© 1996 Oxford University Press
Nucleic Acids Research, 1996, Vol. 24, No. 15 **2885–2893**

Transcriptional terminators of RNA polymerase II are associated with yeast replication origins

Shaoxiong Chen, Roxanne Reger, Charles Miller[1] and Linda E. Hyman*

Department of Biochemistry, SL-43, School of Medicine and [1]Department of Environmental Health Sciences, School of Public Health and Tropical Medicine, Tulane University, 1430 Tulane Avenue, New Orleans, LA 70112, USA

Received May 28, 1996; Accepted June 16, 1996

ABSTRACT

The compact organization of the *Saccharomyces cerevisiae* genome necessitates that non-coding regulatory sequences reside in close proximity to one another. Here we show there is an intimate association between transcription terminators and DNA replication origins. Four replication origins were analyzed in a reporter gene assay that detects sequences that direct 3′ end formation of mRNA transcripts. All four replication origins function as orientation-independent transcription terminators in this system, producing truncated polyadenylated mRNAs. Despite this close association, the *cis*-acting elements that confer replication origin function are genetically separable from those required for transcription termination. Several models are explored in an attempt to address how and why the signals specifying transcription termination and replication initiation overlap.

INTRODUCTION

One of the surprising results from the *Saccharomyces cerevisiae* genome sequencing project is that the density of genes on yeast chromosomes is greater than was initially estimated (1). An implication of this finding is that many of the non-coding regulatory DNA sequences must be confined within relatively short intergenic regions. One clear example of this arrangement is exemplified by the HMRE locus. At HMRE there is an association between the components of a genetic silencer and a replication origin (reviewed in 2). Thus, it is possible that there are examples of other distinct regulatory elements of the yeast genome which reside within the same DNA sequence. While attempting to identify possible sites of protein-binding in replication origin sequences by computer search, we noted that several origins contain matches to sequences that may be involved in transcriptional termination. Relatively few reports associating the sequences required for transcriptional termination with those required for DNA replication exist. One of the best studied yeast origins, ARS1, contains a termination site for the TRP1 gene transcript (3), but no conclusion regarding the generality of this coincidence has been made. Interestingly, a reported transcription termination site from a cryptic RNA polymerase II (Pol II) mRNA in the rDNA of yeast (4) maps to the region identified to contain the minimal rDNA replication origin sequence (5). Consequently, the limited biological information regarding coincidental localization of terminators and replication origins compelled us to investigate this relationship further.

The mechanism of mRNA 3′ end formation, that is, cleavage, polyadenylation and transcription termination, is conserved in eukaryotic organisms ranging from the yeast *S.cerevisiae* to mammalian cells (for reviews see 6–8). However, the sequences which direct cleavage and polyadenylation in yeast are not as conserved as in metazoans. In general, sequences that direct 3′ end formation are A–T-rich, which is not surprising given their lack of coding capacity. However the overall A–T richness of the DNA is insufficient to account for termination (9). The lack of a specific termination consensus sequence in yeast suggests that either: (i) the signals are redundant, such that there are several classes of signals which may be recognized by the processing/transcription machinery, or (ii) that other features, such as RNA secondary structure, may be important for mRNA 3′ end formation.

In an attempt to determine which *cis*-acting sequences are important for transcription termination, analysis has focused on the sequences found at the 3′ end of genes. As noted, ARSs (autonomously replicating sequences) are also found in this region of the DNA. In their native chromosomal context, only a subset of ARSs actually function in chromosomal replication (10). Of the 13 ARSs found on chromosome III, all are located in intergenic regions. This pattern is repeated for chromosome VI, where the locations of nine ARSs have been determined (11). Interestingly, the sequences of these ARSs do not preclude their presence in coding DNA, as there are often open reading frames contained within ARSs (12). Like terminators, ARSs share some limited sequence conservation. A near or perfect match to the 11 bp consensus sequence, WTTTAYRTTTW, is common to all ARSs and origin sequences (13,14). An origin recognition complex (ORC) recognizes the ARS consensus and flanking DNA and may be necessary, but not sufficient to direct ARS function (15–20). The ORC binding site extends 3′ from the consensus and partially overlaps an A–T-rich sequence of ~100 bp immediately downstream from the ARS consensus sequence. Although the ARS 3′ flanking region is not well conserved with regard to primary sequence, it is conserved with regard to function, as one ARS 3′ region can substitute for another (21). In addition, this region has the common feature of being

* To whom correspondence should be addressed

easily unwound and the ease of unwinding has been correlated with ARS replication efficiency (5,22,23). The ARS 3′ flanking region is sensitive to linker scanning mutations, but generally insensitive to point mutations (20,21). Some ARS elements contain sequences that bind transcription factors that function as replication enhancers, the most notable being the Abf1 protein (24,25). Interestingly other transcription factor binding sites, such as the GAL 4 or RAP 1 sites, can substitute for the ABF1 site (24).

Comparison of the components of ARSs with those of transcriptional terminators reveals many similarities. ARSs and transcriptional terminators share the features of A–T richness, poor sequence conservation and intergenic location. ARSs and transcription terminators may utilize these features to specify function with limited regard to primary sequence identity. The usage of such features may account for the difficulty in readily identifying the critical *cis*-acting components of replication origins and transcriptional terminators.

It has been postulated that there is a requirement for ARSs to be located in transcriptionally silent regions of the chromosome (for review see 12). In support of this hypothesis, several groups have demonstrated that transcription from a strong promoter into ARS1 impairs its function (3,26,27). Interestingly, when ARS1 is downstream of the GAL 1 promoter, transcripts end within the ARS, suggesting a dual role in directing both transcription termination and plasmid replication (3). However, the mechanism of termination and how the termination signal relates to sequences known to be important for replication are unknown. In order to examine the generality of the relationship between sequences directing replication and those signaling transcription termination, we tested the ability of four ARSs to stop transcription using an *in vivo* termination assay. In the present study we demonstrate that termination activity is associated with each of the ARSs tested.

MATERIALS AND METHODS

Reagents

Culture media components were obtained from Difco. Amino acids 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X gal) and o-nitrophenyl-β-D-galactosidase (ONPG), as well as most other chemicals, were from Sigma. Restriction endonucleases, T3 and T7 RNA polymerases, *Taq* DNA polymerase, AMV reverse transcriptase, RNasin and calf intestinal alkaline phosphotase (CIAP) were obtained from Promega and used according to manufacturer's specifications. T4 DNA ligase was obtained from New England Biolabs. Sequencing was carried out using the Sequenase Kit (US Biochemicals) according to the manufacturer's instructions. Radiolabelled nucleotides were obtained from Dupont/NEN.

