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Mutational analysis of a yeast transcriptional terminator

(linker substitution/*Saccharomyces cerevisiae*)

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ABSTRACT **We have isolated and mutagenized a DNA fragment from *Saccharomyces cerevisiae* that specifies mRNA 3′ end formation for the convergently transcribed *CYC1* and *UTR1* genes. An *in vivo* plasmid supercoiling assay previously showed that this fragment is a transcriptional terminator, and "run-on" assays shown here are consistent with this interpretation. The poly(A) sites in the mRNAs formed by the fragment are the same whether the fragment resides at the native location or at a heterologous location. No single linker substitution abolishes the fragment's activity, whereas certain large, nonoverlapping deletions have strong, deleterious effects. Therefore, the yeast terminator behaves more like *rho*-dependent bacterial terminators than terminators of higher eukaryotes. That a number of deletions or substitutions have different effects in the two orientations suggests that the fragment contains the sequences of two, unidirectional terminator elements.**

The primary structure of the eukaryotic mRNA is due to precise sites of transcriptional initiation and RNA processing events, including splicing and end formation (reviewed in refs. 1–4). The clearest models of mRNA 3′ end formation emerge from genetic and biochemical experimentation in metazoan cells. Three general events have been described. (*i*) Transcription by RNA polymerase II appears to terminate hundreds of base pairs downstream from the eventual junction of the template-derived RNA and the poly(A) tract (5–8). DNA fragments from those downstream regions that act as terminators have been isolated, but their fine structure and their mode of action has not yet been elucidated (9). (*ii*) The sequence AAUAAA in the RNA directs cleavage 10–30 bases 3′ to the hexanucleotide element (10–15). Extensive mutagenesis of this sequence and its immediate environment indicates that AAUAAA is essential for proper processing and that sequences immediately 3′ to the cleavage site are also required (16–19). (*iii*) The cleaved RNA serves as a substrate for processive polyadenylylation (20, 21). Macromolecular complexes that carry out the latter two reactions have been isolated, and small nuclear ribonucleoproteins are thought to participate (12, 21, 22). Remarkably, evidence has been provided that indicates that all of these processes are coupled *in vivo*, as mutations within the AATAAA strongly decrease the efficiency of distant transcriptional termination (23–25).

How similar is 3′ end formation in yeast to what occurs in higher cells? The consensus element TAG. . .TAGT. . .TTT has been proposed as a key element in termination for many genes, including *CYC1* (iso-1-cytochrome *c*) of *Saccharomyces cerevisiae* (26). To begin an analysis of termination in yeast, we chose to study an 83-base-pair (bp) fragment past the 3′ end of *CYC1* that includes the consensus element. *CYC1* and an adjacent gene, *UTR1*, are convergently transcribed and 3′ ends for both mRNAs fall within the 83-bp region (Fig. 1).

We showed previously that transcription of *CYC1-lacZ* on a plasmid in *S. cerevisiae* resulted in an increase in negative supercoiling of the plasmid (27). By inserting the terminator fragment into various positions in *CYC1-lacZ*, we showed that truncated transcripts of the expected sizes were generated, showing that the terminator fragment was sufficient to generate 3′ ends. Further, the degree of relative, negative supercoiling of these plasmids was proportional to the lengths of truncated transcripts. We interpreted these changes in negative supercoiling as resulting from changes in the numbers of transcribing RNA polymerase molecules, suggesting that the fragment encoded not simply an RNA processing site but a true transcriptional terminator.

Here, we provide further evidence consistent with this view, using a transcriptional "run-on" assay in permeabilized yeast cells. Further, we provide a deletion and linker scanning mutational analysis of the terminator fragment with an aim to identify particular sequence elements involved in termination and 3′ processing. The results show that no single substitution is a potent mutation. Rather, deletion of large segments of the terminator is required to abolish function. These findings are reminiscent of studies on *rho*-dependent terminators in *Escherichia coli*.

MATERIALS AND METHODS

Strains and Medium. BWG1-7a is *MATα leu2-3,112 his4-519 adel-100 ura3-52*. BWG1-7a was grown at 30°C in SD minimal medium with Casamino acids added, 2% lactate, and 0.004% adenine.

Enzymes. Restriction enzymes, BAL-31 exonuclease, and T4 DNA ligase were supplied by New England Biolabs and were used according to the manufacturer's instructions.

