of these fragments on transcription from the actin promoter was examined by genetic complementation and by RNA analysis experiments. Efficient polyadenylation sites were expected to prevent read-through transcripts into the *URA3* gene and to result in truncated actin-specific RNAs (Fig. 1A, lane III). This assay allows a comparison of

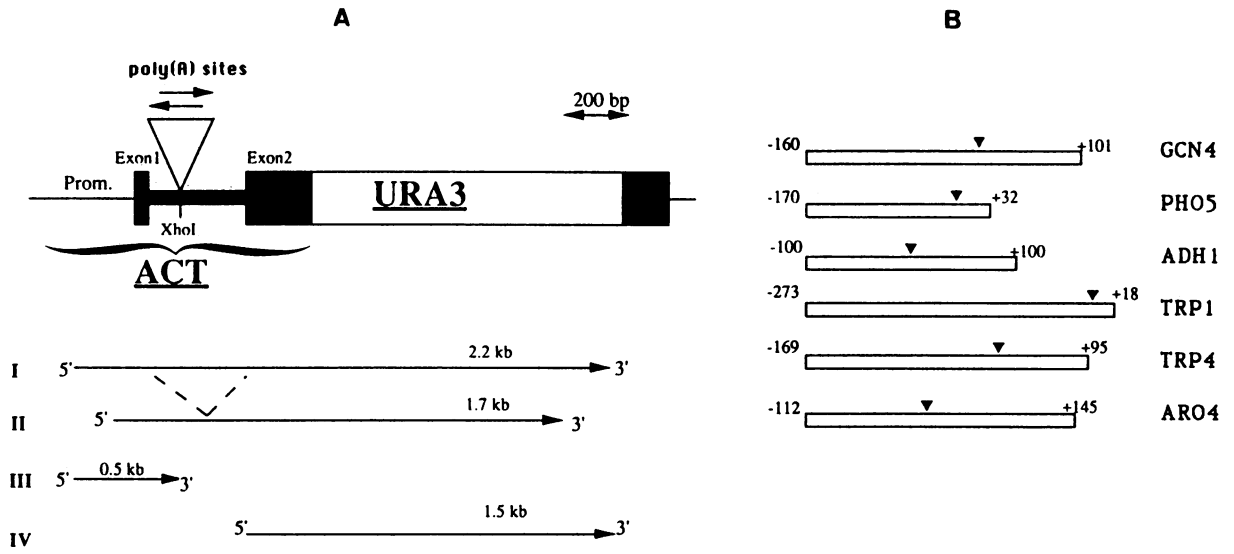

FIG. 1. Test system for polyadenylation sites. (A) Test gene on the 2μm-derived plasmid pME621. A part of the actin gene including the promoter, exon 1, intron, and a portion of exon 2 is fused to an *URA3* cassette. The first six amino acids of the *URA3* gene are missing, and the reading frame is maintained. DNA fragments containing the various polyadenylation sites are inserted in both orientations into the *Xho*I restriction site of the intron of the fusion gene. Expected transcripts are indicated below as follows: I, read-through transcription to the end of the *URA3* gene results in a 2.2-kb transcript that is spliced to II, a 1.7-kb RNA; III, 3′ end formation in the inserted fragment results in a truncated transcript of approximately 0.5 kb; IV, an additional 1.5-kb downstream transcript derives from a cryptic promoter and is discussed in the text. (B) Inserted DNA fragments containing the polyadenylation sites of six different yeast genes. All fragments were introduced in both orientations and contain DNA sequences from the end of the coding region of the corresponding gene to at least 18 bp downstream of the mapped 3′ ends of the transcripts. The locations of the major poly(A) addition sites, an mapped at the chromosomal wild-type locus, are indicated by triangles, and the numbers represent the base pairs upstream and downstream of these sites.

the efficiency and the orientation dependence of the different polyadenylation sites in a single test system.

The suitability of the test system was first examined by inserting a control fragment from the coding region of the *TRP1* gene (232 bp) into the *Xho*I site of pME621. Plasmids containing this fragment as well as empty pME621 were able to complement an *ura3* mutation when transformed into the *ura3* mutant strain RH1367, indicating that a functional URA3 protein was produced. Analysis of the mRNA by Northern hybridization revealed, as for pME621 without insert, only the read-through transcripts from the actin promoter to the end of the *URA3* gene (Fig. 2). The *Xho*I cloning site in the *ACT-URA3* test cassette is located between the 5′ splice site and the TACTAAC box of the *ACT* intron in a region not necessary for lariat formation (20), and therefore splicing was not expected to be abolished. In fact, most of the read-through transcripts were spliced to a 1.7-kb RNA, although a 2.2-kb unspliced RNA was also present, indicating that splicing efficiency was reduced. Accumulation of unspliced precursor mRNA was also observed when the analyzed polyadenylation sites were inserted into the test system. For the evaluation of the test system, one has to take into account that the insertion of polyadenylation sites might have additional effects beyond that of 3′ end formation; such effects might include splicing efficiency and mRNA stability.

The polyadenylation sites we used in the test system originated from the lowly expressed *GCN4* gene encoding a transcriptional activator protein (25), the moderately expressed amino acid biosynthetic genes *TRP1* (42), *TRP4* (19), and *ARO4* (35a), and two highly derepressible genes, the acid phosphatase-encoding gene *PHO5* (5) and the alcohol dehydrogenase-encoding gene *ADH1* (7).