Plasmid constructions

The reporter plasmid, pHZ18Δ2, was constructed as described previously (28,29). pL101 was constructed as described in (30). Each of the plasmids described below were generated by cloning *Xho*I-digested, gel purified, PCR products into the *Sal*I site of pHZ18Δ2. pL601 and pL602 were derived by amplifying a 368 bp H4ARS fragment from the parent plasmid, YCpMM3, using the primers shown in Table 1. YCpMM3 is the same as the plasmid YRp14/CEN4/H4ARS described previously (31).

The plasmids pL603 and pL604 were created by the same strategy except the parental plasmid was pVHA74X36. pVHA74X36 was obtained by ligating the L74 and R36 *Bam*HI linker-deletion derivatives (32) and subcloning the resulting H4ARS derivative. The 74X36 plasmid was then subcloned as an *Eco*RI–*Hin*dIII fragment into pVHA (14). This 74X36 derivative of the H4ARS contains an internal linker deletion that removes a 33 bp region that includes the entire ARS consensus and substitutes in a 10 bp *Bam*HI linker sequence for a net deletion of 23 bp. 74X36 is ars$^-$ in high frequency transformation assays in YIp5 and pVHA (C. Miller and D. Kowalski, unpublished results). The plasmids pL701 and pL702 were derived by inserting a 293 bp fragment obtained by amplification of plasmid YCpMM1 using the primers shown in Table 1. YCpMM1 is a derivative of YRp14/CEN4 with a 1.4 kb TRP1ARS1 *Eco*RI fragment insertion (C. Miller, unpublished). The plasmids pL703 and pL704 were derived by inserting a 32 bp (5′-GTCGAGAATAATCGTTAAACGAAACTCGACAT-3′) oligonucleotide into the test vector. This oligonucleotide contains a high affinity Abf1p binding site, 5′-AATAATCATGTTAAACGAAA-3′, from the *SPT2* gene promoter (33). The plasmids pL705 and pL706 are identical to pL703 and pL704 respectively, with the exception that the oligonucleotide inserted has a double point mutation in the Abf1p binding site (5′-AATAATCATGTTAAA*GC*AAA-3′).

The plasmids pL801 and pL802 were derived by insertion of a 250 bp PCR fragment containing the ARS305 sequence obtained after amplification of the parent plasmid p305BP (23) with the primers shown in Table 1. The plasmids pL901 and pL902 were derived by inserting a 110 bp PCR fragment containing the rDNA ARS, obtained by amplification of the parent plasmid pVHAr8 (5) with the primers shown in Table 1.

The yeast integrating plasmid YIplac128 (34) was used to generate the plasmids YIpHZ, YIp701 and YIp801. The parent plasmids pHZ18Δ2, pL701 and pL801 were digested with *Eco*RI and the resulting 6 kb fragment (approximately) was gel purified. This fragment was ligated to YIplac128 that had also been digested with *Eco*RI to create the YIp derivatives.

The plasmids used to synthesize the RNA probes were derived by cloning the same PCR fragments described above into the plasmid pT7T319U (Pharmacia). The orientations were determined by DNA sequencing and the sense and anti-sense RNA probes were synthesized using either T7 or T3 RNA polymerase and [^{32}P]UTP, according to the manufacturer's instructions.

RNA preparation

Total RNA was prepared by a modification of a previously described method using glass beads and hot phenol (35). Concentrations were determined spectrophotometrically. Poly(A) RNA was isolated on oligo (dT) cellulose (Collaborative Biomedical Products). RNA was separated by electrophoresis on 1.5% agarose–formaldehyde gels and Northern analyses were carried out as described (35). Blots were exposed to autoradiographic film with an intensifying screen and also to a phosphorimaging plate and processed on a Fuji BAS1000 PhosphorImager.

Mapping polyadenylation sites

The 3′ ends of mRNAs were mapped according to a modification of the methods of Russo *et al.* (36). Briefly, 2 μg of total RNA were used in a reverse transcription reaction containing 50 pmol RT primer (Table 1), 1 mM each of the dNTPs, 10 U AMV reverse

transcriptase, 1× AMV buffer (supplied by manufacturer) and 40 U of RNAsin in a final reaction volume of 50 µl. The reaction mixture was incubated at 24°C for 5 min and then 1 h at 37°C. For the PCR reaction, 2 µl of the RT products were added to a PCR reaction mixture containing 20 pmol of each PCR primer (Table 1), 3 mM $MgCl_2$ and 2.5 U *Taq* DNA polymerase. A second round of PCR was performed using primers specific to the individual ARSs (listed in Table 1). The PCR products were gel purified and digested with *Xho*I and *Kpn*I. DNA fragments were ligated to the vector pT7T319U and sequenced.

β-galactosidase plate assay

Cells were grown on complete media plates without uracil and with galactose. Plates were overlaid with a 4% molten agar solution containing 0.1% SDS and 0.02% X-gal, and monitored for the development of blue color.

Quantitative assay of β-galactosidase activity

Yeast cultures were grown in complete medium lacking uracil with galactose as a carbon source to an OD_{600} of 1.0. Cells were harvested from 5 ml cultures by centrifugation and crude extracts were assayed for β-galactosidase activity using ONPG as the substrate (37). Protein concentrations were determined by a Bradford assay (BioRad). At least two colonies from each strain were assayed in duplicate. The results of the trials are presented as the mean ± standard deviation of β-galactosidase specific activity in nmol/min/mg protein.

RESULTS

Termination activity of ARSs

In order to assay for sequences that signal transcription termination, a reporter plasmid was used which allowed us to correlate β-galactosidase activity as a measure of 3′ end formation *in vivo* (30). The salient features of this plasmid, pHZ18Δ2, are shown schematically in Figure 1. The key component of the vector is a fusion gene which consists of part of the ribosomal protein (rp) 51A gene fused in frame to the lacZ gene. The fusion gene is controlled by the GAL upstream activating sequence (UAS). When cells are grown on galactose the fusion gene is transcribed, the pre-mRNA is spliced and the mRNA translated to produce β-galactosidase.

Insertion of a sequence that directs transcription termination, polyadenylation or both into the intron prevents the completion of full length transcripts and thus reduces or abolishes β-galactosidase activity. This is demonstrated by the plasmid pL101, that carriers the 3′ end of the *ADH2* gene inserted into the intron of the rp 51A gene of pHZ18Δ2 (30). When this plasmid is introduced into yeast cells, very low levels of β-galactosidase activity are produced in comparison with the vector without an insert. Analysis of mRNA isolated from this strain confirms that the absence of β-galactosidase activity is due to the presence of a truncated mRNA whose 3′ end maps within the *ADH2* termination sequence, upstream of the lacZ gene. Thus, measuring β-galactosidase activity in strains containing plasmids that have terminators inserted in the intron of pHZ18Δ2 serves as a convenient and simple means of assessing the ability of a given sequence to terminate transcription *in vivo*.

