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Characterisation of 3′ end formation of the yeast *HIS3* mRNA[1]

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Abstract

The nucleotide (nt) sequence of the 3′ end of the yeast *HIS3* mRNA was determined by PCR amplification of the 3′ end. Analysis of 28 individual clones revealed that at least 13 distinct polyadenylation sites are present. The sites of polyadenylation are extremely heterogeneous and do not show any obvious similarity other than that they occur after pyrimidine residues in most cases. Most mutants carrying internal deletions of the 3′ untranslated region (3′ *UTR*) did not abolish 3′ end formation and showed polyadenylation at normal sites. Deletion of a 90-nt region that contains an A+T-rich sequence close to the 3′ end of the *HIS3* coding sequence and a subset of processing sites resulted in a drastic reduction in the levels of full-length *HIS3* mRNA and concomitant transcription past the normal *HIS3* 3′ end. The 90-nt region appears to be sufficient to direct the formation of at least a subset of the *HIS3* 3′ ends since mutants that carry deletions of flanking regions of this sequence show detectable levels of *HIS3* mRNA. Spacing between the upstream A-T sequence and the site of processing is variable. In the light of the extreme heterogeneity of the sites, a possible mechanism for 3′ processing is discussed.

Keywords: *Saccharomyces*; 3′ Processing; Polyadenylation; Transcription termination

1. Introduction

The molecular events involved in termination of transcription and 3′ end formation, though significant in terms of gene expression, are poorly understood in the lower eukaryotes such as yeast. Investigations by different research groups during the past decade have accumulated some amount of information on the sequences directing 3′ end formation in *Saccharomyces cerevisiae*. Unlike the higher eukaryotes for which the sequence AAUAAA is ubiquitous for proper 3′ processing, a large proportion of yeast genes lack this sequence. Among genes that carry this sequence motif, point mutations within the sequence have been shown to have no appreciable effect on 3′ processing (Hyman et al., 1991). Several additional sequences, such as TAGNNNTATGTA, TATATA, $(AT)_9$, TTTTTATA and $(A)_8$, have been implicated in directing 3′ end formation (Zaret and Sherman, 1982; Russo et al., 1991; Abe et al., 1990; Henikoff and Cohen, 1984; Heidmann et al., 1994). 3′ Processing has been shown to occur at varying distances downstream from these motifs. Based on extensive mutational analysis of the *CYC1* 3′ *UTR*, Russo et al. (1993) have proposed the presence of three classes of signals: an 'upstream' sequence such as TAGNNNTATGTA or TATATA that directs 3′ end formation, a downstream element such as TTAAGAAC or AAGAA that specifies the 3′ end, and finally the site of polyadenylation itself, which usually occurs after cytidine residues. Saturation mutagenesis of the canonical element TAGNNNTATGTA has revealed that the variants that are functional have the general consensus of TAYRTA (Irniger and Braus, 1994). This is reminiscent of the polyadenylation signal AAUAAA of the higher eukaryotes.

Several experiments have suggested that termination

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Abbreviations: bp, base pair(s); *CYC1*, gene encoding iso 1-cytochrome *c*; *DED1*, gene encoding a putative RNA helicase; *Δ*, deletion; *HIS3*, gene encoding imidazole glycerol phosphate dehydratase; kb, kilobase(s) or 1000 bp; N, A or C or G or T; nt, nucleotide(s); ORF, open reading frame; PCR, polymerase chain reaction; Py, pyrimidine; R, A or G; RT, reverse transcription(tase); SDS, sodium dodecyl sulfate; ss, single strand(ed); UAS_{gal}, upstream activating sequence responding to the Gal4 activator; *UTR*, untranslated region; wt, wild type; Y, C or T.

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of transcription and 3′ processing are coupled. In one of the earliest studies on 3′ processing and transcription termination, a 38-bp deletion within the 3′ *UTR* of the *CYC1* gene of *S. cerevisiae* was shown to simultaneously affect 3′ processing and transcription termination (Zaret and Sherman, 1982). Several studies have subsequently shown that termination is related to 3′ processing in higher eukaryotes (Connelly and Manley, 1988; Logan et al., 1987). Different models have been proposed for the interdependence of 3′ processing and transcription termination (Connelly and Manley, 1988; Logan et al., 1987; Proudfoot, 1989). A strong processing site followed by a polymerase pause site may constitute an ideal termination signal (Proudfoot, 1989). In rare cases, termination has been shown to occur in the absence of a processing site (Sato et al., 1986). Protein bound to DNA at the 3′ end may be involved in termination in some cases. The main difficulty in understanding the relation between 3′ processing and termination of transcription is the short life of the pre-mRNA. Several studies have documented termination downstream from the polyadenylation site(s) in yeast (Hyman and Moore, 1993; Russo and Sherman, 1989).