Determination of mRNA 3′ Ends by S1 Nuclease Protection. The hybridization buffer was 80% deionized formamide/60 mM Pipes hydrochloride, pH 7.0/0.4 M NaCl/1 mM EDTA. The S1 nuclease reaction solution was 0.3 M sodium acetate, pH 5.2/1 mM $ZnCl_2$/250 mM NaCl/1 mM EDTA/20 μg of calf thymus DNA per ml. Poly(A)$^+$ RNA was used in this experiment, isolated from total RNA using oligo(dT)-cellulose according to the manufacturer's instructions (New England Biolabs). RNA from 10–25 ml of culture was used for each lane. Probes consisting of 365-bp *Mae* III fragments spanning the *Sac* I site of *lacZ* were prepared from plasmids bearing the terminator in the *CYC1* or *UTR1* orientation. Probes were labeled by filling in with DNA polymerase Klenow fragment. RNA, probe, and 5 μl of a 1 mg/ml solution of yeast tRNA (Sigma) were mixed in 50 μl of 1 M LiCl and precipitated with 200 μl of ethanol. The pellets were resuspended in 20 μl of hybridization buffer, overlaid with paraffin oil, heated to 100°C for 3 min, then immediately transferred to 53°C, and incubated for 3 hr. The hybridizations were transferred to chilled tubes containing 180 μl of S1 nuclease reaction solution. Three hundred to 1000 units of S1 nuclease (Boehringer Mannheim) was added and the tubes

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were incubated at 37°C for 30 min. The reaction mixtures were phenol-extracted, precipitated, and run on 6% sequencing gels, using ^{32}P-labeled *Msp* I-cut pBR322 as a size standard.

Run-on Transcription in Permeabilized Yeast Cells. This protocol is essentially as described (28). Less than 5% of the total, extracted radioactivity was trichloroacetic acid-precipitable after NaOH treatment.

Dot Blots Using Radiolabeled RNA as Probe. Five micrograms of single-stranded recombinant M13 DNA was used per dot. Each dot equivalent was resuspended in 400 μl of TE (10 mM Tris, pH 8.0/0.1 mM EDTA) and 40 μl of 3 M NaOH was added to each. Tubes were incubated at 65°C for 30 min and then allowed to cool to room temperature. Four hundred microliters of 2 M NH_4OAc (pH 7.0) was added to each. The solutions were applied slowly to a nitrocellulose sheet (Schleicher & Schuell), held in a dot blot manifold (Bio-Rad), and the strips were baked *in vacuo* at 80°C for 2 hr. Individual strips were slid into 12-ml plastic tubes and prehybridized as described (27). After 1 hr of prehybridization at 65°C, one-half of the radiolabeled RNA (about 5×10^5 cpm) was added to each tube. The strips were hybridized 12–24 hr at 65°C and then washed as described at 55°C (27). Exposure to SB5 film was at −70°C for 2 days with an intensifying screen.

Plasmids. Plasmid LG-312 has been described (29). The plasmid pBIO60R was derived from pLG-312. The 600-bp *Hin*cII–*Hin*cII fragment from the 3′ end of *LEU2* (30) was ligated into the unique *Tth*I111 site of pLG-312, in the same transcriptional orientation as *CYC1-lacZ*. A *Sal* I 8-mer (New England Biolabs) was ligated into this intermediate plasmid at *Sac* I, making pBIO60R. Plasmid pBIO2 has been described (3).

To generate linker substitutions and deletions, pBIO2 was cut with either *Sal* I or *Xho* I and then treated with BAL-31 exonuclease. Ends were rendered blunt by incubation with Klenow fragment (Boehringer Mannheim) and dNTPs, followed by ligation to *Bgl* II 8-mer linkers (New England Biolabs). Appropriate deletions were sequenced by the Sanger method using dATP[α-^{35}S] according to the manufacturer's instructions (Amersham). Linker substitutions and deletions were made by recombining appropriately spaced BAL-31 endpoints. The *Xho* I–*Sal* I fragment, linker substitutions, and deletions were ligated into *Sal* I-cut pBIO60R. Plasmids were transformed into BWG1-7a as described (31).

