An artificial messenger RNA containing a derivative of a tobacco ringspot virus ribozyme was expressed in the bakers' yeast *Saccharomyces cerevisiae*. This mRNA was able to cleave itself efficiently *in vitro*. Using this system, the two steps of mRNA 3' processing, i.e. cleavage and the addition of a poly(A) tail, can be separated in yeast *in vivo*. The ribozyme-cleaved transcript was shown to be polyadenylated. The poly(A) tail length was similar to the poly(A) tail length of an endogenous yeast mRNA. Therefore, cleavage of the precursor RNA at the polyadenylation site and the addition of adenosine residues to the 5' product require independent cellular machineries in yeast and can be separately analyzed. This is in contrast to higher eukaryotes where both processes are coupled.

The yeast *Saccharomyces cerevisiae* represents a feasible model system to study eukaryotic transcription because many aspects of this process are conserved from yeast to man. From *in vitro* systems in mammals and in yeast, it can be concluded that the 3'-ends are formed by endonucleolytic cleavage of precursor transcripts followed by the addition of A residues at the polyadenylation site (Moore and Sharp, 1984, 1985; Butler et al., 1990). An additional step is the actual termination of transcription, which seems to require a pausing signal for the RNA polymerase II and which takes place in various distances downstream of the processing site. Beside these common aspects, there are differences within the eukaryotes concerning these processes. The length of the poly(A) tail varies from about 60 A residues in yeast (Groner et al., 1975) to approximately 200 residues in higher eukaryotes (for reviews, see Humphrey and Proudfoot 1988, Manley (1988), and Wickens (1990)). Furthermore, whereas in higher eukaryotes histone mRNAs do not contain a poly(A) tail, all mRNAs seem to be polyadenylated in yeast (Fahrner et al., 1980).

Higher eukaryotes require the highly conserved hexanucleotide AUUAAG sequence on the nascent RNA as a signal for 3'-end formation. This canonical element is not functional in yeast and shows no homology to the yeast signal sequences that have been proposed so far (Hyman et al., 1991; Proudfoot, 1991; Irniger et al., 1993). A decade ago, the degenerate tripartite sequence motif TAG...TA/GT(A)...TTT had been suggested as polyadenylation signal because a deletion of this sequence had resulted in a CYC1 mutant defective in mRNA 3'-end formation (Zaret and Sherman, 1982). Further analysis of intragenic CYC1 revertants that had restored proper mRNA 3'-end formation revealed that two of six revertants had acquired the bipartite motif TAG...TATGTA and that four revertants had created the modified sequences TATATA or TACATA (Russo et al., 1991). Irniger et al. (1992) found that the nonanucleotide sequence TAGTATGTA, similar to the bipartite CYC1 sequence motif was required for function of the cauliflower mosaic virus polyadenylation site in *S. cerevisiae*. Mutational analysis of the yeast GAL7 polyadenylation signal demonstrated that a sequence element of 26 bp containing an alternating TA stretch was essential for 3'-end formation (Abe et al., 1990). A similar sequence element, i.e. the octanucleotide TTGTTTGA, has also been suggested as a signal sequence (Henikoff and Cohen, 1983). However, the absence of a unique processing and polyadenylation signal suggested that there is no single mechanism for 3'-end formation in *S. cerevisiae* (Yu and Elder, 1989; Irniger et al., 1991).

Cleavage and polyadenylation are performed by similar multicomponent complexes that are partially equivalent in mammals and in yeast. In higher eukaryotes, there are five known factors in addition to the poly(A) polymerase that are required for this process. The poly(A) polymerase (Raabe et al., 1991; Wahle et al., 1991) and two protein factors, i.e. the cleavage and polyadenylation specificity factor and the poly(A) binding protein II, are required for specific polyadenylation (Christofori and Keller, 1988; Takagaki et al., 1988, 1989; Gilman and Nivins, 1989; Bienroth et al., 1993). Three factors involved in endonucleolytic cleavage, designated cleavage factors I and II and cleavage-stimulation factor have been separated from HeLa nuclear extracts (for review, see Wahle and Keller (1992)).