All plasmids containing these various polyadenylation sites inserted into the test gene complemented an *ura3* mutation when transformed into strain RH1367. Four plasmids, however, complemented on minimal medium with a growth rate reduced by 70% compared with that of the control plasmid, indicating that only a small amount of URA3 protein is synthesized. These plasmids contain the *GCN4* and *PHO5* polyadenylation sites in forward orientation as well as the *ADH1* fragment in either orientation (data not shown).

The direct influence of the different fragments on the transcription of the test gene was investigated by Northern hybridization and S1 nuclease mapping the mRNAs of plasmid-carrying yeast cells. Northern analysis with poly(A)$^+$ RNA and with total RNA showed no difference, indicating that all RNAs, including the truncated ones, are polyadenylated (data not shown).

Read-through transcription could be detected by hybridization with an *URA3* probe and is represented by a 2.2-kb unspliced RNA and a 1.7-kb spliced RNA. Transcripts with 3′ ends within the inserted fragment had lengths of 0.4 to 0.6 kb and could be detected by hybridization with a DNA probe from the actin 5′ untranslated region (Fig. 1A). As an independent control for the total amount of loaded RNA, a DNA probe of the chromosomally encoded *ARO1* gene was used.

Two polyadenylation sites act efficiently in a strict orientation-dependent manner, and four polyadenylation sites are functional in both orientations with various efficiencies. The *GCN4* and the *PHO5* 3′ end fragments inserted in forward orientation as well as the *ADH1* 3′ region inserted in either orientation prevented the formation of any read-through transcripts (Fig. 2). Stable truncated RNAs complementary

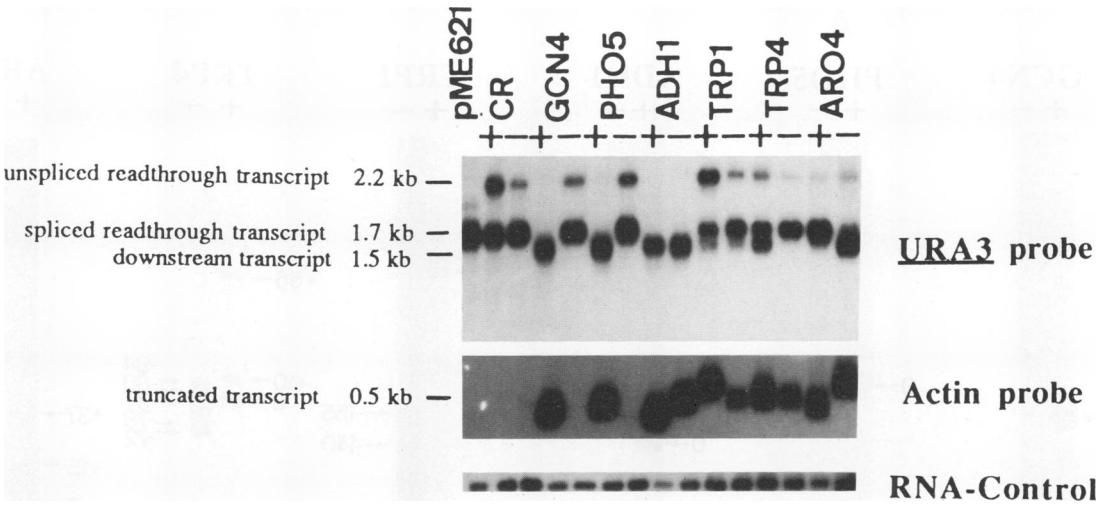

FIG. 2. Analysis of polyadenylation sites by Northern hybridization. Poly(A)$^+$-enriched RNAs isolated from the *S. cerevisiae* strains carrying the test plasmid with the six inserted polyadenylation sites (*GCN4*, *PHO5*, *ADH1*, *TRP1*, *TRP4*, and *ARO4*; + signifies forward insertion, − signifies reverse orientation) were separated on denaturing formaldehyde agarose gels, transferred to nylon membranes, and hybridized to a 1.1-kb *Hin*dIII *URA3* DNA fragment (*URA3* probe) to detect read-through transcripts into the *URA3* gene (900-bp homology to the read-through transcript). After removal of the radioactivity, the filters were hybridized with a 370-bp *Mlu*I-*Hin*fI DNA fragment from the actin 5' untranslated region (actin probe) to detect the truncated transcripts with 3' ends within the inserted polyadenylation sites (130-bp homology to the truncated transcripts). The filters were hybridized with a DNA fragment from the chromosomal *ARO1* gene in order to standardize the amount of loaded RNA (RNA control). As controls for the test system, RNAs from yeast strains carrying the test plasmid without insert (pME621) or with a fragment of comparable size from the *TRP1* coding region (CR) were also analyzed.

to the *ACT* probe were formed instead, indicating that these DNA fragments contain efficient signals for 3' end formation. In reverse orientation, however, the *GCN4* and *PHO5* fragments revealed only the read-through transcripts, indicating that these polyadenylation sites function exclusively in one orientation.

The three polyadenylation sites from the *TRP1*, *TRP4*, and *ARO4* genes were functional in either orientation but had a reduced efficiency compared with the *GCN4*, *PHO5*, and *ADH1* fragments. Besides the formation of the truncated transcripts, the read-through transcripts into the *URA3* gene were also present, with levels varying between 20 and 50% for *TRP1* and *TRP4*, depending on the orientation of the inserted fragments. The *ARO4* site showed significantly less read-through transcription and was therefore more efficient in reverse than in forward orientation (Fig. 2).

