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In vivo analyses of RNA polymerase I termination in *Schizosaccharomyces pombe*

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ABSTRACT

Recent studies on the termination of rDNA transcription by RNA polymerase I in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have suggested a more complex mechanism then previously described in higher eukaryotes. Termination appears to occur when a DNA-bound Reb1 protein molecule induces polymerase to pause in the context of a release element [see Reeder,R.H. and Lang,W. (1994) *Mol. Microbiol.*, 12, 11–15]. Because these conclusions in yeast were based entirely on *in vitro* analyses, we have examined the same termination process in *S.pombe* by expressing targeted mutations *in vivo*. S_1 nuclease protection studies indicate three tandemly arranged termination sites with most transcripts very efficiently terminated at the first site, 267 nt after the 3′ end of the mature 25S rRNA sequence. Termination at each site is mediated by conserved terminator elements which bear limited sequence homology with that of mouse and also can be identified in *S.cerevisiae*. Removal of the first terminator element transfers dominance to the second site and construction of a new single terminator element at +150 still results in efficient termination and rRNA processing without a need for an additional upstream element. Genomic 'footprint' analyses and gel retardation assays confirm a process mediated by a strongly interacting protein factor but implicate an alternate binding site. Removal of the 5′ flanking sequence or structure also had no effect on the site or efficiency of termination. Taken together the results *in vivo* suggest that the termination process in this fission yeast more strongly resembles the single element-mediated mechanism initially reported in mouse and is not dependent on additional upstream sequence as first reported in *S.cerevisiae* and postulated to function in general.

INTRODUCTION

In the rRNA genes of higher eukaryotes, including man, transcription termination by RNA polymerase I (pol I) is dependent on a short DNA sequence, the 'Sal box' in mouse (1). This 18 bp sequence element, (AGGTCGACCAGA/TT/ANTCCG), which is repeated eight times within the 3′-terminal spacer interacts with a nuclear transcription termination factor (TTFI) that mediates the termination process. The precise mechanism of pol I termination remains unclear although it appears certain that the stop in elongation is not the result of simple protein blockage but depends on specific interactions between TTFI and pol I as well as protein-induced conformational changes in the DNA structure (2). A similar sequence has been observed to function in *Xenopus* (3) and has even been suggested in a thermophilic fungus, *Thermomyces lanuginosus* (4) and *Schizosaccharomyces pombe* (5). But with other types of signal also having been proposed (6–8) and a 'Sal box'-like sequence element even suggested in *Saccharomyces cerevisiae* (4), it has been surprisingly difficult to unequivocally establish a specific termination signal in yeast.

At present, the majority of evidence has indicated that a single protein factor, Reb1, may act as both an enhancer and termination factor in *S.cerevisiae* (9,10) but until recently, it was generally accepted that only the Reb1p binding site and a few surrounding nucleotides are needed for termination, *in vitro* (11). Lang and co-workers (12), however, have now presented new data which suggest that ~46 bp of 5′ flanking sequence is also required, with the Reb1p binding site representing a 'pause' element and the extra sequence representing a 'release' element. A similar proposal also has been made for *S.pombe* when recently (13), the gene encoding the Reb1 factor in this fission yeast was isolated and the effect of a derived polypeptide was examined *in vitro*. As was the case in *S.cerevisiae*, the protein factor could terminate transcription *in vitro*, but was only effective in combination with an upstream sequence.

In view of these somewhat conflicting observations in *Saccharomyces*, and also to clarify the termination signal in other fungi, we have examined the role of the Sal box-like sequence elements in *S.pombe* using targeted mutagenesis *in vivo*. A convenient system for the efficient expression of mutant rRNA in *S.pombe* was recently developed in the course of studies on the function of the ribosomal 5.8S rRNA (14), a strategy which also has been used effectively in the study of rRNA processing (5) and ribozyme function (15). When applied in the present study, specific mutations indicate that unlike studies *in vitro*, *in vivo* the Sal box-like elements do function directly to efficiently terminate transcription as has been shown for higher eukaryotes (16).

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MATERIALS AND METHODS

Construction and expression of mutant rRNA genes

Site-specific mutations were introduced into a *S.pombe* rDNA using a genomic 10.4 kb genomic *Hin*dIII digestion fragment which contained one complete rDNA transcriptional unit that had previously been subcloned into the pFL20 yeast shuttle vector (17) and 'tagged' with a unique *Not*I restriction site in the last variable sequence of the 25S rRNA (5). Further mutations based on a two-step PCR-amplification procedure (18) were introduced into the 3′ ETS region using the unique *Not*I/*Pvu*II sites as described earlier (5). For these experiments, the *Bgl*II/*Pvu*II restriction digestion fragment of *S.pombe* rDNA containing the 3′ ETS region was subcloned into the pTZ19R vector (19) and used as a template for PCR amplification. All mutations were confirmed by DNA sequencing based on the dideoxy methods of Sanger and co-workers (20). Recombinant DNAs containing the appropriate changes were subsequently amplified using *Escherichia coli* strain C490 as the bacterial host and used to transform *S.pombe*, strain h⁻ leu1-32 ura 4-D18 by the method of Prentice (21).