M13 probes for dot blot were made by ligating these fragments into mp18 or mp19: the *Stu*–*Hin*dIII fragment of *URA3* from pLG-312, the 2.0-kilobase (kb) *Bam*HI–*Sac* I fragment of pLG-312 (the 5′ probe), or the 1.0 kb *Sac* I–*Eco*RI fragment of pLG-312 (the 3′ probe). The 445-bp fragment containing *GAL1* coding sequence inserted into m13 was a gift of John Warner and Stuart Johnson (Albert Einstein College of Medicine).

Other Methods. Isolation of total yeast RNA has been described (27). Northern transfer and hybridization protocols have been described (27).

RESULTS

The Sequence in the *Ava* II–*Fnu*DII Restriction Fragment Generates the 3′ Ends of the *CYC1* and *UTR1* mRNAs. We wished to verify that the 83-base *Ava* II–*Fnu*DII fragment (Fig. 1) was sufficient to specify correct 3′ ends in either orientation when inserted into *CYC1-lacZ*. Thus, we compared the locations of the poly(A) sites within the *CYC1* and *UTR1* mRNAs with poly(A) sites within the mRNAs generated by insertion of the *Ava* II–*Fnu*DII fragment into the *CYC1-lacZ* gene.

The poly(A) sites of the *CYC1* and *UTR1* mRNAs were determined by sequencing a number of independent cDNAs from a yeast cDNA library constructed in λgt10 (gift of R. Young, Massachusetts Institute of Technology). Six sequences for *CYC1* and three for *UTR1* were obtained. The junction sequences for all *CYC1* phages as well as all *UTR1* phages were identical and are depicted in Figs. 4 and 5. We note that the junctions cannot be determined to the exact base in these sequences as three (for *CYC1*) or one (for *UTR1*) of the adenines in the junction region of the cDNAs may either be template-derived or added posttranscriptionally. However, the identification of all of the independent *CYC1* and *UTR1* poly(A) site sequences suggests that 3′ end formation in yeast is highly precise.

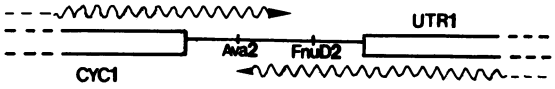


FIG. 1. Convergently transcribed *CYC1* and *UTR1* genes. Wavy arrows are the mRNAs. The 83-bp *Ava* II–*Fnu*DII fragment is shown.

The poly(A) sites in the mRNAs formed by insertion of the fragment into the *CYC1-lacZ* gene were determined by S1 nuclease analysis. Probes were prepared from constructs bearing the terminator in the *CYC1* or *UTR1* orientation (see *Materials and Methods*). S1 nuclease cleavage of these particular DNA probes at the poly(A) sites predicted by the cDNA sequences would generate products of 99 and 125 nucleotides for the *CYC1* and *UTR1* orientations, respectively (see *Materials and Methods*). As shown in Fig. 2, the sizes of the cleavage products are in agreement with the predicted sizes. This result is a strong argument that the *Ava* II–*Fnu*DII fragment contains those sequences actually used for generation of the *CYC1* and *UTR1* mRNA 3′ ends *in vivo*.

Transcriptional Run-On Analysis Suggests that the *Ava* II–*Fnu*DII Fragment Is a Terminator. As discussed above, we employed a plasmid supercoiling assay to show that the *Ava* II–*Fnu*DII fragment was a transcriptional terminator (27). To further this interpretation we have analyzed labeled RNA generated by provision of radiolabeled UTP to detergent-

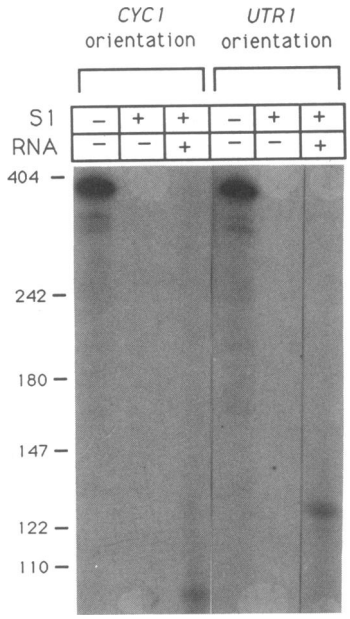


FIG. 2. S1 nuclease analysis of transcripts truncated at the *CYC1* terminator. The terminator fragment was inserted into the *Sac* I site of *CYC1-lacZ*. The probe was an internal *lacZ* fragment prepared from a construct bearing the terminator in the *CYC1* orientation (left side) or the *UTR1* orientation (right side). RNA was extracted from cells bearing the fragment inserted in the *CYC1* orientation (left side) or *UTR1* orientation (right side) and probed (see text). Size markers (given in nucleotides) were pBR322 *Msp* I fragments labeled with ^{32}P.