An additional 1.5-kb RNA complementary to the *URA3* probe was found for all strong polyadenylation sites and for some inefficient polyadenylation sites. Its formation correlated with the reduction of the read-through transcripts and with the formation of the truncated transcript (Fig. 2). We demonstrate below that this RNA represents a downstream transcript which starts within the actin intron.

The 3' ends of the truncated RNAs were mapped with S1 nuclease in order to determine their precise location. For all polyadenylation sites inserted into the test system in their natural forward orientation, the 3' ends were mapped at the same positions as those found for the 3' ends of the chromosomal genes (Fig. 3A).

When the orientation of the *ADH1*, *TRP1*, *TRP4*, and *ARO4* polyadenylation sites was reverse, transcription extended approximately 70 to 150 bp downstream of the normal 3' ends of these genes. Therefore, the new 3' ends were all mapped close to the ends of the corresponding coding regions (Fig. 3B). The mapping of the 3' end formed by the reversely inserted *ARO4* polyadenylation site revealed that it was located not within the inserted fragment itself but immediately downstream in adjacent actin intron sequences that contain three stretches of T residues (4, 7, and 5 bp) in series. These actin intron sequences might be responsible for the rather efficient function of the *ARO4* polyadenylation site in reverse orientation.

The tripartite sequence TAG...TA(T)GT...TTT is necessary but not sufficient for one class of polyadenylation sites. Sequence comparison of the six investigated polyadenylation sits revealed that the efficient *GCN4*, *PHO5*, and *ADH1* genes all contain the sequence element TTTTTAT in the coding strand of their 3' untranslated DNA region within 60 bp upstream of the mapped mRNA 3' ends (Fig. 3B). On the noncoding strand, this sequence is present only in the bidirectional *ADH1* polyadenylation site. The sequence deviates slightly from the previously proposed Henikoff-Cohen consensus sequence TTTTTATA (22). The three inefficient *TRP1*, *TRP4*, and *ARO4* fragments do not contain this sequence motif but contain the tripartite sequence TAG...TA(T)GT...TTT proposed by Zaret and Sherman as the consensus sequence for 3' end formation (45). This sequence is present on the coding strand as well as on the noncoding strand within 140 bp upstream of the mapped mRNA 3' ends.

The putative role in 3' end formation of the sequence TTTTTAT in the three efficient polyadenylation sites and of the sequence TAG...TA(T)GT...TTT in the three bidirectional polyadenylation sites was further investigated.

We first tested whether these two sequence elements alone are sufficient to direct 3' end formation. Two synthetic oligonucleotides were inserted into the intron of the *ACT-URA3* fusion gene: a 26-bp oligonucleotide that mimics the DNA region around the sequence TTTTTAT of the *PHO5* gene and a 37-bp oligonucleotide corresponding to the DNA region containing the sequence TAG...TAGT...TTT of the *TRP1* gene. Northern hybridization experiments showed