In *S. cerevisiae*, the gene for the poly(A) polymerase has been cloned by Lingner et al. (1991a). In addition, there are at least three trans-acting factors that are involved in 5'-end formation. Chen and Moore (1992) showed that *in vitro* cleavage of the precursor transcript occurred upon the combination of fractions containing cleavage factor I and cleavage factor II without requiring the poly(A) polymerase or the polyadenylation factor I. These two factors in addition to the cleavage factor I were required for the specific polyadenylation reaction of a pre-cleaved GAL7 RNA. Both processes, the cleavage and the polyadenylation, were dependent on the UA repeat within the poly(A) signal (Chen and Moore, 1992).

While in mammals, cleavage and polyadenylation of the precursor transcript are coupled *in vivo* (for review, see Wahle and Keller (1992)) the situation is unclear in yeast (Butler and Platt, 1988; Zaret and Sherman, 1982). Cleavage and polyadenylation can be uncoupled *in vitro* using yeast extracts (Chen and Moore, 1992; Sadhale and Platt, 1992).
This situation prompted us to ask whether it is possible to uncouple cleavage and polyadenylation in yeast in vitro. We present here the first in vivo data showing that, in yeast as a simple eukaryotic model system, cleavage and polyadenylation can be separated into two processes. Separation of the processes was shown by expressing a heterologous ribozyme sequence under the control of a RNA polymerase II promoter.

**EXPERIMENTAL PROCEDURES**

Yeast Strains, Media for Yeast Strain Cultivation, and Methods—The *S. cerevisiae* strain used for these studies was RH 1416 (MATa ura3-52), a derivative of the *S. cerevisiae* laboratory standard strain X2180-1A (MATa gal2 SUC2 mal CUP1). Yeast strains were cultivated in YEPD complete medium (2% bactopeptone, 1% yeast extract, 2% glucose) or MV minimal medium (0.145% yeast nitrogen base, 0.526% ammonium sulfate, 2% glucose, 1% succinic acid) (Miozzari et al., 1978).

**Northern Hybridization Experiments**

For the mapping of 3'-mRNA termini (RACE)—Mapping of the 3'-ends was carried out by a procedure modified from Frohman et al. (1988) and illustrated by Rassoulzadegan et al. (1991). We used the oligonucleotide with the sequence CCGAAGCTGAGCTCGGGATCCTGATAG (T) for the reverse transcription. For the specific amplification of the cDNA, the two used oligonucleotides were GGGAAATTCGTCGACATTCGTTTGAAGAATGG, with a 5'-overlapping EcoRI restriction site and binding in the ACT1 promoter, and the oligonucleotide CCGAAGCTGAGCTGCGACGTCCGATC binding the flanking sequence of the oligonucleotide used for the production of the cDNA. The amplified fragments were isolated over an 0.8% low melting agarose gel, restricted with the enzymes EcoRI and BamHI, cloned into pGEM7ZA(+), restricted with the same enzymes, and sequenced using the dideoxy method (Sanger et al., 1977).

**RESULTS**

**Construction and Expression of an Artificial Yeast Test Gene Containing a Hammerhead Ribozyme**—An artificial mRNA was constructed that contained the potential of its own cleavage. A yeast test cassette for 3' processing consisted of the strong *ACT1* promoter for RNA polymerase II, a multiple cloning site, and the ADH1 polyadenylation signal was used as a basis. Putative 3' processing elements cloned into the multiple cloning site (MCS) of the test cassette resulted in short truncated (T) transcripts in case of functionality and in long readthrough transcripts (RT) in case of no functionality (Fig. 1). A modified plant ribozyme sequence was introduced into the multiple cloning site of the test cassette. The used ribozyme sequence was designed by Eckner et al. (1991) and was based on the results of Haseloff and Gerlach (1988). It is a derivative of satellite RNAs of tobacco ringpot virus, which is of the hammerhead type and which directs intramolecular RNA self-cleavage in plants. For proper function the cleavage site (cs) including the sequence GUC and the catalytic domain (cd) are necessary (Haseloff and Gerlach, 1988, Fig. 1). The ribozyme was cloned into the multiple cloning site of the test cassette resulting in construct 1. The various constructs were integrated at the URA3 locus to avoid multicopy effects and were expressed in yeast.