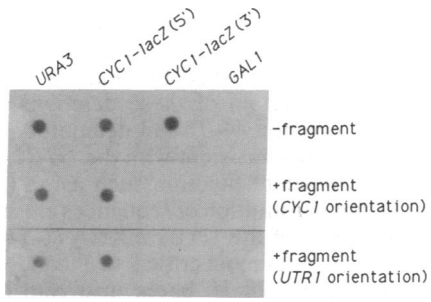
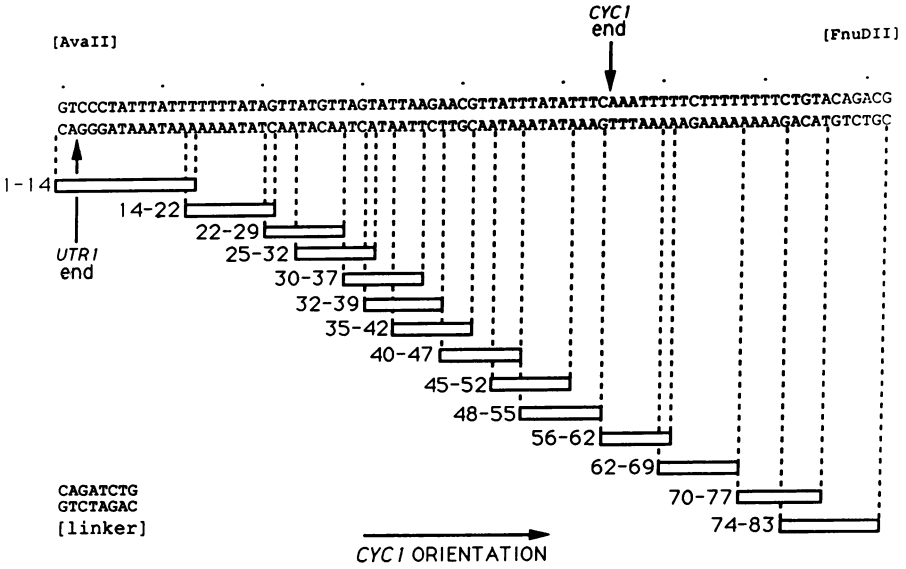


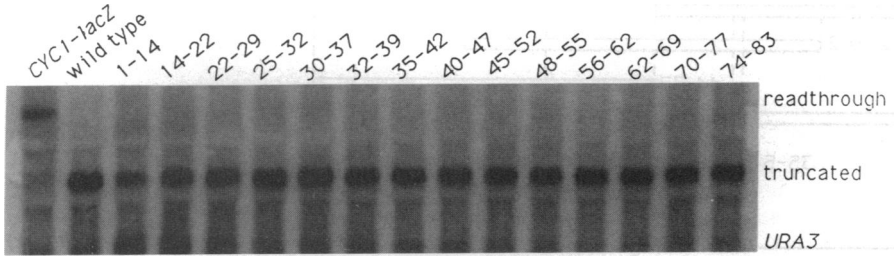
FIG. 3. Nuclear run-on analysis of transcripts terminated at the *CYC1* terminator. [^{3}H]Uridine-containing RNA was prepared from three cultures—one bearing *CYC1-lacZ* (minus fragment), one bearing *CYC1-lacZ* with the terminator inserted into the *Sac* I site in the *CYC1* orientation, and one bearing *CYC1-lacZ* with the terminator inserted in the *UTR1* orientation. These RNAs were hybridized to three nitrocellulose strips spotted with a *lacZ* probe 5′ to the *Sac* I site or a *lacZ* probe 3′ to the *Sac* I site. Probes for a transcribed gene (*URA3*) and a nontranscribed gene (*GAL1*) were used as positive and negative controls. Strips were washed, dried, and autoradiographed.

permeabilized yeast cells (28). In this experiment, labeled RNA made in short, 2-min pulses was hybridized by dot-blot to DNAs containing sequences both upstream and downstream from the site of insertion of the *Ava* II–*Fnu*DII fragment into the *CYC1-lacZ* gene. If the fragment were a transcriptional terminator no hybridization should be detected between labeled RNA and sequences downstream of the site of insertion. Fig. 3 shows that only the sequences 5′ to the site of insertion are transcribed. We note that this experiment does not rule out the existence of very short-lived RNAs or very small RNAs that could be generated by processing events dictated by the fragment.