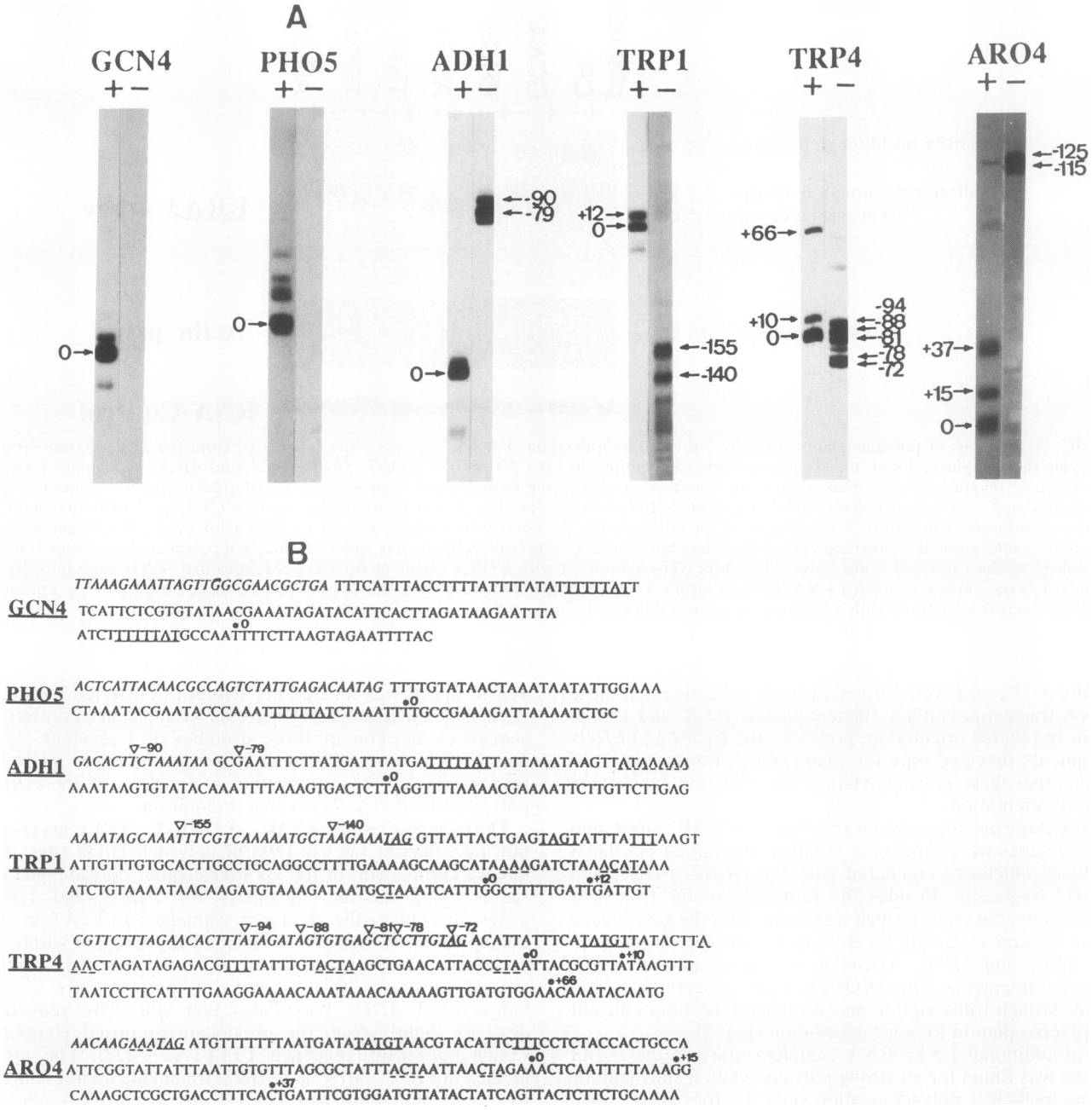

FIG. 3. Analysis of 3′ ends of various polyadenylation sites. (A) Nuclease S1 mapping of the 3′ ends of the truncated transcripts. Poly(A)$^+$-enriched RNAs from *S. cerevisiae* strains carrying the test plasmid with the polyadenylation sites were hybridized to complementary radiolabeled DNA fragments (ranging from the *Bst*YI restriction site in the actin 5′ leader region to the *Cla*I site in the intron). S1 nuclease-resistant hybrids were analyzed on denaturing polyacrylamide gels by using pBR322 digested with *Hin*fI or *Hpa*II and a G+A sequencing ladder as size standards. The 3′ ends of the forward (+) or reverse (−) inserted polyadenylation sites are indicated relative to the mapped transcript ends of the corresponding chromosomal genes (0). (B) DNA sequences of analyzed polyadenylation sites. DNA sequences in italics represent the ends of the open reading frames, and dots and triangles mark the transcript 3′ ends found when the DNA fragments were inserted in forward and reverse orientation, respectively. Numbering corresponds to that in panel A. The 3′ ends for the reverse inserted *ARO4* poly(A) site are not indicated, because they are located in adjacent actin intron sequences. The DNA sequences TTTTTAT and TAG...TA(T) GT...TTT are underlined, and the corresponding sequences on the complementary DNA strands are underlined with broken lines.

that the oligonucleotides neither caused the formation of a truncated transcript nor reduced the amount of the read-through transcripts, indicating that both of the two putative signal sequences are not sufficient to direct 3′ end formation alone (Fig. 4A).

In addition, we investigated whether these sequences are essential for 3′ end formation as a part of a polyadenylation signal. This was tested by oligonucleotide-directed mutagenesis of both sequences. The *PHO5* TTTTTAT sequence was mutagenized to GTCTTGT and the TAG...TATGT...TTT