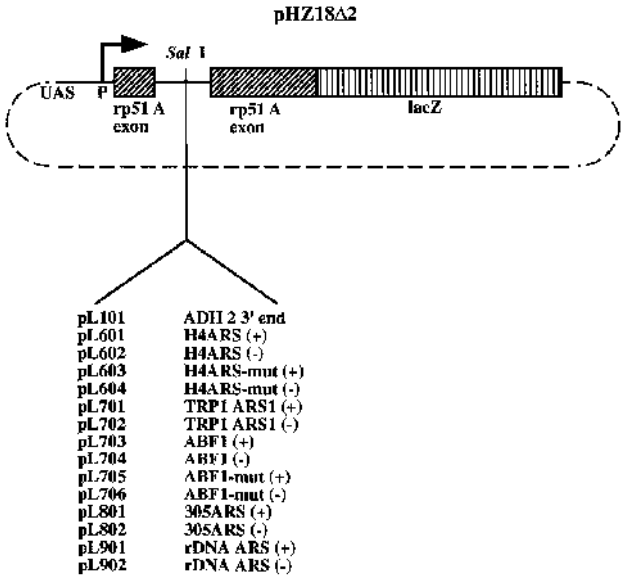

Figure 1. Schematic representation of the transcription termination reporter plasmid. The pHZ18Δ2 plasmid contains the GAL UAS upstream from the *CYC1* promoter (P) that drives transcription of the ribosomal protein 51A–lacZ fusion gene. A unique *Sal*I site in the intron of the ribosomal protein gene was used to insert ARSs, and other related sequences, into the reporter construct.

In order to establish the ability of ARSs to act as transcriptional terminators, we first focused on the sequence containing the H4ARS. This ARS is located 3′ from the copy I histone H4 gene, just downstream of the natural polyadenylation site (32,38). The ARS sequence we examined does not contain the sequences that specify where the poly(A) tail is added on the histone H4 gene mRNA. Any termination activity observed from this construct is not due to the presence of the H4 mRNA 3′ end formation signals. A 368 bp H4ARS fragment was amplified by PCR using the primers indicated in Table 1. The H4ARS PCR fragment was inserted into the reporter plasmid in both orientations to create pL601 (+) and pL601 (–). The (+) orientation refers to the T-rich ARS Consensus Sequence (ACS) on the non-transcribed strand, while the reverse orientation (–) refers to the T-rich ACS on the transcribed strand. The ARS orientation was determined by restriction digestion and/or DNA sequencing. Both plasmids were individually introduced into yeast. Individual transformants were grown in medium containing galactose as a carbon source, in order to induce transcription from the reporter gene construct. β-galactosidase activity was first assessed using a plate assay which demonstrated that the pL601 and pL602 plasmids produced little or no β-galactosidase, as the colonies remained white on plates containing X-gal. This result was confirmed by utilizing a quantitative assay which demonstrated that β-galactosidase activity was <1% of the level observed for the pHZ18Δ2. Little, if any, read-through transcription of the ARS insert was observed. This demonstrates that efficient transcription termination is caused by the H4ARS sequence in either orientation. These results are summarized in Figure 2 and Table 2.

Table 1. Summary of oligonucleotides used as primers

(**A**) Primers used to generate the ARS-containing reporter plasmids

Construct	5′ primer	3′ primer	Parent plasmid	Fragment size
pL601	CCCGCTCGAGCGCCAACAAATACTACC	CCCGCTCGAGTCGCTTGCCTGTAAC	YCpMM3	368 bp
pL602	same	same	same	same
pL603	same	same	p74x36 H4ARS	same
pL604	same	same	same	same
pL701	CCCGCTCGAGCTGGTGGACTGACGCC	CCCGCTCGAGACAATCAATCAAAAAGCC	YCpMM1	293 bp
pL702	same	same	same	same
pL703	GTCGAGAATAATCATGTTAAACGAAACTCGAC	GTCGAGTTTCGTTTAACATGATTATTCTCGAC	N/A	32 bp
pL704	same	same	same	same
pL705	GTCGAGAATAATCATGTTAAAGCAAACTCGAC	GTCGAGTTTGCTTTAACATGATTATTCTCGAC	N/A	32bp
pL706	same	same	same	same
pL801	CCCGCTCGAGCGGCCAGTTTGAATGC	CCCGCTCGAGGCCCCCCGTGTAAGTTAC	p305BP	250 bp
pL802	same	same	same	same
pL901	CCCGCTCGAGCCATTTTGATTGTTTATG	CCCGCTCGAGCTTAACTATTCTATG	pVHAr8	110bp
pL902	same	same	same	same

(**B**) Primers used in RT–PCR for fine structure mapping of 3′ ends (**Q**, Randomized at this position with A/C/G; #, Randomised at this position with A/C/G/T)

Reverse transcriptase primer	GACGAGCGGTACCTCTGCAGTTTTTTTTTTTTTTTT**Q#**
PCR 5′ primer	GGCGCGCTCGAGGGAGGCTTTTAAGGACACG
PCR 3′ primer	GACGAGCGGTACCTCTGCAGTTTTTT

The sequences are shown in a 5′→3′ orientation.

Table 2. β-galactosidase activity in strains containing the reporter plasmids

Plasmid	Terminator	β-gal units	Plate assay
pHZ18Δ2	vector	n/a	blue
pL101	ADH 2 3′ end	4 ± 1.8 (259 ± 133)	white
pL601	H4ARS (+)	1 ± 0.6 (512 ± 52.1)	white
pL602	H4ARS (–)	1 ± 0.2 (512 ± 52.1)	white
pL701	Trp1ARS1 (+)	21 ± 6.5 (54 ± 45.6)	lt blue
pL702	Trp1ARS1 (-)	5 ± 2.0 (54 ± 45.6)	lt blue
pL801	ARS 305 (+)	2 ± 0.1 (251 ± 5.1)	white
pL802	ARS 305 (–)	30 ± 2.0 (251 ± 5.1)	white
pL901	rDNA ARS (+)	155 ± 2.0 (249 ± 6.8)	lt blue
pL902	rDNA ARS (–)	153 ± 3.0 (249 ± 6.8)	lt blue

The plasmid names and the termination sequence inserted into the parent vector pHZ18Δ2 are as indicated. The β-galactosidase activity is calculated as specific activity normalized to protein concentration. Each strain was measured at least four times and the numbers shown represent the mean± the standard deviation. The last column displays the results of a colorimetric assay in which yeast strains, grown on plates containing galactose as the carbon source, were overlaid with an X-gal mixture.