S1 nuclease mapping of RNA precursors

The termini of mature and precursor ribosomal RNA transcripts were determined by digesting RNA:DNA hybrids with S1 nuclease as previously described (5,22). Appropriate 3′ end labelled probes, overlapping the 25S rRNA/3′ ETS junction in either the host cell or plasmid-associated rDNA, were incubated with whole cell extracted RNA (20 µg) at 30°C for 12 h in 50 µl of 3 M NaTCA, 5 mM Na_2EDTA, 50 mM PIPES (pH 7.0), rapidly chilled on ice and digested for 30 min at 37°C with S_1 nuclease (50–200 U) in 15 mM $ZnCl_2$, 250 mM NaCl, 40 mM NaAcO (pH 5.5; 200 µl total volume). Digestions were terminated with SDS/EDTA, extracted with phenol/chloroform and analyzed on 6% polyacrylamide sequencing gels. Standardized chain termination sequence reaction products were also applied to the analytical gels as fragment length markers. For the 3′ end labelled DNA probes, normal or mutant rDNA was digested with an appropriate restriction endonuclease and the protruding single-stranded ends were used to extend the 3′ ends with Klenow enzyme and [α-^{32}P]dCTP (23). In these instances the DNA was initially purified plasmid or PCR amplified DNA. Each labeled fragment was incubated for 5 min at 90°C to separate the strands for hybridization.

Genomic footprint analysis

Genomic footprints were determined by methods based on those described by Huibregtse and Engelke (24) and modifications by Diffley and Cocker (25). Cultures of *S.pombe*, strain h⁻ leu1-32 ura 4-D18, growing exponentially with aeration at 30°C in broth (0.67% yeast nitrogen base without amino acids), were harvested, washed and resuspended in 3 ml of 1 M sorbitol, 1 mM EDTA, 3 mM dithiothreitol containing 2 ng (~3000 U) Lyticase (Sigma Chemical Co., St. Louis, MO). The resulting spheroplasts were lysed in buffer containing 10 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulphanyl fluoride, 2 µg/ml leupeptin and 1 µg/ml pepstatin; 300 µl aliquots were treated with 1–16 µl of DNase I (5 mg/ml) for 5 min at room temperature. Following the addition of an equal volume of 50 mM Tris–HCl pH 7.5, containing 1 M NaCl, 1% sodium dodecylsulfate and 50 mM EDTA, the DNA was extracted twice with phenol/chloroform (1:1), precipitated with 2-propanol, treated with RNase A and extracted further with phenol/chloroform. After precipitation with ethanol, the DNA was dissolved in water (2 µg/µl). For control DNA digestions, genomic DNA was prepared as described above without DNase I treatment.

The cleavage sites were detected by repeated primer extension with Taq polymerase using 20 cycles of 1 min at 94°C, 1 min at 42°C and 2 min at 72°C, and 10 µg of template DNA. The oligonucleotide primers which were specific either for the host cell or the plasmid-associated rRNA gene sequence, were labelled using T_4 polynucleotide kinase and [γ-^{32}P]ATP (7000 Ci/mmol) and primers of equal specific activity was prepared by polyacrylamide gel electrophoresis (26). Standard dideoxy sequencing reactions using Taq polymerase and the same primers and thermal cycle were used as chain length markers.

Electrophoretic mobility shift assay

A nuclear protein extract was prepared from *S.pombe*, strain h⁻ leu1-32 ura 4-D18, cells and used for gel retardation studies as described by Henninghausen and Luban (27) and Jazwinski (28). A DNA probe containing the termination region (nt +267 to +338) was prepared by PCR amplification and labelled at the 5′ ends using [γ-^{32}P]ATP and T_4 polynucleotide kinase in 10 µl of 10 mM $MgC1_2$, 5 mM DTT, 0.1 mM spermidine and 50 mM Tris–HC1 pH 7.5. An oligonucleotide probe corresponding to only the Sal box-like sequence region was prepared by annealing and 5′ end labelling two complementary synthetic oligonucleotides (1 µg) with polynucleotide kinase (pAGGTAAGGGTAATGCAC and pGTGCATTACCCTTACCT) for 12 h at 37°C and purifying the hybrid on a neutral 10% polyacrylamide gel. Aliquots of labelled DNA probe (10 000 c.p.m.) or of labelled oligonucleotide probe (10 000 c.p.m.) were incubated for 20 min with 30 µg of protein extract in 12.5 µl of binding buffer (12 mM HEPES pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 8% glycerol) containing 2 µg of pBR322 plasmid DNA to eliminate non-specific DNA interactions. After incubation samples were applied to a neutral 5% polyacrylamide gel in low ionic strength buffer (30 mM Tris–HCl pH 8.0, 30 mM boric acid, 1 mM EDTA) and fractionated at 4°C for 2.5 h using 12 V/cm; following electrophoresis, the gel was dried and the bands were detected by autoradiography.