The *Hammerhead Ribozyme Is Active in the Heterologous Host S. cerevisiae*—The part of the ribozyme sequence of the artificial mRNA was 84 nucleotides in length. The test construct containing this element was expected to result in potential truncated transcripts caused by ribozyme cleavage of a length of 266 bp. Northern hybridization revealed that the RNA from construct 1 was exclusively of the short truncated type with a length of approximately 260 bp, indicating that the transcript was cleaved within the ribozyme sequence (Fig. 2, lane 2). No readthrough transcript of 550 bp could be detected, indicating that the ribozyme was highly efficient in the yeast cells. The finding of the full activity in vitro reflected the in vitro situation where the ribozyme-containing transcript, transcribed by the T7 RNA polymerase, was entirely cleaved (data not shown). In case of the test cassette without any DNA cloned into the MCS (TC), the truncated transcript was not present, but a readthrough transcript of 470 bp appeared on the Northern blot (Fig. 2, lane 1). This indicated that in construct 1 the cleavage was directed by the ribozyme sequence. Quantification of five different Northern blots revealed that the amount of the truncated transcript was between 30% and 35% in comparison with the readthrough transcript. The control construct 2 with the ribozyme sequence in the negative orientation was created to further confirm this finding. RNA from this control construct 2 was exclusively of the readthrough type with 550 bp in length (Fig. 2, lane 2). These results demonstrate (i) that the

**Ribozyme Expression and 3'-End Formation in Yeast**

27379
ribozyme causes a specific truncated transcript in vivo, and (ii) that no readthrough transcript could be detected, suggesting that the ribozyme cleaves with an efficiency of close to 100%.

*The Hammerhead Ribozyme Cleaves Specifically Its Own Transcript in Yeast Cells*—Two elements, the cleavage site with the conserved nucleotides GUC and the catalytic domain, are required for ribozyme activity. Two additional constructs were generated to test whether the truncated transcript was the result of the ribozyme self-cleavage activity or caused by either a cryptic 3' processing signal or by cleavage of an endogenous yeast nuclease. In construct 3, the cleavage site was destroyed by point mutations in all four nucleotides from position -2 to +1. In construct 4, the catalytic domain was deleted (Fig. 1). Both alterations resulted in complete loss of the cleavage activity (Fig. 2, lanes 3 and 4). Two additional methods were applied to further confirm the ribozyme activity and to exclude the presence of cryptic yeast polyadenylation signals. The 3'-end of the cleaved transcript was analyzed either by nuclease S1 mapping (Fig. 3) or by sequencing of cDNA clones obtained by RACE experiments (Fig. 4). Both methods revealed that the 3'-end of the truncated transcript was located within the ribozyme cleavage site, indicating that the cleavage reaction was ribozyme-dependent. The polyadenylation sites and the transcripts were named as -1 for transcript A and +1 for transcript B.

*The Hammerhead Ribozyme-cleaved Transcripts Are Polyadenylated*—Two independent methods showed that the short self-cleaved transcript from construct 1 was polyadenylated. Firstly, the transcript was still present on the Northern blot after oligo(dT) affinity chromatography (Fig. 2, lane 2). Ten different Northern blots with total RNA and poly(A) RNA were compared by scanning analysis. The relative amounts of the truncated transcript were the same on the Northern gel with...
Ribozyme Expression and 3'-End Formation in Yeast