A prediction of the hypothesis that β-galctosidase activity is a reflection of the RNA synthesized from the reporter gene is that the RNA produced from these strains should contain a truncated transcript that ends within the H4ARS sequence. Northern blot analysis was performed to assess the presence of such transcripts. In order to test both the pL601 and the pL602 plasmids, RNA probes were synthesized for both strands of the corresponding transcripts. However, only one orientation of the H4ARS should

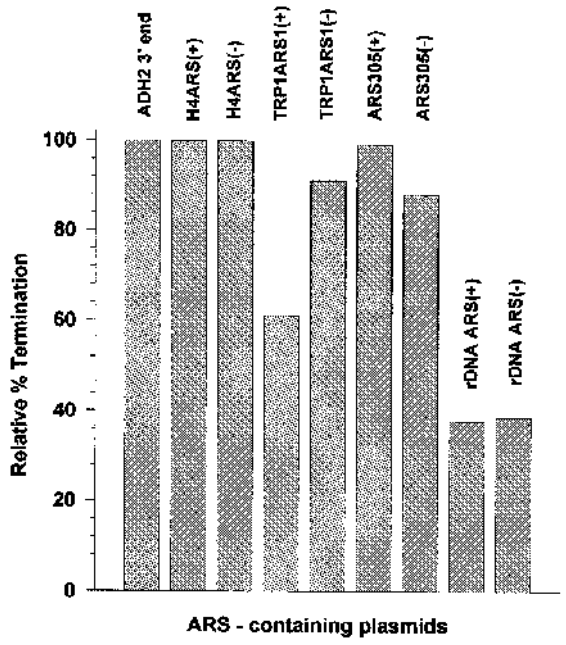

Figure 2. ARSs are associated with transcriptional terminators. The level of β-galactosidase produced in strains containing the test plasmids were compared with the pL101 strain, containing the *ADH2* termination sequence, and to the plasmid without a terminator (pHZ18Δ2). The results are expressed as a relative percentage of the termination activity of the vector without a terminator.

hybridize to the sense probe, while the opposite orientation H4ARS transcripts should be positive for hybridization with the anti-sense probe. The data in Figure 3A shows the presence of a small transcript containing H4ARS sequence, in an orientation-dependent fashion. Thus, only pL601 demonstrated positive hybridization for the low molecular weight RNA with the probe in the (+) orientation (lanes 1 and 2) while the pL602 plasmid shows hybridization to the probe made of RNA from the anti-sense strand (lanes 3 and 4). The remaining signal observed is independent of the probe used and reflects non-specific hybridization to high molecular weight transcripts, most likely rRNAs.

As all mRNAs are polyadenylated in yeast, we next asked if the truncated RNA fragment contained a poly(A) tail. Thus, total RNA from strains containing pL601 was fractionated on oligo d(T) cellulose and analyzed by subsequent Northern analysis. The results shown in Figure 3B demonstrate that most of the RNA containing the H4ARS sequence has a poly(A) tail (compare lane 1 with lane 2). The RNA remaining in the poly(A)$^-$ fraction probably results from incomplete fractionation rather than the absence of poly(A).

We examined next whether other ARSs could terminate transcription in a manner analogous to the H4ARS. We chose to look at three other ARSs—ARS1, ARS305 and the rDNA ARS. ARS1 is an efficient replication origin that has previously been shown to interfere with transcription when present in the (–) orientation (3,26). We confirmed this observation in our assay system by introducing ARS1 into the reporter construct to create plasmids pL701 and pL702 (Table 1). Termination activity was determined using the assays described above. The data presented in Table 2 confirms the results of Tanaka *et al.* (3) who also demonstrated termination activity in a 1453 bp fragment of DNA containing ARS1. Additionally these results show that this ARS sequence can direct proper 3′ end formation in an orientation-independent manner. In the case of ARS1, the termination activity is stronger in the (–) orientation than in the (+) orientation. In general, the ARS1 fragment we tested is a less efficient terminator than the H4ARS fragment. The results with β-galactosidase were confirmed by Northern analysis that demonstrated that transcription terminated within the ARS1 fragment (Fig. 3A, lanes 5–8).

ARS305 is a very efficient replicator in a plasmid context and acts as an origin of replication on the chromosome (20,23). A 250 bp ARS305 fragment was amplified using the primers shown in Table 1 and cloned into pHZ18Δ2. Both orientations were isolated, and the resulting plasmids are referred to as pL801 (+) and pL802 (–). Similar to H4ARS and ARS1, ARS305 terminated transcription in an orientation-independent manner. As with the H4ARS, the ARS305 fragment was very efficient at directing transcription termination, as the β-galactosidase levels were very low (0–12%) compared with the parent reporter construct. These data are summarized in Figure 2 and Table 2. Northern blot analysis confirmed that transcription from the CYC 1 promoter terminates within the ARS305 insert (Fig. 3A, lanes 9–12).

Finally, we examined the effectiveness of a 110 bp fragment containing the rDNA ARS as a transcriptional terminator. Notably, the rDNA locus is transcribed by RNA Pol I and Pol III between the divergently oriented 37S and 5S RNAs (39). A sequence that resembles that of other terminators has been identified in the rDNA ARS region on chromosome XII. This terminator may actually function *in vivo* because a Pol II transcript termination site has been mapped to the rDNA ARS (4). The Pol II transcript in the rDNA appears to be a source of 37S rRNA in petite strains of yeast (40). Thus, if it is some characteristic of the ARS that confers termination function, this ARS should affect the process in much the same way as the ARSs found associated with Pol II transcripts. It is noteworthy that the rDNA ARS does not contain sequences that would suggest its functioning as a Pol I terminator (41).