RESULTS

As summarized in Figure 1, previous studies on the processing of rRNA in *S.pombe* (5) revealed three tandemly arranged Sal box-like sequence elements beginning ~281 bp downstream of the mature 25S rRNA sequence. S_1 nuclease protection studies indicated that all three were associated with extended termini; the first element was associated with most of the termini, a modest amount was observed with the second and only traces were associated with the third element (see Scp in Fig. 2). These data were entirely consistent with an efficient termination signal, most transcripts being terminated at the first site. Still longer transcripts were not observed, eliminating the possibility that these actually were intermediate processing sites. Initially to provide direct evidence for this conclusion, in this study, the first Sal box-like element was removed using a two-step PCR strategy (Fig. 1) and the termini of nascent transcripts were again characterized by S_1 nuclease digestion. As previously demonstrated (5,14), the mutant rDNA could be efficiently expressed *in vivo* using a high

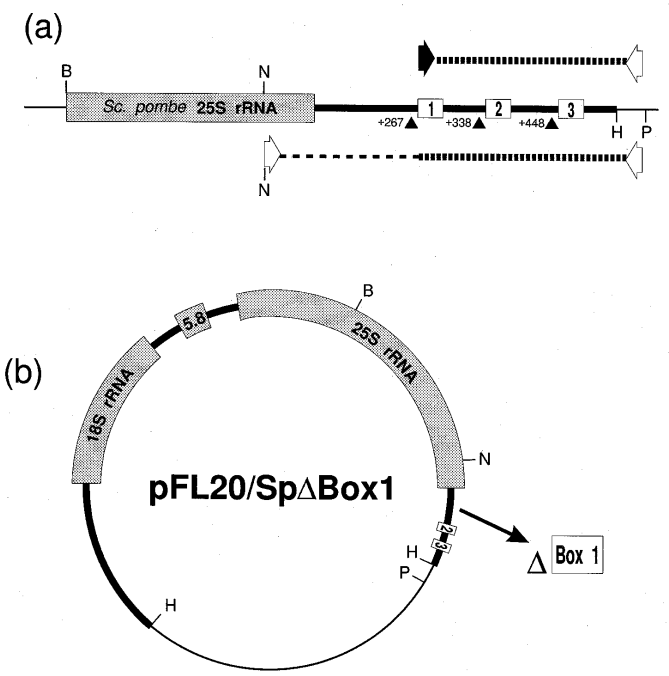

Figure 1. PCR mediated Box 1 mutation in the 3′ ETS of rDNA from *S.pombe*. (**a**) A *Bgl*II (B)/*Hin*dIII (H) endonuclease digestion fragment of *S.pombe* rDNA containing the 3′ end of the mature 25S rRNA sequence (stippled box) and the 3′ spacer region (thick continuous line) with three Sal box-like elements (1–3) previously shown to be associated with termination sites (5) 267, 338 and 448 nt distal (arrowheads) to the mature 3′ end was subcloned in pTZ19R (thin continuous line). A mutagenic primer (GAAGAGATAATCTAGAG-CACTTTTGAAG) which overlaps the first Sal box-like element (filled arrow) and a universal sequencing primer (open arrow) were used to PCR amplify an intermediate mutagenic primer (thick broken line) which was again used with a second 25S rRNA-specific primer to PCR amplify the full mutant sequence. (**b**) Mutant DNA was digested with *Not*I (N) and *Pvu*II (P) restriction endonucleases and the mutant rDNA sequence was subcloned into a pFL20 yeast shuttle vector (thin line) containing an *S.pombe* rDNA transcription unit (pFL20/Sp25NotI) which also contained a *Not*I restriction site (20). Recombinants which contained the mutated Box 1 sequence were selected by differential hybridization (32) using the mutagenic oligonucleotide primer; as indicated, the mutated construct was designated pFL20/SpΔBox1.