Fig. 4. Sequence analysis of the cloned cDNA of construct 1. RACE analysis exhibited two different 3'-ends of ribozyme-cleaved transcripts which contain poly(A) tails of comparable length to endogenously generated transcripts. Panel A, the cDNAs of the ribozyme-cleaved transcripts were amplified, cloned into an EcoRl/BamHI-cleaved pGEM7'Zf(+) vector and sequenced. Nine cDNAs were analyzed, two corresponded to transcript A, seven to transcript B. The transcripts were amplified using the RACE method (Russo et al., 1991). For the cDNA of both transcripts, one example is given with a corresponding poly(A) tail of 27 (A) and 28 A residues (B), respectively. Panel B, the corresponding ribozyme sequence and the transcripts 3' -ends. The nucleotides from the cleavage site (cs) and from the catalytic domain (cd) are written in bold. The 3'-ends of the transcripts are assigned as [-1] and [+1] in agreement to the earlier described cleavage site (Haseloff and Gerlach, 1988).

While the two steps of 3' processing, i.e. cleavage of the precursor transcript and addition of a poly(A) tail, seem to be strongly coupled in higher eukaryotes (for review, see Wahle and Keller (1992)), we show that it is possible to separate the two reaction steps in vivo in the yeast S. cerevisiae.

The rationale of our assay was to separate the polyadenylation from the cleavage reaction in vivo by using a self-cleaving ribozyme. Thus, the cellular 3' processing machinery was not involved in the cleavage reaction. The ribozyme element contained all of the required sequence elements to form a hammerhead structure with the active cleavage site. Expression in yeast, the ribozyme was surprisingly fully active, which is rare for hammerhead ribozymes in heterologous systems. The cleavage site of the ribozyme in the heterologous yeast system was shown to be located at the sequence GUC, which is a peculiarity for this type of hammerhead ribozymes. By sequencing cDNA clones and by nuclease S1 mapping, two polyadenylation sites differing in two nucleotides were identified. It is most likely that ribozyme cleavage happens at the cleavage site and that the two different 3'-ends are generated total and with poly(A) RNA, demonstrating that the cleaved transcript was polyadenylated. Secondly, we used the RACE method and produced cDNA of the truncated transcripts using an internal oligonucleotide and an additional (dT, ) oligonucleotide. Afterwards the cDNA was amplified and cloned. Sequencing of nine independent ribozyme-induced RACE products gave an average poly(A) tail length of 21 A residues. This finding indicated that the transcripts were polyadenylated. The endogenous yeast mRNA of the GCN4 gene was simultaneously investigated by using the same procedure. Interestingly the poly(A) tail length of the GCN4 RACE product was with 24 A residues in the same range as the measured poly(A) tail length of the ribozyme-induced RACE product.

DISCUSSION

Although the process of 3'-end formation is much better understood in higher than in lower eukaryotes, most information in this field suggests that the principal mechanisms involved are conserved in all eukaryotes. We show here one of the differences in 3'-end formation between yeast and mammals.
after the cleavage reaction by cellular activities. The first polyadenylation site (−1) was located one nucleotide upstream of the cleavage site and is thought to be generated by an endonucleolytic activity. The second polyadenylation site (+1) was located just downstream of an A residue. Therefore this A residue can be either encoded by the DNA template or it can be the first nucleotide of the poly(A) tail added by poly(A) polymerase. This two-band pattern was observed earlier in the work of Eckner et al. (1991) and seems to be a feature of this special ribozyme. The specificity of this cleavage reaction was further confirmed by destroying the cleavage site by point mutagenesis in the cleavage site and by deletion of the catalytic domain. Each of these modifications turned this sequence into a completely inactive element. By these results, the accidental generation of a cryptic yeast cleavage site could be excluded.

The ribozyme activity cleaved the precursor transcript of the test gene, which resulted in a 5'- and a 3'-cleavage product. The 5'-cleavage product corresponds to the truncated short transcript detectable on the autoradiographs in Fig. 2. This transcript should be capped and was shown to be polyadenylated. The amount of the ribozyme-cleaved transcript was lower than that of the corresponding readthrough transcript. Since these results indicated that the 3'-product was rapidly degraded, presumably due to the lack of the Cap structure. This is in agreement with earlier findings that a Cap structure is crucial for mRNA stability (Gerstel et al., 1992).