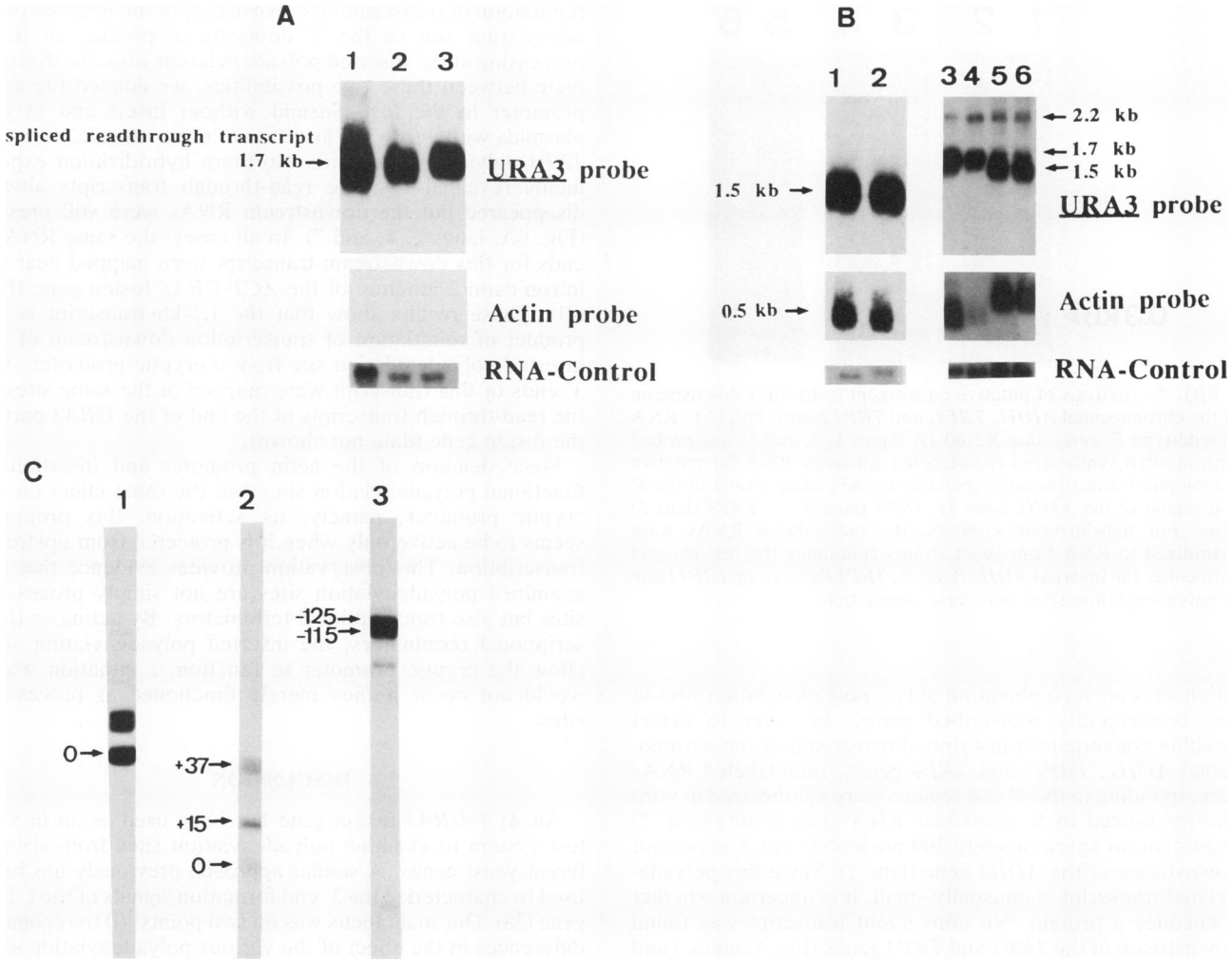

FIG. 4. Effect of the insertion of synthetic oligonucleotides and point-mutated polyadenylation sites into the test system. (A) Northern hybridization with poly(A)$^+$ RNAs from *S. cerevisiae* strains carrying the test plasmid without insert (lane 1) and the test plasmid with two inserted synthetic oligonucleotides (lanes 2 and 3). The oligonucleotide 5'-TACCCAAATTTTTTATCTAAATTTTGC-3' (lane 2) corresponds to a part of the *PHO5* polyadenylation site (−23 to +3 with respect to the mapped 3' end) containing the sequence TTTTTAT; the oligonucleotide ATAGGTTATTACTGATAGTATTTATTTAAGTATTGTTT (lane 3) corresponds to part of the *TRP1* polyadenylation site (−135 to −98 with respect to the mapped 3' end) containing the sequence TAG...TAGT...TTT. Northern hybridization with the *URA3* probe, the actin probe, and the RNA control were performed as described in the legend to Fig. 2. (B) Effect of mutations within the *PHO5* and the *ARO4* polyadenylation sites on transcript formation. The sequence TTTTTAT (*PHO5*) was converted to GTCTTGT and the sequence TAG..TATGT...TTT (*ARO4*) was converted to TAG...TCGAT...GTA by oligonucleotide-directed mutagenesis. Northern hybridization was performed as described in the legend to Fig. 2. Transcripts of the test gene containing the wild-type *PHO5* (lane 1) and mutated *PHO5* (lane 2) polyadenylation sites are compared at left; transcripts of the test gene containing wild-type *ARO4* (forward insertion in lane 3 and reverse insertion in lane 5) and mutated *ARO4* (forward insertion in lane 4 and reverse insertion in lane 6) polyadenylation sites are compared at right. (C) Nuclease S1 mapping of the 3' ends of the truncated transcripts of the mutated *PHO5* polyadenylation site (lane 1) and of the mutated *ARO4* polyadenylation site (forward and reverse insertion in lanes 2 and 3, respectively), performed as described in the legend to Fig. 3. The numbers mark the distance (in base pairs) from the major poly(A) addition site (0).

sequence of the *ARO4* gene was converted to TAG...TC GAT...GTA. The mutated *PHO5* and *ARO4* 3' end fragments were introduced into the test plasmid in place of the wild-type fragments, and the influence of the mutations was analyzed by Northern hybridization and S1 nuclease experiments. Figure 4B shows that there are no read-through transcripts for the mutation in the putative *PHO5* signal sequence and therefore no reduction in efficiency (Fig. 4B, lanes 1 and 2). The location of the mRNA 3' end was also not altered (Fig. 4C, lane 1). The mutations in the Zaret-Sherman sequence of the coding strand of the *ARO4* gene, however, caused a significantly reduced amount of truncated transcript (less than 10%) when inserted in forward orientation (Fig. 4B, lanes 3 and 4) but had no effect when inserted in reverse orientation (lanes 5 and 6). Nuclease S1 mapping of the precise 3' ends revealed no alterations of the locations of the *ARO4* mRNA 3' ends (Fig. 4C, lanes 2 and 3) compared with the wild-type mRNA 3' ends (Fig. 3).

The bidirectional *ADH1* polyadenylation site is located between two convergently transcribed genes. The nature of the bidirectional function of four of the six 3' end fragments was further examined. We tested whether these polyadeny-

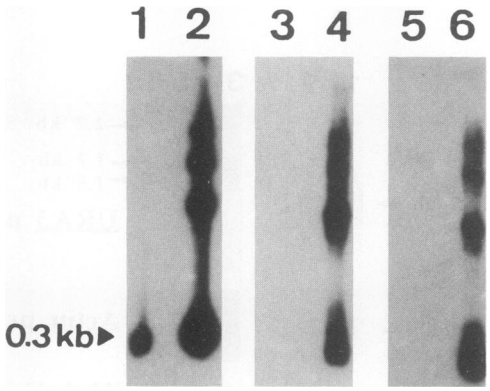

FIG. 5. Analysis of putative convergent transcripts downstream of the chromosomal *ADH1*, *TRP1*, and *TRP4* genes. Poly(A)$^+$ RNA of wild-type *S. cerevisiae* X2180-1A (lanes 1, 3, and 5) was probed with in vitro synthesized radiolabeled antisense RNA for putative convergent transcripts corresponding to the coding strand of the 3′ end region of the *ADH1* (lane 1), *TRP1* (lane 3), or *TRP4* (lane 5) gene. For hybridization controls, the radiolabeled RNAs were hybridized to RNA from yeast strains containing the test plasmid with either the inserted *ADH1* (lane 2), *TRP1* (lane 4), or *TRP4* (lane 6) polyadenylation sites in reverse orientation.