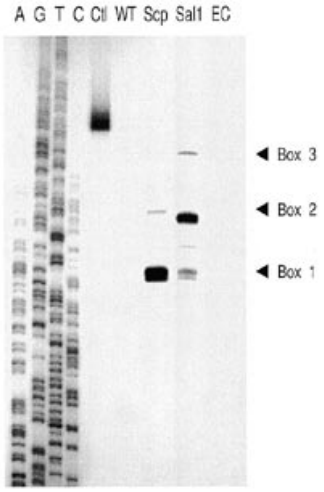

Figure 2. Effect of the Box 1 sequence on the termination of rRNA transcripts in transformed *S.pombe* cells. Whole cell RNA was prepared by sodium dodecyl sulphate/phenol extraction from *S.pombe* transformed with pFL20/Sp25NotI (Scp) or pFL20/SpΔBox1 (Sal1) as described in Materials and Methods. One kb *Not*I/*Hin*dIII digestion fragments of DNA overlapping the terminal regions (Fig. 1) in each of the plasmids were labelled at the 3′ end of the coding strand with [α-^{32}P]dCTP and Klenow fragment for plasmid-specific probes. Labelled fragments were incubated at 90°C for 5 min to separate the strands and then incubated with RNA aliquots (20 µg) at 30°C for 12 h. The resulting hybrids were digested with 100 U of S_1 nuclease at 37°C for 30 min and after extraction with phenol/chloroform, the fragments were fractionated on a 6% polyacrylamide sequencing gel. An undigested mutant-specific probe (Ctl) and digests with RNA from untransformed *S.pombe* (WT) or *E.coli* (EC) were also fractionated together with standard dideoxy sequencing reaction products (A, G, T, C) for a standardized sequence (5) as residue markers. The positions of the major termini (Fig. 1) are identified at the right.

copy number autonomously replicating yeast shuttle vector, pFL20 (12), and plasmid derived transcripts could be specifically assayed using a probe with a plasmid-specific internal *Not*I site mutation (5). Host cell transcripts are efficiently cut at this site and removed from the assay.

As shown in Figure 2, the *in vivo* analyses clearly indicate that the Sal box-like element does function as an efficient termination signal (Box 1). In the normal gene (Scp), most transcripts are terminated ~11 bp upstream of the first element (Fig. 1), but when the first element is changed (Sal1) most transcripts are now terminated at the equivalent position for the second element (Box 2) and the third termination site (Box 3) is much more apparent. The Box 2 site migrates slightly faster due to the deletion of residues in Box 1. A slight amount of termination appears to continue at the first sight but this trail of slight cleavages likely reflects some non-specific cleavage by S_1 nuclease which is often observed in AT-rich clusters of the type that is now present within this modified region (5). In any case, the second site is obviously now the dominant termination signal.

Since terminations at 'Sal box' elements (16,29,30) in mammals and amphibians are known to be mediated by a protein factor (TTF-I) and in yeast Reb1 protein has been shown to cause termination *in vitro* (12,13), the *S.pombe* element was further examined with respect to protein interactions using genomic 'footprint' analysis and gel retardation. For DNase I 'footprinting,' yeast cell lysates or purified yeast genomic DNA were treated with a wide range of nuclease concentration to determine effective and equivalent levels of digestion and the cleaved fragments were detected by PCR amplified DNA sequencing (24). As shown in the example autoradiograph of an analytical gel (Fig. 3, left), under appropriate digestion conditions, differences due to protein protection could be detected in the termination region. The sequence bias in DNase I digestion which is clearly evident with naked DNA (lane N), however, made a precise assessment of differences difficult, so the gels were scanned and the data was normalized at each residue to eliminate this bias. As shown in the resulting histogram (Fig. 3, right), when the degree of cleavage was examined as a percentage of that which is observed with naked DNA, an extended region of protection was present in the Sal box region. Furthermore, when a nuclear protein extract was prepared from gently lysed cells (24) and incubated with a purified and labelled DNA fragment containing the Box 1 sequence, a deoxyribonucleoprotein complex also could be

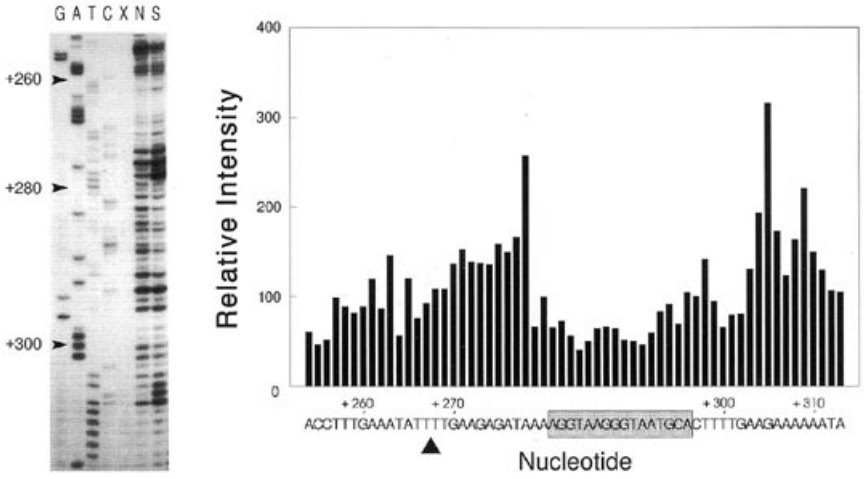

Figure 3. Comparison of the DNase I digestion footprint in a genomic and naked rDNA termination site from *S.pombe*. Spheroplast lysates were prepared from exponentially growing cultures of *S.pombe*, treated with 20 µg of DNase I and the resulting DNA fragments were primer extended with Taq polymerase in 20 cycles of 1 min at 94°C, 1 min at 42°C and 2 min at 72°C as described in Materials and Methods before fractionation by polyacrylamide gel electrophoresis (left). Treated genomic (S) and naked (N) DNA fragments were applied together with an undigested control (X) as well as standardized dideoxy sequencing reaction products (G, A, T, C) as chain length markers. The autoradiographs for replicated footprint analyses were scanned and the extent of digestion at each residue in the first termination region is shown in the histogram (right) as a percentage of the digestion observed with naked DNA. Residues corresponding to the histogram are identified together with the first termination site at +268. The shaded block of sequence corresponds to the previously identified Sal box-like sequence (5).