The length of the poly(A) tail was estimated from the sequence of the RACE products. Since the (dT) oligonucleotide was able to bind at any position within the poly(A) tail, this method does only allow to determine the relative length of the poly(A) tails. The poly(A) tail lengths of all sequenced RACE products of the test construct and of all GCN4-derived transcripts used as control were in the same range varying from 17 to 28 A residues. These data suggest that the poly(A) tail of the truncated transcript was of a comparable length as the poly(A) tail of the GCN4 mRNA.

The most interesting result of this report is that the yeast poly(A) polymerase is able to recognize the 3'-ends of a ribozyme precleaved transcript and to add a regular poly(A) tail in vivo in a yeast cell. The exact chemical mechanism of the addition of the first A residue is difficult to explain. The poly(A) polymerase requires a free 3'- hydroxyl end, whereas the ribozyme cleavage should result in a 2',3'-cyclic phosphate. Therefore we cannot exclude the possibility that in addition to the cleavage and the polyadenylation activity, a third cellular activity is required. The polyadenylation reaction does not require and is not coupled to the regular cleavage reaction, which normally takes place in the cell. We cannot discriminate whether the poly(A) polymerase requires only a free 3'-end and is otherwise unspecific or whether it is able to recognize additional specific features on a pre-mRNA. Several data support the idea that there is an unspecific polyadenylation activity in the yeast cell. Zaret and Sherman (1982) have found that the CYC1-512 deletion in the 3' region of the gene resulted in aberrant longer transcripts that all seemed to be polyadenylated. In contrast to other eukaryotes, the yeast histone genes encode for transcripts that are polyadenylated (Fahrner et al., 1988). There are also in vitro data with purified enzymes and unspecific RNA including Escherichia coli tRNA or rRNA (Lingner et al., 1991b) which suggest that the yeast poly(A) polymerase is an enzyme that recognizes unspecifically the 3'-end of a RNA molecule. However, there are also reports that suggest that there is a certain specificity in the polyadenylation reaction in yeast. Butler and co-workers (Butler et al., 1990) have found that an ammonium sulfate fraction of a yeast extract did not polyadenylate unprocessed precursor RNAs, whereas the 5' cleavage products were efficiently polyadenylated. Even in mammalian cell, an unspecific and a specific polyadenylation reaction can be found in vitro. In the presence of Mg\textsuperscript{2+}
ions, the mammalian enzyme has an unspecific polyadenylation activity. However, the cleavage and polyadenylation specificity factor that is dependent on the AAUAAA signal sequence is required for the specific addition of the poly(A) tail and the poly(A) binding protein II for its elongation (Christofori and Keller, 1988; Takagaki et al., 1988; 1989; Gilmartin and Nhevins, 1989; Bienroth et al., 1993).

It might be possible that the difference between yeast and mammals is a more stable and tight complex of the poly(A) polymerase, specificity factors, stimulating factors, and cleavage factors in mammalian cells in comparison to yeast. In mammalian polyadenylation does not seem to be required for cleavage. In yeast it might therefore be possible that the poly(A) polymerase can easily dissociate from the other factors and can provide a higher unspecific polyadenylation activity in the yeast cell.

In summary, we have expressed a heterologous ribozyme in yeast and have found that this ribozyme is highly active in yeast. The ribozyme-cleaved transcript was stable and polyadenylated and is therefore the first transcript where it was possible to separate the cleavage and the polyadenylation of a 3'-end processing of a mRNA in vivo. This system might be useful to study polyadenylation and RNA cleavage independently from each other and might facilitate the understanding of mRNA 3'-end formation in eukaryotes.

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Ribozyme Expression and 3'-End Formation in Yeast

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