Linker Substitution Analysis of the *Ava* II–*Fnu*DII Fragment. Linker substitutions (32) were constructed (*Materials and Methods*) and all alter at least four of the eight bases substituted. These mutagenized fragments were ligated into the *Sal* I site of a plasmid-borne *CYC1-lacZ* fusion gene on a high-copy plasmid, in both orientations. Northern analysis of total cellular RNA was then carried out on strains bearing the various plasmid constructs. As can be seen in Fig. 4, the unmutagenized fragment truncates the *CYC1-lacZ* mRNA and prevents read-through transcription in both orientations. A loss of function would be seen as a diminution in levels of the truncated transcript and as an appearance of a read-through transcript.

It is clear from Fig. 4 that no single linker substitution abolishes the fragment's activity in either orientation, al-





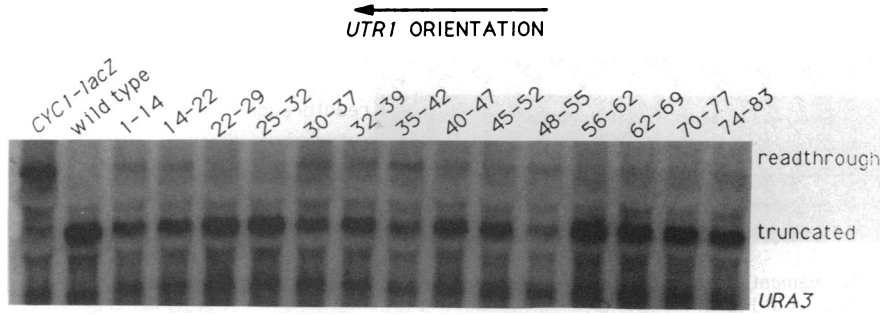


FIG. 4. Linker scanning of the *Ava* II–*Fnu*DII fragment. The sequence of the linker is shown. The position of linker substitutions under the DNA sequence of the region is also indicated. The 3′ ends of *CYC1* and *UTR1* mRNA were obtained by sequencing λgt10 clones and are shown by arrows. Fragments bearing each linker substitution were inserted into the *Sac* I site of *CYC1-lacZ* in both the *CYC1* and *UTR1* orientations and Northern analysis of total RNA was carried out as described (27). The full-length *CYC1-lacZ* transcribed (read-through), the transcript truncated at the terminator (truncated), and a control *URA3* transcript are indicated.

though some allow some read-through transcription in the *UTR1* orientation. This result is in sharp contrast to the results from metazoan cells, where the sequence 5'-AATAAA-3' is conserved and essential for proper 3' end formation (10–15, 33, 34).

Further, this analysis suggests that previously proposed consensus sequences for yeast 3' end formation are not essential. One sequence proposed, 5'-TAG. . .TAGT. . .TTT-3', is present in only the upper strand, as noted by Zaret and Sherman (26). The substitutions 14–22, 22–29, 25–32, and 30–37 mutagenize one or two parts of this tripartite consensus, with no discernible effect in the *CYC1* orientation. Substitution of another proposed consensus, 5'-TTTTTATA-3' (35), is a neutral substitution in the *CYC1* orientation. Overall these data strongly argue against any model for a terminator, mono- or multipartite, that invokes the existence of any essential sequence that only occurs once.

Also noteworthy is the observation that only certain of the substitutions in the *UTR1* orientation allow partial read-through transcription. These substitutions are clustered into two groups, in the region from 1 to 22 and in the region from 30 to 48. What distinguishes these regions from the remainder of the sequence is not immediately apparent.

In conclusion, because no single substitution eliminated termination in either orientation, we assume that no particular sequence element specifies 3' end formation for *CYC1* or *UTR1* mRNAs. Rather, transcription of a specific sequence block high in A-T content may trigger termination. Further, the finding that certain substitutions allowed partial read-through in the *UTR1* orientation, but not the *CYC1* orientation, indicates that there is not one bidirectional terminator for the two mRNAs.