demonstrated by gel retardation (27). As shown in Figure 4A, the slowly migrating complex was only evident with nuclear extract (lane b); no complex was observed when extract was not added (lane a) or when a large excess of competing unlabelled DNA fragment was present (lane c). The interaction was still further confirmed when a synthetic Sal box-like element was substituted for the DNA fragment. As shown in Figure 4B, when a 17 bp duplex consisting only of the Sal box-like repeating sequence element (5) was substituted for the DNA fragment, a strong and distinctly retarded band was clearly evident after fractionation by gel electrophoresis (lane a). Again this band was absent (lane b) when a large excess of competing unlabelled oligonucleotide was added. In addition, mutations in this competing unlabelled sequence had dramatically different effects depending on the nucleotides which were altered. As also shown in Figure 4B, nucleotide changes in the centre of the sequence (lanes c and e) essentially eliminated its ability to compete with the labelled sequence while a similar change at only the proximal end of the element (lane d) had no effect.

While both types of experiment were entirely consistent with previous models in higher eukaryotes (16,29,30) or yeasts (12,13), the competitive effect with a Sal box-like sequence from *S.cerevisiae* (lane e), raises further questions about the role of the Reb1 protein *in vivo*. As shown in Figure 5, this sequence is similar to the termination box elements in *S.pombe*, but is associated with a previously reported termination site (6) rather then Reb1 protein binding domain. If proteins from both yeasts are equivalent, it appears that the protein from *S.cerevisiae* cannot bind to its own sequence but the factor from *S.pombe* can.

In vitro studies in both yeasts indicate that the transcription termination site for *S.cerevisiae* RNA polymerase I requires not only an 11 bp binding site for Reb1p, but also ~46 bp of 5′ flanking sequence. To access this requirement *in vivo*, the entire termination region in *S.pombe* including the three copies of the Sal box-like element and the 5′ flanking sequence was deleted, and a single new element was inserted much earlier in the 3′ external transcribed sequence (pFL20/SpT_{150}) as indicated in Figure 6a. Both changes were again made using two-step PCR

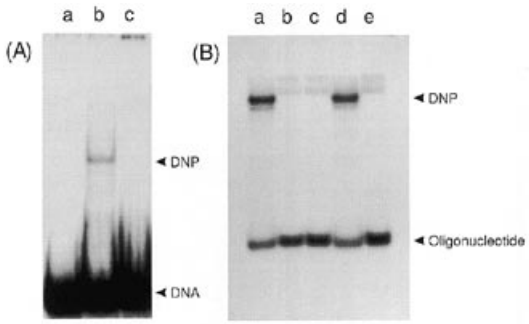

Figure 4. Binding of nuclear protein to the Box 1 *S.pombe* termination region in rDNA. (**A**) A 72 bp fragment containing the first termination signal from +267 to +338 was labelled at the 5′ end and incubated for 15 min at room temperature with 30 µg of nuclear protein extract in 12.5 µl of binding buffer before fractionation on a neutral 5% polyacrylamide gel (lane b). Labelled DNA incubated in the absence of protein (lane a) and in the presence of 2 µg of unlabeled DNA fragment (lane c) were included as controls. The positions of the free labelled fragment (DNA) and protein-associated complex (DNP) are indicated at the right. (**B**) A 17 bp synthetic oligonucleotide duplex (pAGGTAAGGGTAATGCAC/TCCATTCCCATTACGTGp) was incubated with nuclear protein extract and fractionated by gel electrophoresis as described above in the absence (lane a) or presence (lane b) of 2 µg of unlabelled oligonucleotides of alternate sequence; AGGTAAGGGTCCGGCAC in lane c, CAACAAGGGTAATGCAC in lane d or AGAGAAGGGCTTTCAC in lane e. The positions of the labelled synthetic duplex (oligonucleotide) and protein-associated complex (DNP) are indicated at the right.

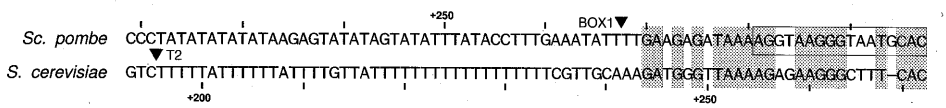

Figure 5. Comparison of the Box 1 termination site in *S.pombe* with the T2 termination site in *S.cerevisiae*. Shaded regions indicate identical residues in the putatively equivalent termination signal; the boxed residues are identical in two of the Sal box-like sequence elements in *S.pombe* (5).

amplification. As shown in Figure 6b, when *S.pombe* cells were transformed with this construct, plasmid-derived RNA clearly could be demonstrated. S_1 nuclease digestion again revealed significant amounts of mature plasmid-derived 25S rRNA. Both the mature 25S rRNA and the immediate processing intermediate remained essentially constant over a range of nuclease concentrations (50–200 U) while no RNA was detected in the absence of plasmid (WT).