The results presented here show that the polyadenylation sites of the *HIS3* mRNA are highly degenerate and heterogeneous and the signals directing 3′ end formation are redundant. Shorter internal deletions of the *HIS3 UTR* do not abolish 3′ processing. Interestingly, in the mutants, processing occurs at normal sites that are not covered by the deletion. These studies also show that the spacing between the upstream element and the site of processing is variable. Deletion of all sequences involved in 3′ processing leads to transcription past the normal 3′ end. A possible role of RNA secondary structure in directing mRNA processing is also considered.

2. Results and discussion

2.1. Mapping of the HIS3 mRNA 3′ end

As the first step in understanding the molecular events involved in mRNA 3′ end formation, the sites of 3′ processing and polyadenylation of steady state *HIS3* mRNA were determined. Total RNA isolated from a

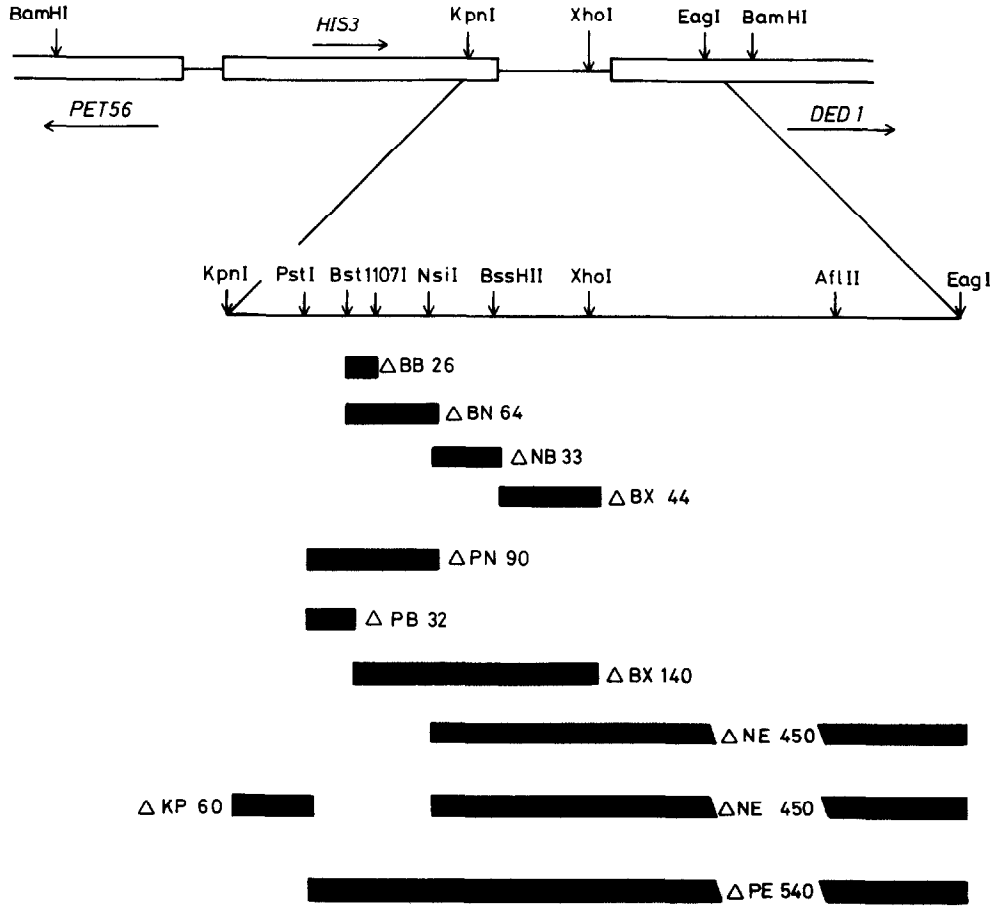

Fig. 1. Organisation of the *HIS3* gene. The open boxes represent ORFs. Arrows represent their orientation. The intergenic region between the *HIS3* and the *DED1* ORFs is amplified and some of the restriction sites are indicated. The bottom part shows the 5′ and 3′ boundaries of the internal deletions generated using the restriction sites.