lation sites are a combination of two polyadenylation sites of two convergently transcribed genes. In order to detect possible convergent transcripts downstream of the chromosomal *ADH1*, *TRP1*, and *TRP4* genes, radiolabeled RNAs corresponding to the 3′ end regions were synthesized in vitro and hybridized to *S. cerevisiae* RNA. The results (Fig. 5) show that an approximately 300-nucleotide RNA is present downstream of the *ADH1* gene (lane 1). Since this polyadenylated transcript is unusually small, it is uncertain whether it encodes a protein. No convergent transcript was found downstream of the *TRP1* and *TRP4* genes (Fig. 5, lanes 3 and 5). No convergent transcript has been found for the *ARO4* gene, but it has been previously shown that immediately downstream of the polyadenylation site another gene, *HIS7*, is transcribed in the same direction as *ARO4* (35a). The three inefficient *TRP1*, *TRP4*, and *ARO4* polyadenylation sites containing the tripartite Zaret-Sherman sequence are therefore apparently bidirectional without a convergent transcript on the chromosome, whereas the reverse function of the *ADH1* site is caused by the combination of two polyadenylation sites of two genes. The 3′ ends of this convergent chromosomal transcript were localized at the same positions (data not shown) as the 3′ ends found for the reversely inserted *ADH1* 3′ end fragment in our test system (Fig. 3).

The efficiency of the polyadenylation sites corresponds to the activation of transcription from a cryptic promoter within the actin intron. Besides the expected transcripts, insertion of a functional polyadenylation site into our test system resulted in an additional 1.5-kb transcript of unknown origin (Fig. 2). The formation of this transcript correlated with the reduction of the read-through transcript and with the formation of the truncated transcripts and was dependent on functional polyadenylation sites. This transcript hybridized to the *URA3* probe and was therefore expected to be a transcript downstream of the inserted polyadenylation sites. No unspliced pre-mRNA could be detected for the efficient polyadenylation sites. Therefore, it is very unlikely that the 1.5-kb transcript is the result of incorrect splicing (Fig. 2).

The 1.5-kb transcript could be either the product of reinitiation of transcription downstream of the inserted polyadenylation site or the 3′ downstream product of RNA processing at the inserted polyadenylation sites. To discriminate between these two possibilities, we deleted the actin promoter in the test plasmid without insert and in test plasmids with a forward insertion of either the *GCN4* or the *TRP4* polyadenylation site. Northern hybridization experiments revealed that the read-through transcripts always disappeared but the downstream RNAs were still present (Fig. 6A, lanes 2, 4, and 7). In all cases, the same RNA 5′ ends for this downstream transcript were mapped near the intron-exon 2 junction of the *ACT-URA3* fusion gene (Fig. 6B). These results show that the 1.5-kb transcript is the product of reinitiation of transcription downstream of the inserted polyadenylation site from a cryptic promoter. The 3′ ends of this transcript were mapped at the same sites as the read-through transcripts at the end of the *URA3* part of the fusion gene (data not shown).

Since deletion of the actin promoter and insertion of functional polyadenylation sites had the same effect on the cryptic promoter, namely, its activation, this promoter seems to be active only when it is protected from upstream transcription. This observation provides evidence that the examined polyadenylation sites are not simply processing sites but also transcriptional terminators. By acting as transcriptional terminators, the inserted polyadenylation sites allow the cryptic promoter to function, a situation which would not occur if they merely functioned as processing sites.

DISCUSSION

An *ACT-URA3* fusion gene has been used as an in vivo test system to examine polyadenylation sites from six different yeast genes. A similar approach previously has been used to characterize the 3′ end formation signals of the *CYC1* gene (38). Our main focus was on two points: (i) to recognize differences in the effect of the various polyadenylation sites and (ii) to investigate the involvement of putative signal sequences in this process. We have identified two classes of polyadenylation sites which differ in their degrees of efficiency and orientation dependence. There are no obvious sequence similarities between the two classes, but there are some common features within each of the two classes of polyadenylation sites. Our findings may suggest the existence of different mechanisms for mRNA 3′ end formation in *S. cerevisiae* and of different sequence elements directing this process.

Three polyadenylation sites are able to completely shut down read-through transcription from the *ACT* promoter into the *URA3* gene. The genes of two of these sites (*PHO5* and *ADH1*) contain efficient inducible promoters, whereas the third gene (*GCN4*) encodes a transcript with an unusual 5′ leader for sophisticated translational regulation of gene expression of a transcriptional activator protein (25). Two of these sites (originating from the 3′ end of the *GCN4* and *PHO5* genes) are functional in a strictly orientation-dependent manner, i.e., only in their natural forward orientation. The 3′ end of the *ADH1* gene contains a combination of two efficient polyadenylation sites (*ADH1* and an additional convergently transcribed gene of unknown function) and is therefore efficient in either orientation.