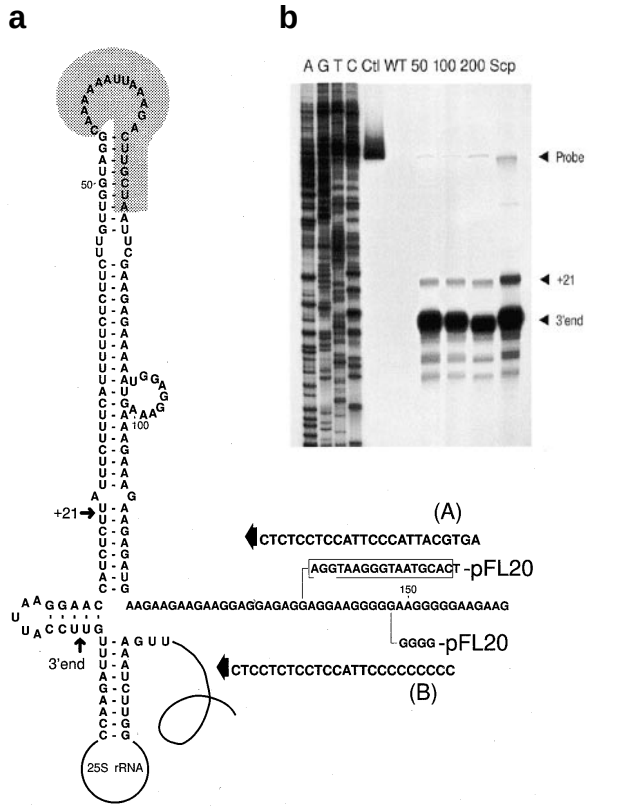

Figure 6. Effect of a relocated Box 1 sequence on the termination of rRNA transcripts in transformed *S.pombe* cells. Truncated *S.pombe* rDNA transcriptional units were prepared by PCR amplification using the 25S rRNA-specific primer described in Figure 1 and two primers which totally deleted the termination region as indicated above and below the normal sequence (**a**). One primer (A) added a new Sal box-like sequence element at approximately +150 ($pFL20/SpT_{150}$) and the other (B) resulted in a rDNA construct that contained no known termination signal (pFL20/SpΔ3T). The shaded region identifies the Δ22 deletion which fully inhibits 3′ end maturation (5). Whole cell RNA was prepared from exponentially growing *S.pombe* transformed with $pFL20/SpT_{150}$ or pFL20/Sp25NotI and the 3′ end termini were mapped by S_1 nuclease digestion as described in Figure 2. Samples of $pFL20/SpT_{150}$-derived RNA digested with 50–200 U of enzyme (50, 100, 200) were fractionated (**b**) together with undigested probe (Ctl) as well as RNA from untransformed cells (WT) and cells transformed with pFL20/Sp25NotI (Scp) which was digested with 100 U of S_1 nuclease. Standard dideoxy sequencing reaction products (A, G, T, C) were also fractionated as residue markers.

Because RNA processing might produce mature 25S rRNA from unterminated transcripts, a study was also made of RNA from cells transformed with rDNA that contained neither the three original Sal box-like elements nor the new element (pFL20/SpΔ3T). As shown in Figure 7, in this case, S_1 digestion indicated that little or no mature RNA was present. Although some mature 25S rRNA and shorter fragments of further degradation were observed with a lower concentration of enzyme (50 U), this signal clearly was not stable to higher concentrations of enzyme showing it to be the result of less specific protection, probably due to the host cell 25S rRNA. In contrast, both the new single terminator element construct (Sal) and the normal three box construct (Scp) resulted in much higher amounts of mature RNA, underlining the efficiency of termination at the new site.

While mature plasmid derived RNA was easily demonstrated by S_1 nuclease digestion in Figure 6, because the greatly abbreviated 3′ ETS region is processed much more rapidly, the actual new termination site was only slightly visible when gels were strongly overexposed. In previous studies, the complete or partial removal of the highly conserved extended hairpin structure shown in Figure 6, (A), substantially inhibited RNA processing and greatly elevated the amount of unprocessed terminus (5). As shown in Figure 8, when this deletion was introduced into a new rDNA construct with the new Sal box-like element as well as the normal three elements ($pFL20/SpT_{150}$Δ22), the new terminus was clearly visible (T150) and now the predominant termination site, accounting for at least 90% of the termini. Again no band was evident in the absence of plasmid (WT) or with non-specific DNA (EC), and only the normal termini were evident when the new Sal box element was not present (Δ22). Furthermore, the position of this new terminus prior to the Sal box-like element was entirely consistent with that which was previously observed (5) at the normal elements.