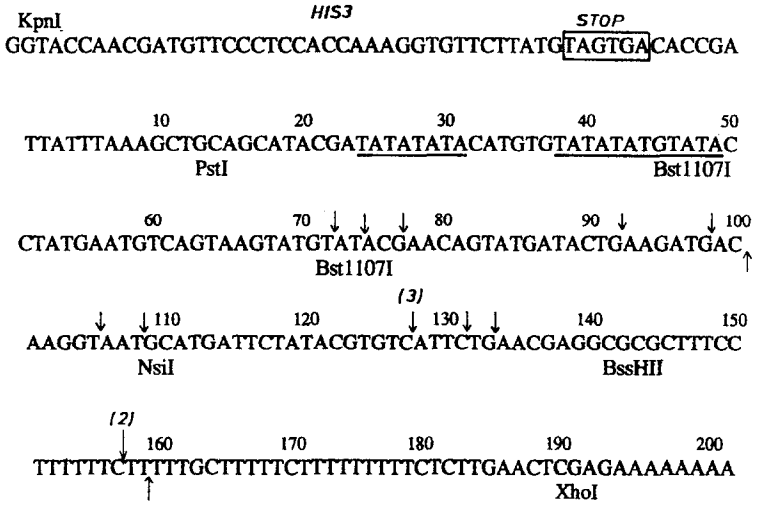

Fig. 2. Location of the sites of polyadenylation in the wt *HIS3* gene. Numbering of the bases is arbitrary. Position 1 corresponds to nt 1693 of the published *HIS3* sequence (Struhl, 1985, GenBank Accession No. X03245). Stop codon at the 3′ end of the *HIS3* ORF is boxed. The arrows indicate the alternative sites of processing and polyadenylation. Numbers in parentheses (*2* and *3*) indicate the number of independent clones (if more than one) showing processing at the site. **Methods:** The 3′ end of the *HIS3* gene was amplified using a procedure similar to the RACE protocol (Frohman, 1990). RNA was isolated from yeast cells carrying the wt *HIS3* allele and subjected to reverse transcription. Cell pellets were resuspended in a solution containing 10 mM Tris pH 7.5/10 mM EDTA/0.5% SDS and incubated with an equal volume of acid phenol at 65°C for 60 min with periodic mixing. The aqueous phase was re-extracted with phenol. RNA was precipitated with ethanol after removing phenol and resuspended in water. RT was carried out in 20 μl volume using Superscript RT (Gibco-BRL) and the primer 5′-GACTCGAGTCGACATCGA$(T)_{17}$. The cDNA synthesised was diluted to 50 μl and 5 μl was used in PCR reactions using the following two primers. *HIS3* primer: 5′-ATG-GTACCAACGATGTTCCCTCC; adapter primer: 5′-GACTCGAGTCGACATCG. PCR products were analysed on a 3% agarose gel (FMC-Nusieve). DNA fragments were purified from the gel and ligated with pUC19 vector after digestion with *Kpn*I + *Sal*I. The nt sequences of individual clones were determined by the chain termination method using T7 DNA polymerase (Sequenase Version 2, US Biochemical).

yeast strain carrying a wt copy of the *HIS3* gene (Fig. 1) was subjected to reverse transcription using a $(dT)_{17}$ adapter primer. The 3′ end of the *HIS3* mRNA was specifically amplified by PCR using an internal primer close to the 3′ end of the *HIS3* gene and the adapter used in cDNA synthesis. The PCR products were cloned and the sites of polyadenylation determined by nt sequencing of individual clones. Sequence analysis of 16 individual clones showed the presence of 13 different sites where polyadenylation occurred. In two cases, processing at the same site was seen in multiple clones (Fig. 2). The 13 sites were spread over a distance of 90 bp. The polyadenylation sites did not exhibit any apparent homology. The only feature that was common to these sites is that polyadenylation occurred next to a pyrimidine residue in nine out of 13 cases.