Deletion Analysis of the *Ava* II–*Fnu*DII Fragment. To examine the effects of more extensive mutation on the fragment, deletions were constructed and analyzed (Fig. 5). These conclusions may be drawn from this analysis. (*i*) Deletion 55–77, which removes 24 bases including the last template-derived base of the *CYC1* mRNA, did not affect 3' end formation in either orientation. Thus, a 59-base fragment extending from the *Ava* II site to just upstream of the last template-derived base of the *CYC1* mRNA will suffice as a terminator in either orientation. (*ii*) Any deletion encroaching on this 59-base region abolished termination in the *UTR1* orientation. Because certain of these deletions were non-overlapping (e.g., deletion 44–77 and deletion 7–43 or deletion 14–35) the data are consistent with the *UTR1* terminator having a length-dependent function such as an (A+T)-rich block. We note that substitution of sequences close to the last template-derived base of the UTR1 mRNA (1–14) had little effect. (*iii*) The sequences most critical to 3' end formation in the *CYC1* orientation lie >20 bases upstream of the last template-derived base of the normal *CYC1* mRNA.

Deletion of sequences close to the last bases (deletion 44–77 or deletion 35–54) has little effect on activity, whereas deletion of bases further upstream (deletion 14–35, deletion 27–62, or deletion 7–43) allowed substantial read-through. These data indicate that for both the *CYC1* and *UTR1* terminators the most critical regions lie tens of bases upstream of the last template-derived base of the mRNAs.

DISCUSSION

Comparative molecular studies indicate that mechanistic details of basic cellular processes, such as transcription, RNA splicing, translation, and protein sorting into organelles, are shared in eukaryotes from yeast to mammals (36–39). In contrast, this and other work on transcription termination suggest that yeast termination may be similar to termination in bacteria rather than to the AAUAAA-dependent RNA processing found in higher cells (11, 14). It has been noted that the AAUAAA element signaling polyadenylylation in higher cells is not found at the 3' ends of yeast genes (35, 40). Further, in metazoan cells, the actual sites of termination lie far downstream, at least several hundred nucleotides from the poly(A) sites (5–8). The results presented here and other recent work (41, 42) show that the sites of termination for RNA polymerase II-transcribed genes in *S. cerevisiae* are close to the ends of the coding regions.

Previous work using the supercoiling assay indicated that the 83-bp fragment from a region 3' to *CYC1* was a bona fide

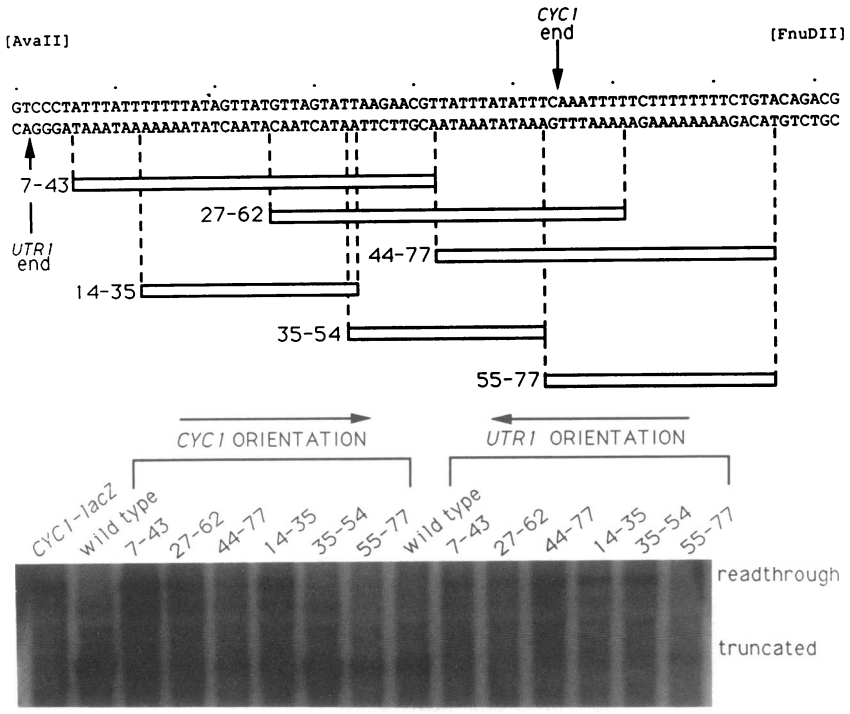


FIG. 5. Deletion analysis of the *Ava* II–*Fnu*DII fragment. Terminator fragments bearing the deletions shown were inserted into the *Sac* I site of *CYC1-lacZ* and analyzed by Northern analysis as in Fig. 4.