In vitro analyses of pol I termination in *S.cerevisiae* (12) have suggested that efficient termination is dependent on two sequence elements, a binding site for the Reb1 protein as well as ~46 bp of 5′ flanking sequence which acts as a release element. As shown in Figure 9, this region in *S.pombe*, also is likely to form a substantial amount of secondary structure. To further examine the possibility that this sequence or structure affects the efficiency of termination, the region was deleted including all of the equivalent sequence which was reported to affect termination in *S.cerevisiae*. As shown in Figure 9, when this truncated construct was expressed, *in vivo*, the termination efficiency remained unchanged.

DISCUSSION

Taken together, the results presented here indicate that the termination signal for RNA polymerase I in *S.pombe* is very similar to that which has been observed in higher eukaryotes. A tandemly repeated Sal box-like sequence constitutes the primary

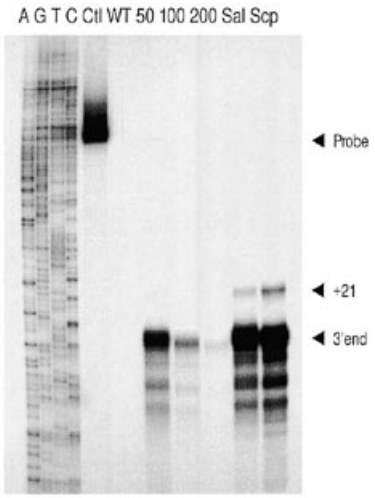

Figure 7. Comparison of rRNA transcripts in the presence and absence of Sal box-like termination sequence elements. Whole cell RNA was prepared from exponentially growing *S.pombe* cells, transformed with normal (pFL20/Sp25NotI) and truncated (pFL20/SpΔ3T and pFL20/SpT_{150}) *S.pombe* rDNA transcriptional units (Fig. 5, left) and the 3′ end termini were mapped by S_1 nuclease digestion as described in Figure 1. Samples of pFL20/SpΔ3T-derived RNA digested with 50–200 U enzyme (50, 100, 200) were fractionated together with undigested probe (Ctl) as well as RNA from untransformed cells (WT) and cells transformed with pFL20/SpT_{150} (Sal) and pFL20/Sp25NotI (Scp) which were digested with 100 U S_1 nuclease. Standardized dideoxy sequencing reaction products (A, G, T, C) were also fractionated as residue markers.

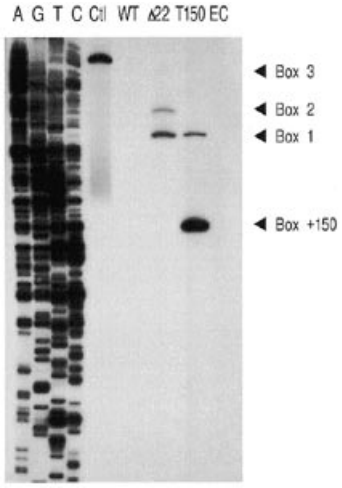

Figure 8. S_1 nuclease mapping of rRNA transcript termini using a relocated Box 1 sequence and a truncated 3′ ETS region. A construct unit containing a new Sal box-like sequence at +150 (Fig. 6) and a deletion in the highly conserved extended hairpin structure (Fig. 6a) was prepared as previously described resulting in a rDNA transcriptional unit that contained a premature termination site and a truncated 3′ ETS sequence (pFL20SpT_{150}Δ22). Whole cell RNA was prepared from exponentially growing *S.pombe* transformed with pFL20/SpT_{150}Δ22 and the 3′ end termini were mapped by S_1 nuclease digestion (100 U enzyme). The digest (T150) was fractionated by gel electrophoresis together with undigested probe (Ctl) as well as digested RNA from untransformed cells (WT), cells transformed with pFL20/Sp3′ETSΔ22 and from *E.coli* cells (EC). Standardized dideoxy sequencing reaction products (A, G, T, C) were also fractionated as residue markers.

signal and the termination event is mediated through at least one protein factor which strongly interacts with the terminator element. Unlike the recent reports in *S.cerevisiae* (12) and *S.pombe* (13), termination *in vivo* appears not to be critically dependent on an extended upstream release element. As shown in Figures 2 and 5, effective termination is observed when the termination signals is moved 71 bases downstream or 140 bases upstream, respectively. As further shown in Figure 9, the efficiency also is not affected when the normal upstream region is simply removed. All of these observations remain fully consistent with analyses in higher eukaryotes (16) which also did not indicate a need for additional flanking sequence. Unless new 'release elements' have been added fortuitously in every instance, a very unlikely possibility, the Sal box-like element clearly is sufficient for efficient termination *in vivo*.