The target fragment was amplified and cloned into pHZ18Δ2 as described above and the plasmids pL901 (+) and pL902 (–) were transformed into yeast. As with the other ARSs, we observed transcription termination by the β-galactosidase assay (Table 2), and confirmed this phenotype by Northern blot analysis that demonstrated the presence of a terminated transcript (Fig. 3A, lanes 13–16). The relative level of termination in pL901 and pL902 is less than the other ARSs tested and the *ADH2* control terminator. However, variation in termination efficiency is common among different terminators and the termination efficiency reported in this study is similar to some known terminators (42). It has been suggested that terminators that work in an orientation independent manner are often less efficient than those that work in one orientation only (43,44). However, we find that with the ARS/terminators, orientation dependency is not a valid indicator of termination efficiency. There is also no correlation between efficiency in replication initiation and efficiency of termination. ARS1 is an efficient replicator in a plasmid context and on chromosome IV, yet it is not as efficient in termination as ARS305 and the H4ARS (21,24). Conversely the rDNA ARS is not an efficient replicator (5) nor is it an optimal terminator.

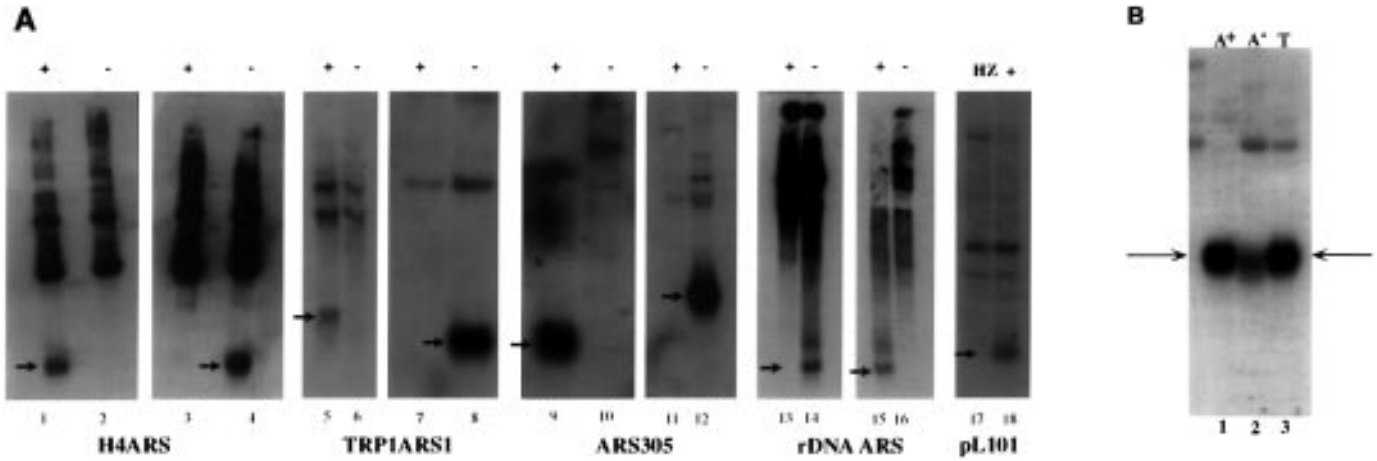

Figure 3. Northern blot analysis of ARS/terminator strains. RNA was isolated from strains harboring the different ARS-containing reporter plasmids, as indicated. (**A)** Autoradiograph of total RNA hybridized to either a sense (+) or an antisense (–) RNA probe. As each ARS is present in the plasmid in one orientation, only one of the probes should be complementary to a given ARS-containing RNA. Lanes 1, 3, 5, 7, 9, 11, 13 and 15 contain the ARS in the (+) orientation, while lanes 2, 4, 6, 8, 10, 12, 14 and 16 contain the ARS in the (–) orientation. The arrows point to the truncated RNA product representing the terminated transcript within the intron. The RNA derived from the pL101 (lane 18) and pHZ18Δ2 (lane 17) strains were hybridized to an *ADH2* antisense probe. (**B**) RNA from the pL601 strain (containing the H4ARS) was fractionated into poly(A)-containing (lane 1) and poly(A)-lacking RNA (lane 2) and examined using an antisense (–) H4ARS RNA probe. Unfractionated total RNA is shown in lane 3. Arrows indicate the truncated ARS-containing RNA.

Table 3. β-galactosidase activity in strains containing reporter plasmids that contain ARS mutations and associated sequences

Plasmid	Terminator	β-gal units	Plate assay
pHZ18Δ2	vector	n/a	blue
pL101	ADH 2 3′ end	4 ± 1.8 (259 ± 133)	white
pL603	H4ARS mut (+)	0.4 ± 0.3 (249 ±6.3)	white
pL604	H4ARS mut (–)	0.4 ± 0.3 (249 ± 6.3)	white
pL703	ABF1 (+)	186 ± 7.5 (249 ± 6.8)	blue
pL704	ABF1 (–)	176 ± 6.1 (249 ± 6.8)	blue
pL705	ABF1 mut (+)	183 ± 0.6 (249 ±6.8)	blue
pL706	ABF1 mut (–)	190 ± 18 (249 ± 6.8)	blue

The plasmid names and the termination sequence inserted into the parent vector pHZ18Δ2 are as indicated. The β-galactosidase assays were performed as described for Table 2.

Analysis of ARS sequences for termination

To determine those characteristics of the ARS that cause transcription termination, we examined the termination phenotype of a mutant version of the H4ARS [pL603 (+) and pL604 (–)]. The 74X36 mutation consists of *Bam*HI linker insertion/deletion that alters a 33 bp region that includes the entire ACS, which is responsible for the binding of the large protein complex required for replication (ORC). Deletion of this sequence completely abolishes ARS function (C. Miller and D. Kowalski, unpublished). One possible mechanism for ARSs that act as terminators is that upon ORC binding, a roadblock to transcription is established, such that the transcription complex cannot proceed through an ARS sequence, or its movement is somehow impaired. This type of termination mechanism may be mediated by a specific DNA–protein interaction that has been shown to be operative in the adenovirus major late promoter transcription unit where the CCAAT binding interaction is important for termination (45). As ORC is thought to be bound tightly to the DNA throughout most, if not all of the cell cycle, a roadblock mechanism is viable (46). However, we found that the mutated H4ARS sequence is fully functional with regard to transcription termination, despite its inability to function as a replication origin (Fig. 4). As shown in Table 3, there is no difference in β-galactosidase activity when comparing the ars$^-$ mutant versus the wild-type H4ARS. In addition, the orientation of the ars$^-$ derivative has no effect on termination function. Thus, an ORC roadblock alone does not appear to be responsible for transcription termination.