terminator and specified the 3′ ends of the divergently transcribed *CYC1* and *UTR1* mRNAs (27). Nuclear run-on analysis reported here confirms this contention and S1 nuclease analysis shows that the fragment specifies correct 3′ ends when moved into the body of a *CYC1-lacZ* transcript. Therefore, the 83-bp fragment encodes termination and correct 3′ end formation for both the *CYC1* and *UTR1* mRNAs.

Most notably, linker substitution analysis presents a picture of the yeast terminator, described in detail below, that is reminiscent of the *rho*-dependent terminators of *E. coli*. This analysis shows that no single 8-bp substitution abolishes the ability of the fragment to specify 3′ end formation in either orientation. Thus, the consensus elements suggested as critical in earlier studies cannot be essential for 3′ end formation. Deletions encompassing larger blocks of sequences on the fragment can abolish its activity. A 59-base sequence roughly spanning the region between the bases specifying the 3′ ends of *CYC1* and *UTR1* mRNAs is sufficient for 3′ end formation of both. Deletions encroaching into this region can abolish activity. For the *CYC1* terminator, a region between 43 and 22 bases upstream of the 3′ end site is defined as important by deletional analysis. Similar results are observed for the *UTR1* terminator. Sequences between 12 and 53 bases upstream of the 3′ end site are important as seen in the deletion analysis, but linker substitutions have, at most, partial effects. Interestingly, deletions in the region that is close to the *CYC1* 3′ end but well upstream of the *UTR1* end (deletion 35–54 or deletion 44–47) selectively inactivate the *UTR1* terminator. Thus, the *CYC1* and *UTR1* terminators do not overlap entirely. Since no linker substitution in this region totally abolishes termination, we suggest that a more general feature of the region, such as its high A-T content, is a critical determinant in termination.

However, not all (A+T)-rich sequences signal termination. Such sequences are found in yeast introns and at the 3′ ends of RNA polymerase II-transcribed genes. For example, the *CYC1* gene contains a block of (A+T)-rich sequence immediate 5′ to the fragment described here, yet this block appears not to dictate 3′ end formation. Furthermore, yeast terminators have been described here and in other work that function only in one orientation, meaning that only one of the two strands of an (A+T)-rich block may contain sequences that dictate termination (41).

There is no evidence from our results that termination sites and RNA 3′ processing sites are separable by mutation. Mutations that selectively altered a 3′ processing site and left intact a termination site would be expected to result in a truncated, poly(A)$^-$ transcript. Comparison of results from total and poly(A)$^+$-selected RNA did not reveal any RNA fraction lacking poly(A) (unpublished data). If poly(A)$^-$ RNA were unstable, mutations that selectively affected a processing site would abolish accumulation of truncated RNA without a concomitant increase in read-through RNA. Such mutations were not found. Conversely, mutations that selectively altered a termination site and left intact a processing site would be expected to give rise to both truncated transcripts and read-through RNA that extended from the processing site to the 3′ end of *CYC1-lacZ*. This read-through transcript would be about 2 kb and easily seen in Northern analysis. Again, such mutations were not found, although it is not clear whether the read-through product would be stable *in vivo*. Our inability to separate termination and processing by mutation suggests that the terminated transcript itself is a substrate for polyadenylylation or that termination and 3′ processing occur at separate sites but are necessarily coupled.

The finding that termination is signaled by no specific sequence element but rather an (A+T)-rich block of sequence is reminiscent of the *rho*-dependent terminators of *E. coli*. These terminators are pyrimidine-rich tracts, in which small substitutions or deletions have little or no effect (43–46). In some of these cases, the terminated transcript is processed at a site several hundred bases upstream of the termination site. The stem–loop hairpin structure at the processing site may well stabilize the final product. In yeast, such stabilization may be provided by rapid polyadenylylation at the site of the primary termination event.

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