All efficient polyadenylation sites contain the sequence element TTTTTAT, a slight derivation of the putative signal sequence TTTTTATA proposed by Henikoff and Cohen (22). This sequence is present in the coding strand of the

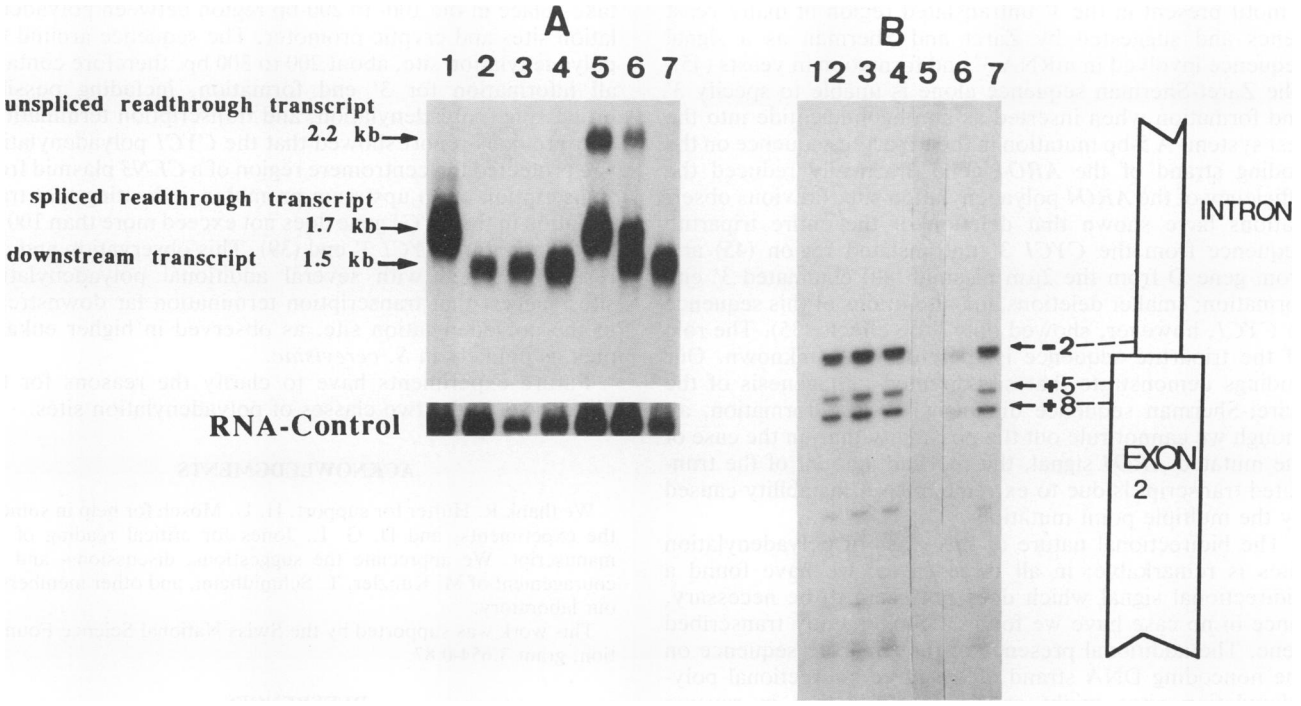

FIG. 6. Analysis of the 1.5-kb downstream transcript. Northern hybridization (A) and primer extension (B) experiments were performed with poly(A)$^+$ RNA from *S. cerevisiae* strains carrying the following plasmids: test plasmid pME621 without insert (lane 1), test plasmid with deleted actin promoter and without insert (lane 2), test plasmid with *GCN4* poly(A) site inserted in forward orientation (lane 3), test plasmid with *GCN4* poly(A) site inserted in forward orientation and with deleted actin promoter (lane 4), test plasmid with *GCN4* poly(A) site inserted in reverse orientation (lane 5), test plasmid with *TRP4* poly(A) site inserted in forward orientation (lane 6), and test plasmid with *TRP4* poly(A) site inserted in forward orientation and with deleted actin promoter (lane 7). The actin promoter was deleted with nuclease *Bal*31 (Boehringer Mannheim); deletions covered the DNA regions from 200 to 300 bp upstream to 100 to 200 bp downstream of the actin transcriptional start site. (A) Northern hybridization was performed as described in the legend to Fig. 2 by using an *URA3* DNA fragment and an *ARO1* DNA fragment as controls for the loaded RNA. (B) Primer extension experiments were performed by using the 5'-radiolabeled oligonucleotide 5'-GCTTCATCACCAACGTAGGA-3', corresponding to bp 143 to 163 of the noncoding strand of actin exon 2. At right is shown the region of the intron-exon 2 junction, and the locations of the detected primer extension signals in this region are indicated by numbers representing the distances from the intron-exon 2 junction.

unidirectional *GCN4* and *PHO5* polyadenylation sites and is also present in both the coding and the noncoding strand of the bidirectional polyadenylation site between the *ADH1* and its convergently transcribed gene. Insertion of an oligonucleotide containing the Henikoff-Cohen sequence into the test system does not direct 3' end formation. On the other hand, site-directed mutations in the sequence of the *PHO5* polyadenylation site do not reduce the efficiency of 3' end formation. Therefore, although this sequence is present in all of these polyadenylation sites, it is neither sufficient nor necessary for correct 3' end formation. If this sequence is at all involved in 3' end formation, it has to be part of a redundant complex signal which is recognized not only on the primary sequence level.