The roadblock mechanism might be mediated by DNA–protein interactions other than ORC. With this in mind, we further investigated the mechanism of termination function by focusing

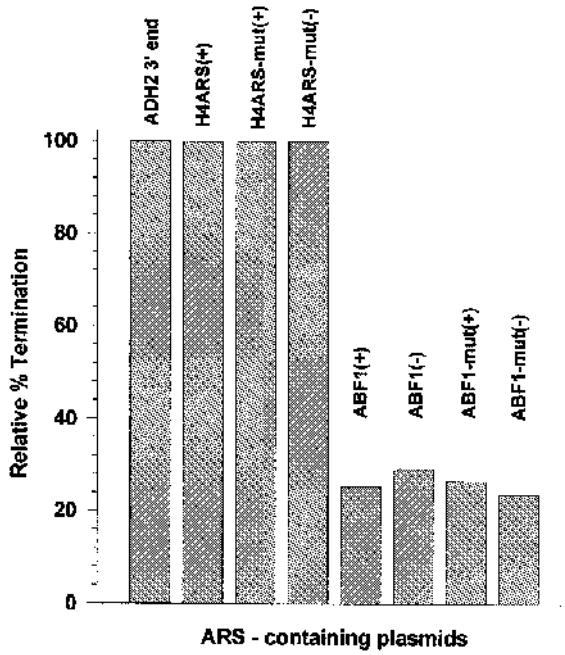

Figure 4. Effect of ARS mutations and associated sequences on transcription termination. The level of β-galactosidase produced in strains containing the test plasmids was compared with the pL101 strain, containing the *ADH2* termination sequence. The results are expressed as a relative % of the termination activity compared with the vector.

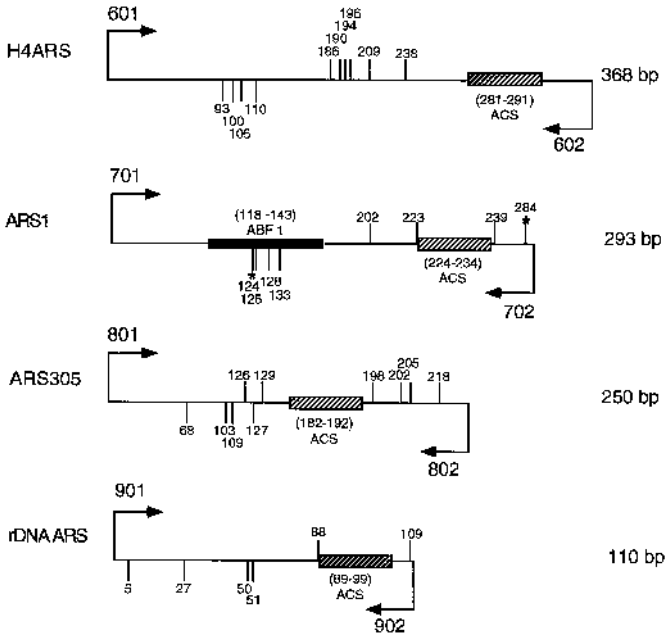

Figure 5. Fine structure mapping of polyadenylation sites. The exact position of polyadenylation sites were determined for each of the ARS/terminator constructs, in both the (+) and the (–) orientations (see text), and are as indicated. The poly(A) sites are indicated by a vertical line. The numbering is in the 5′→3′ direction relative to the (+) orientation. The arrows depict the direction of transcription. The position of the 11 bp ARS concensus sequences (ACS) is indicated by the striped box. The T-rich strand of the ACS is on the bottom stand and the 'B' domain is to the left of the ACS as depicted here. The Abf1p binding site is shown for the ARS1 construct (solid box). The * marks the position of previously identified polyadenylation sites in ARS1.

on ARS1 because the ARS1 sequence is one of the ARSs that has been extensively analyzed by linker scanning analysis (24). In addition to the 11 bp ACS, there is a region of DNA 3′ to the ACS, called the B region, which has been further defined as B1, B2 and B3 elements. The transcription factor Abf1p binds at the B3 region. Tanaka and co-workers showed that when ARS1 is placed downstream of a promoter, transcripts terminated in the B3 region (3). This is consistent with a model that suggests a role for Abf1p in the termination mechanism. Although not all ARSs contain Abf1p binding sites, other unidentified transcription factor binding sites may be present as a general feature of ARS composition. Interestingly, the transcription factor, Reb1p, has been shown to play a role in transcription termination of RNA Pol I (47). Therefore the presence of the Abf1p binding site in the ARS sequences led us to examine the role of Abf1p in transcription termination of RNA Pol II. A 32 bp double-stranded oligomer containing a high affinity Abf1p binding site (see Methods) was inserted into the intron of pHZ18Δ2, to create plasmids pL703 (+) and pL704 (–). After introduction into yeast cells, we determined that the Abf1p site caused a small reduction in β-galactosidase activity (Table 3). The slight increase in termination caused by the Abf1 site is probably not due to binding, as an inactivated version of this construct (pL705 and pL706), containing a double point mutation in the Abf1p binding site which does not bind Abf1p (33), also showed similar levels of activity (Table 3). Therefore, although a previous study suggested that a protein–DNA mediated by Abf1p binding may be important for terminating transcription, we show here that the Abf1 site acts weakly as a terminator in a manner that is independent of Abf1p binding. From this we conclude that the Abf1p binding to DNA is insufficient by itself to create a roadblock to transcription.

The Northern blot data shown in Figure 3 allows us to conclude that termination occurs within the ARSs tested. However, a more detailed analysis of the polyadenylation site(s) might help elucidate the relationship between the sequences important for ARS function and the sequences specifying 3′ end formation. To address this issue we examined the polyadenylation sites of all four ARS sequences, in both the (+) and the (–) orientations. This was accomplished by sequencing cDNA clones derived from reverse transcription and PCR amplification of RNAs derived from each of the test strains. The results are depicted in Figure 5. The polyadenylation sites are heterogeneous. In general, termination occurs 3′ to the ACS when the inserts are present in the (–) orientation, but appear to be less specific in the constructs in which the ARS is in the (+) orientation. However, in all cases poly(A) sites never map within the ACS itself. In the case of the TRP1ARS1 terminator (pL702) there is a notable preference for termination within the B3 sequence. This result confirms the observation of Tanaka *et al.*, and thus validates this method for mapping the 3′ ends of mRNAs. No obvious correlation between the sequence elements defined for ARS305 function (20) and poly(A) sites in the pL801 and pL802 constructs is apparent. An additional aspect of this study points to the heterogeneity of poly(A) site selection, which has also been observed for other yeast genes (9,42).