When compared with previous studies in *S.cerevisiae*, the present results raise a number of further questions regarding the role of the Reb1 protein in the termination of pol I transcription. Earlier *in vivo* studies of pol I transcription in *S.cerevisiae* (6,31) were consistent with at least two putative termination signals, T2 which mapped at +210, the beginning of a T-rich stretch, and a second apparently fail-safe termination site, ~950 nt distal to the 3′ end of the 35S ribosomal RNA precursor. In contrast, based on *in vitro* analyses Lang and Reeder (12) found the terminator to be located 108 bp downstream of the 3′ end of the mature 25S rRNA, just 17 bp upstream of the Reb1p binding site, a contributing sequence element and an essential enhancer sequence which regulates rRNA transcription (10). Although recent sequence comparisons do reveal some homology between Reb1p and the mammalian termination factor (29), we believe the present results are more consistent with the original findings in four respects: there is striking homology between the termination signals in *S.pombe* and sequence associated with the T2 site in *S.cerevisiae*, an upstream release element is not important to termination in *S.pombe* as it appears to be for Reb1p-induced termination in *S.cerevisiae*, there is no obvious sequence homology between the Reb1 protein binding site in *S.cerevisiae* and the Sal box-like sequence in *S.pombe*, and no Reb1p-associated termination has been detected in RNA from normal *S.cerevisiae* cells. As indicated in Figure 5, a comparison of the alternative termination signals in *S.cerevisiae* with the first Sal box-like sequence in *S.pombe* reveals a marked similarity with sequence following the T2 site but no significant homology with the Reb1p binding site. Furthermore, as shown in Figure 4B, based on gel retardation, the Sal box-like element in *S.cerevisiae* competes effectively with the element in *S.pombe*. The only discrepancy in *S.cerevisiae* is the position of the 3′ end (T2 in Fig. 5) relative to the homologous sequences, but, as pointed out by Lang and Reeder (7), in S_1 nuclease protection assays the T-rich hybrid is likely to be lost, yielding an apparent map position coinciding with T2. This equally may be true for the second termination site in *S.pombe* rDNA which also is positioned immediately upstream of an extended thymidilic acid cluster and may explain why the Box 2 sequence bears much less homology with the Box 1 and Box 3 that are essentially identical (5).

A question which remains is why Reb1p-induced termination functions so efficiently *in vitro*, but has not been observed *in vivo*? As pointed out in studies on the rRNA enhancer in *S.cerevisiae* (10), this sequence functions to regulate rRNA transcription in response to nutritional shifts, an observation which indicates that, unlike studies *in vitro*, the Reb1p binding site is unlikely to be

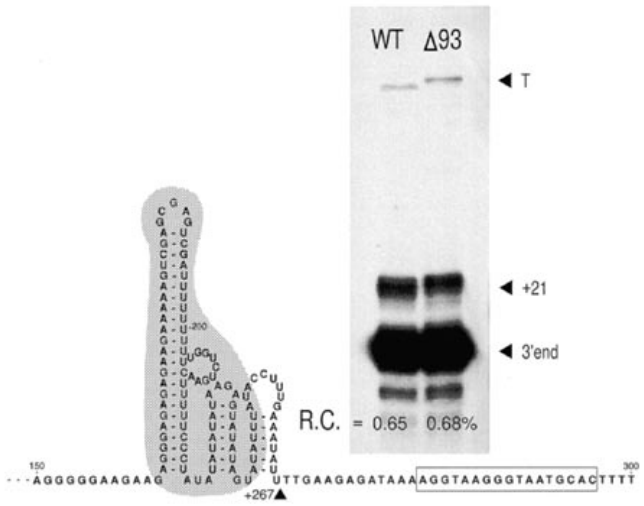

Figure 9. Effect of upstream sequence or structure on the termination of rRNA transcripts in transformed *S.pombe* cells. Sequence (shaded region), upstream to the Box 1 (boxed sequence) termination site, was deleted by two step PCR amplification (5) and the truncated rDNA transcriptional unit was cloned and expressed in *S.pombe*. Whole cell RNA was prepared from cells transformed with normal (pFL20/Sp25NotI) or truncated (pFL20/Sp3′ETSΔ93) transcriptional units and the 3′ end termini were mapped by S_1 nuclease digestion using a plasmid sequence-specific 3′ end labeled probe prepared from pFL20SpΔ93. The major termination sites (T), processing intermediates (+21) and mature 25S rRNA termini (3′ end) are compared in normal (WT) and truncated (Δ93) gene transcripts. The radioactivity in the bands was used to determine the relative concentration (R.C.) of RNA terminating at T1, based on the total amount of 25S rRNA. With truncated RNA the T site corresponds with transcripts terminated by the Box 1 sequence, but with normal RNA, the site corresponds with the beginning of the deletion in the truncated probe sequence.

saturated with Reb1p under physiological conditions while the T2 site may be tightly bound to protein. Although direct evidence was not presented Lang and Reeder (7) assumed such tight interactions as an explanation for the additional termination sites which were observed *in vitro*. Taken together, therefore, all of the studies indicate that in fungi, as in higher eukaryotes, termination can be mediated by a simple Sal box-like protein interaction which does not require additional sequence elements. The physiological significance of a more complex mechanism based on Reb1 protein and two DNA sequence elements remains unclear and not demonstrated *in vivo*.

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