Besides the TTTTTAT motif, the efficient polyadenylation sites have sequences with a high A+T content (about 80%) and A+T stretches of longer than 10 bp at or near the mRNA 3' end. Recently, it has been shown that a 26-bp region with a high A+T content upstream of the mRNA 3' end of the *GAL7* gene is a signal sequence for *GAL7* mRNA 3' processing (1). The strict orientation-dependent function of the *PHO5* and *GCN4* 3' end fragments demonstrates that a high A+T content alone cannot be sufficient for directing 3' end formation. Recent studies showed that transcription termination sites identified by in vitro transcription with purified RNA polymerase II are often located in stretches of T residues (14, 28). The efficient polyadenylation sites might therefore represent real transcriptional terminators. This hypothesis is supported by an analysis of the effect of the polyadenylation sites on a cryptic promoter located about 200 bp downstream of the polyadenylation sites within the actin intron of our test gene. This promoter was activated by the insertion of polyadenylation sites and was shown to function only when it is protected from upstream transcription. This effect was especially strong for the efficient polyadenylation sites.

The other three polyadenylation sites (originating from the 3' ends of the *TRP1*, *TRP4*, and *ARO4* genes) have a reduced efficiency for 3' end formation. We cannot completely rule out the possibility that these poly(A) sites are inefficient in our test system because the inserted 200- to 300-bp DNA fragments do not contain all sequence information for proper functioning and signals far away from the poly(A) addition site may be also required. All three corresponding genes (*TRP1*, *TRP4*, and *ARO4*) are moderately expressed from promoters which are of only limited inducibility. All three polyadenylation sites are functional in a bidirectional manner. The mRNA 3' ends of the reverse orientation are located at different positions compared with the 3' ends of the natural forward orientation.

All weak, bidirectional polyadenylation sites contain the tripartite sequence TAG...TA(T)GT...TTT on both strands,

a motif present in the 3′ untranslated region of many yeast genes and suggested by Zaret and Sherman as a signal sequence involved in mRNA 3′ end formation in yeasts (45). The Zaret-Sherman sequence alone is unable to specify 3′ end formation when inserted as an oligonucleotide into the test system. A 5-bp mutation in the tripartite sequence on the coding strand of the *ARO4* gene drastically reduced the efficiency of the *ARO4* polyadenylation site. Previous observations have shown that deletion of the entire tripartite sequence from the *CYC1* 3′ untranslated region (45) and from gene D from the 2μm plasmid (40) eliminated 3′ end formation; smaller deletions and alterations of this sequence in *CYC1*, however, showed only little effects (35). The role of the tripartite sequence is therefore still unknown. Our findings demonstrate that site-directed mutagenesis of the Zaret-Sherman sequence diminishes 3′ end formation, although we cannot rule out the possibility that, in the case of the mutated *ARO4* signal, the reduced amount of the truncated transcript is due to extreme mRNA instability caused by the multiple point mutations.

The bidirectional nature of this class of polyadenylation sites is remarkable; in all three cases, we have found a bidirectional signal which does not seem to be necessary, since in no case have we found a convergently transcribed gene. The additional presence of the tripartite sequence on the noncoding DNA strand of the three bidirectional polyadenylation sites might cause it to function in reverse orientation. Another possible explanation is that it is a signal for the 3′ processing and polyadenylation complex that also involves RNA secondary or tertiary structure. Mirrorlike RNA structures might be formed when either of the two DNA strands is transcribed, and the two tripartite Zaret-Sherman sequences could be elements which are essential for the formation of a higher-order RNA structure.

The symmetrical function of these sites raises the question about differences in the efficiencies of polyadenylation in forward and reverse orientation. The efficiency of the polyadenylation sites is not only obvious in the decrease of the read-through transcript but also in the appearance of the downstream transcript of the cryptic actin promoter located downstream of the polyadenylation sites. The promoter is less active when less efficient polyadenylation sites are inserted. Whereas the *TRP1* and *TRP4* polyadenylation sites are weaker in reverse than in forward orientation, the *ARO4* polyadenylation site functions with distinct increased efficiency when inserted in reverse orientation. In this case, the mRNA 3′ end is located in a DNA region of the actin intron containing stretches of T residues resembling the mRNA 3′ ends of the efficient polyadenylation sites. This might be a chimeric efficient polyadenylation site consisting of intron sequences together with the *ARO4* 3′ end signal sequence. Therefore, sequences at or near the mRNA 3′ end presumably play a role in 3′ end formation and affect the strength of a polyadenylation site. In agreement with our observations is the identification of signals for 3′ end formation at the very end of the transcript in the Ty element (44) and in the *GAL7* gene (1), which also suggests the involvement of the mature mRNA 3′ end in specifying 3′ end formation.

In conclusion, we suggest that the signals directing 3′ end formation in the class of the bidirectional polyadenylation sites consist of a symmetrical recognition element, including the Zaret-Sherman sequence TAG...TA(T)GT...TTT, and of additional sequences at the mRNA 3′ end.

The appearance of the downstream transcript from the cryptic actin promoter located downstream of the polyadenylation sites demonstrates that termination of transcription takes place in the 100- to 200-bp region between polyadenylation sites and cryptic promoter. The sequence around the polyadenylation site, about 200 to 300 bp, therefore contains all information for 3′ end formation, including possible processing, polyadenylation, and transcription termination.

A previous report showed that the *CYC1* polyadenylation site protected the centromere region of a *CEN3* plasmid from transcription of an upstream promoter, indicating that transcription in the *CYC1* gene does not exceed more than 100 bp past the mature *CYC1* 3′ end (39). This observation and our results obtained with several additional polyadenylation sites suggest that transcription termination far downstream of the polyadenylation site, as observed in higher eukaryotes, is unlikely in *S. cerevisiae*.

Future experiments have to clarify the reasons for the differences in the two classes of polyadenylation sites.

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