The experiments presented above were designed to assess the termination function of the ARS sequence. The additional question remains, however, whether these ARSs can function in replication when present in the context of the reporter gene. The plasmids used in the termination studies contain the 2μ plasmid origin and therefore are not solely dependent on the ARSs within the intron for replication. Thus, a fragment containing the reporter gene and flanking sequences was introduced into the yeast integration plasmid, YIplac128 (34). This plasmid cannot replicate in yeast cells in the absence of a functional ARS. Using a transformation efficiency assay we determined that cells containing the reporter plasmid without an ARS sequence (YIpHZ) did not grow, while cells containing the YIp plasmid and the reporter gene with either the 305ARS (YIp801) or the TRP1 ARS1 (YIp701) were efficiently transformed (data not shown). Thus, these ARSs are functional in an intronic context within a gene.

DISCUSSION

In higher eukaryotes the signal(s) that govern the positioning of the 3′ end of an mRNA molecule are simple and well defined. They consist of the highly conserved hexamer AAUAAA positioned between 10 and 30 nt upstream of the polyadenylation site, and a less well defined downstream region that is usually rich in G and/or U residues (for review see 6,8). The signals for the same process in *S.cerevisiae* are less well defined due to the apparent complexity or redundancy of the sequences involved, although the mechanism of 3′ end formation is conserved. In an attempt to define the sequence characteristics that are important for transcription termination and polyadenylation, we designed an assay to test the ability of specific sequences to direct the termination/polyadenylation reaction (30). In this study we present evidence that within or near the same sequences that direct DNA replication, there is also a separable *cis*-acting element (or elements) that directs transcription termination.

The proximity of the sequences that direct DNA replication and transcription termination is intriguing. At least three explanations for this relationship are possible. The first is that the placement of the signals found within these relatively short regions of the DNA is coincidental and the relationship is insignificant. We consider this unlikely as both sequences can be accomodated within the space provided in a typical intergeneic region. Although formally we have not ruled out this explanation, it is the least interesting of the possibilities, as it lends no insights as to the nature of either a termination or replication signal. The fact that all four of the ARSs we tested contain termination activity argues against a chance co-localization of ARSs and terminators.

The second possibility is that transcription termination and initiation of replication are functionally interrelated. For example, it has been noted that ARSs are located only in nontranscribed regions of the chromosome (12), suggesting this may be a prerequisite for ARS function. Thus, one way to ensure that the ARS region remains transcriptionally inactive is to build termination signals in or around ARSs. It follows that mutations that disturb termination function should also interfere with ARS function. We have isolated termination mutants with the ARS305 sequence but do not yet know if these mutants will have an effect on replication (unpublished results). As termination signals are generally very ill defined it is difficult to assess whether ARS associated termination signals will be different from non-ARS terminators. For example, it remains to be established if there are multiple termination signals or a single signal associated with the ARSs we have examined [with the exception of the H4ARS, which we know has multiple termination functions, i.e., those adjacent to the fragment we examined that are associated with histone H4 transcripts (38)]. If terminators are reiterated to create a transcript-free chromatin organization at replication origins then combined mutagenesis of the redundant terminators to give full transcription through the ARS may be required to visualize replication defects. Interestingly, a similar model of multiple termination signals surrounding yeast centromeric sequences has been proposed (48), and a transcriptional terminator has been mapped to the promoter region of the *URA3* gene (49). Thus, transcriptional terminators may generally be associated with several regulatory sequences in yeast.

It is much less likely that ARS function is required to terminate transcription, as there are far more terminators in the genome than there are ARSs. However, it is possible that different mechanisms may be responsible for termination at different sites, and that ARS-associated termination could represent a particular class of terminator. It is noteworthy that each of the ARSs we have examined terminate transcription in an orientation-independent fashion. It has been suggested that terminators that operate in either orientation constitute a class of terminators that is distinct from those that work in an orientation-dependent manner (42,43). Thus the idea that the differences between these classes could be due to the presence or absence of an ARS is intriguing. Our data eliminate the possibility that ARS function is required for termination, as severe mutations in the ACS have no apparent effect on termination. In addition, the possibility that a simple roadblock mechanism is responsible for the termination activity by ORC or by Abf1p is unlikely. We have not excluded the possibility that Abf1p binding is necessary, but not sufficient, to cause transcription termination. This possibility should be considered in light of what is known about Pol I termination where the transcriptional activator Reb1p plays an important role in stopping transcription, but Reb1p binding alone does not account for the termination effect (47). In fact, replacement of Reb1p with an unrelated DNA binding protein, the lac repressor, also contributed to termination of a yeast Pol I transcript *in vitro* (50). The authors propose that Pol I termination occurs as a result of two stepwise events, requiring the polymerase to pause in the proximity of a release element. Reb1p binding serves as a mediator of the pausing, but the lac repressor can functionally replace the endogenous termination factor.

The third explanation for the proximity of the ARS/terminator signals is that the same features required for initiation of replication may also be important for termination of transcription. For example, the ease of DNA unwinding is an important feature of an ARS (22). It is possible that this same characteristic is important in terminators, and therefore both types of signals have evolved from the same region of the chromosome. Why or how unwinding might facilitate termination is unclear, however several possibilities exist. As the ease of unwinding reflects the strength of DNA–DNA strand interactions, it may also affect the strength of a potential RNA–DNA hybrid and/or intramolecular base pairing and RNA secondary structure formation. The sequence composition, reflected in the overall A–T-richness of the DNA, is not sufficient to direct termination (9). Another characteristic of some ARSs is the presence of a sequence which causes DNA to bend (51). In ARS1, a bent DNA sequence is

found in the B3 region, which is also the region that contains the ABF1 binding site (3). However, the bending function in ARS1 can be eliminated without affecting replication efficiency (24). As DNA bending has also been implicated in intrinsic termination (52), it is possible that this feature is critical in directing termination, but unimportant in replication. Thus, it is not yet fully clear which characteristics of the DNA account for triggering either the initiation of replication or transcription termination in the yeast genome. Further experiments may reveal which, if any, of the components within these short ARS sequences we examined are actually utilized to specify a termination signal.

ACKNOWLEDGEMENTS

We would like to thank Claire Moore, Arthur Lustig, Dean Dawson and William H. Baricos for helpful comments on the manuscript. This work was supported by grants from the NSF (MCB9316701) and the American Cancer Society (JFRA-500) to L.E.H. and to the Tulane Cancer Center for support of S.C.

REFERENCES

1 Oliver, S.G., van der Aart, Q.J.M., Agostoni-Carbone, M.L., Aigle, M., Alberghina, L., Alexandraki, D., Antoine, G., Anwar, R., Ballesta, J.P.G., *et al*. (1992) *Nature* **357**, 38–46.
2 Dillin, A. and Rine, J. (1995) *TIBS* **20**, 231–235.
3 Tanaka, S., Halter, D., Livingstone-Zatchej, M.J., Reszel, B. and Thoma, F. (1994) *Nucleic Acids Res.* **22**, 3904–3910.
4 Parikh,V.S., Conrad-Webb, H., Docherty, R. and Butow, R.A. (1989) *Mol. Cell. Biol.* **9**, 1897–1907.
5 Miller, C.A. and Kowalski, D. (1993) *Mol. Cell. Biol.* **13**, 5360–5369.
6 Wahle, E. (1995) *Biochem. Biophys. Acta* **1261**, 183–194.
7 Proudfoot, N.J. (1989) *TIBS* **14**, 105–110.
8 Keller, W. (1995) *Cell* **81**, 829–832.
9 Heidmann, S., Obermaier, B., Vogel, K., and Domdey, H. (1992) *Mol. Cell. Biol.* **12**, 4215–4229.
10 Newlon, C.S., Collins, I., Dershowitz, A., Deshpande, A.M., Greenfeder, S.A., Ong, L.Y. and Theis, J.F. (1993) *Cold Spring Harbor Sym. Quant. Biol.* **58**, 415–424.
11 Shirahige, K., Iwasaki, T., Rashid, M.B., Ogasawara, N., and Yoshikawa, H. (1993) *Mol. Cell. Biol.* **13**, 5043–5056.
12 Brewer, B.J. (1994) *Curr. Opn. Genet. Dev.*. **4**, 196–202.
13 Broach, J.R., Li, Y.Y., Feldman, J., Jayaram, M., Abraham, J., Nasmyth, K.A. and Hicks, J.B. (1983) *Cold Spring Harbor Symp. Quant. Biol.* **47**, 1165–1173.
14 Van Houton, J.V. and Newlon, C.S. (1990) *Mol. Cell. Biol.* **10**, 3917–3925.
15 Bell, S.P. and Stillman, B. (1992) *Nature* **357**, 128–134.
16 Bell, S.P., Kobayashi, R. and Stillman, B. (1993) *Science* **262**, 1844–1849.
17 Foss, M., McNally, F.J., Laurenson, P. and Rine, J. (1993) *Science* **262**, 1838–1844.
18 Li, J.J. and Herskowitz, I. (1993) *Science* **262**, 1870–1874.
19 Micklem, G., Rowley, A., Harwood, J., Nasmyth, K. and Diffley, J.F.D. (1993) *Nature* **366**, 87–90.
20 Huang, R.Y. and Kowalski, D. (1996) *Nucleic Acids Res.* **24**, 816–823.
21 Rao, H., Marahrens, Y. and Stillman, B. (1994) *Mol. Cell. Biol.* **14**, 7643–7651.
22 Natale, D.A., Umek,, R.M. and Kowalski, D. (1993) *Nucleic Acids Res.* **21**, 555–560.
23 Huang, R.Y. and Kowalski, D. (1993) *EMBO J.* **12**, 4521–4531.
24 Marahrens, Y. and Stillman, B. (1992) *Science* **255**, 817–823.
25 Walker, S.S., Malik, A.K. and Eisenberg, S. (1991) *Nucleic Acids Res.* **22**, 6255.
26 Snyder, M., Sapolsky, R.J. and Davis, R.W. (1988) *Mol. Cell. Biol.* **8**, 2184–2194.
27 Russo, P. and Sherman, F. (1989) *Proc. Natl Acad. Sci. USA* **86**, 8348–8352.
28 Teem, J.L. and Rosbash, M. (1983) *Proc. Natl Acad. Sci. USA* **80**, 4403–4408.
29 Pikielny, C.W., Teem, J.L. and Rosbash, M. (1983) *Cell* **34**, 395–403.
30 Hyman, L.E., Seiler, S.H., Whoriskey, J. and Moore, C.L. (1991) *Mol. Cell. Biol.* **11**, 2004–2012.
31 Hieter, P., Mann, C., Snyder, M. and Davis, R. (1985) *Cell* **40**, 381–392.
32 Bouton, A.H. and Smith, M.M. (1986) *Mol. Cell. Biol.* **6**, 2354–2363.
33 Buchman, A.R. and Kornberg, R.D. (1990) *Mol. Cell. Biol.* **10**, 887–897.
34 Gietz, R.D. and Sugino, A. (1988) *Gene* **74**, 527–534.
35 Ausubel, F.M. *et al*. (1987) *Current Protocols in Molecular Biology.* John Wiley & Sons, Inc., NY.
36 Russo, P., Li, W-Z., Hampsey, D.M., Zaret, K.S. and Sherman, F. (1991) *EMBO J.* **10**, 563–571.
37 Rose, M.D., Winston, F. and Hieter, P. (1988) *Laboratory Course Manual for Methods in Yeast Genetics.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
38 Smith, M.M. and Andresson, M. (1983) *J. Mol. Biol.* **169**, 663–690.
39 Warner, J. (1989) *Microbiol. Rev.* **53**, 271.
40 Conrad-Webb, H. and Butow, R. (1995) *Mol. Cell. Biol.* **15**, 2420–2428.
41 Reeder, R.H. and Lang, W. (1994) *Mol. Micro.* **12**, 11–15.
42 Irniger, S., Egli, C.M. and Braus, G.H. (1991) *Mol. Cell. Biol.* **11**, 3060–3069.
43 Egli, C., Springer, C. and Braus, G.H. (1995) *Mol. Cell. Biol.* **15**, 2466–2473.
44 Irniger, S., Sanfacon, Egli, C.M. and Braus, G.H. (1992) *Mol. Cell. Biol.* **12**, 2322–2330.
45 Connelly, S. and Manley, J. (1989) *Mol. Cell. Biol.* **9**, 5254–5259.
46 Diffley, J.F. and Cocker, J.H. (1992) *Nature* **357**, 169–172.
47 Lang, W.H., Morrow, B.E., Ju, Q., Warner, J. and Reeder, R.H. (1994) *Cell* **79**, 527–534 .
48 Hegemann, J.H. and Fleig, U.N. (1993) *Bioessays* **15**, 451–460.
49 Yarger, J.H., Armilei, G. and Gorman, M.C. (1986) *Mol. Cell. Biol.* **9**, 2431–2444.
50 Jeong, S.W., Lang, W.H. and Reeder, R.H. (1995) *Mol. Cell. Biol.* **15**, 5929–5936.
51 Snyder, M., Buchman, A.R. and Davis, R.W. (1986) *Nature* **324**, 87–89.
52 Reines, D., Wells, D., Chamberlin, M. and Kane, C.M. (1988) *J. Mol. Biol.* **196,** 299–312.