2.2. *Analysis of mRNA from mutants carrying deletions of the 3′* UTR

In an attempt to identify the *cis*-acting elements involved in directing 3′ end formation of the *HIS3* mRNA, deletions were generated using restriction sites present within the 3′ *UTR*. The extent of the deletions is indicated in Fig. 1. These deletions sequentially removed most of the *HIS3* 3′ *UTR*. To determine the effect of these deletions on 3′ end formation, total RNA was isolated from cells carrying the mutations on a centromere plasmid. The chromosomal copy of *HIS3* was deleted in these strains. The *HIS3* mRNA was probed using an internal *HIS3* probe on Northern blots. Initially five mutants carrying deletions spanning the region between the *Pst*I and the *Xho*I sites were analysed. All the mutants with deletions located between the *Bst*1107I and the *Xho*I sites showed transcripts similar in size to the normal *HIS3* message, but at reduced levels (Fig. 3A). The reduction in the levels of *HIS3* signal is likely to be due to the loss of a subset of processing sites in the mutants. When the deletion extended to the *Pst*I site close to the *HIS3* ORF, there was a drastic reduction in the level of *HIS3* mRNA. The ΔPN90 deletion covering the region between the *Pst*I and the *Nsi*I sites almost completely abolished normal *HIS3* message (Fig. 3A, lane 4) as opposed to the detectable levels of *HIS3* signal seen in the other mutants.

Since the ΔPN90 deletion overlaps with the ΔBN64 deletion (a mutation that does not eliminate detectable levels of *HIS3* mRNA) and at the same time leads to loss of *HIS3* mRNA, a shorter deletion that removed specifically the sequences between the *Pst*I site and the proximal *Bst*1107I site was constructed (ΔPB32). The region covered by this deletion contains the canonical sequences TATATA and TATGTA implicated in 3′ end formation (Russo et al., 1991; Abe et al., 1990). This deletion also resulted in a marked reduction of *HIS3*

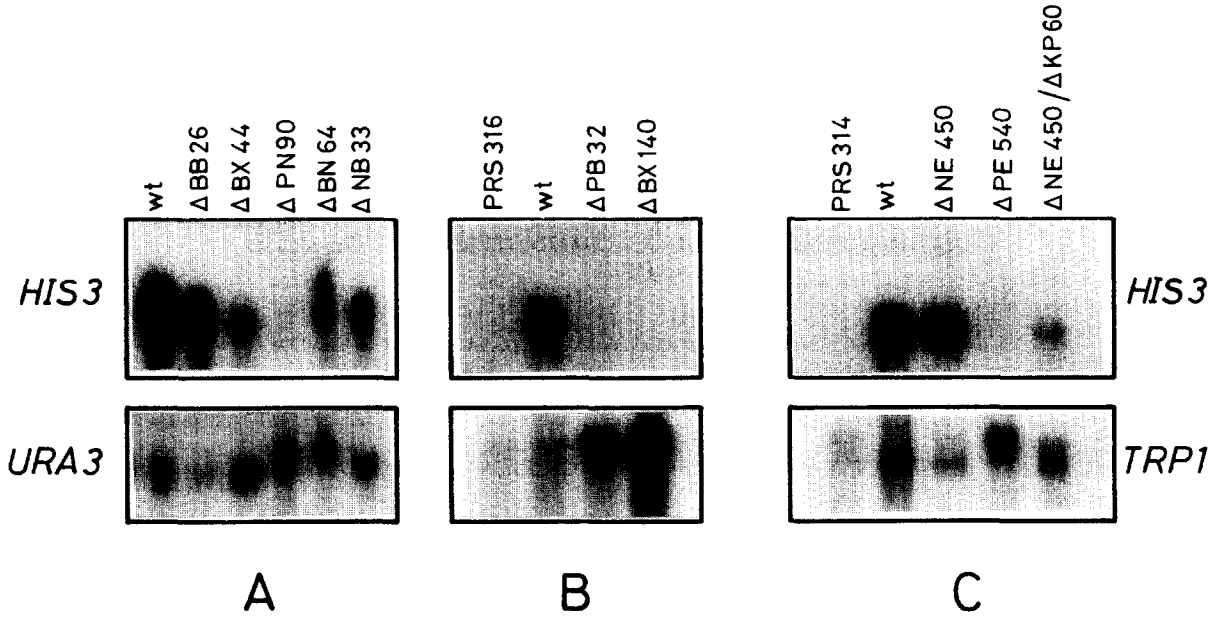

Fig. 3. Northern analysis of mRNA from wt strains and strains harbouring internal deletions of the *HIS3* 3′ end. Panels **A** and **B** depict analysis of RNA from transformants carrying the different *HIS3* alleles on pRS316 plasmid. RNA was normalised to the *URA3* transcript. Panel **C** depicts RNA analysis of mutant alleles present on plasmid pRS314. The *TRP1* transcript is used as an internal control. **Methods:** Plasmid derivatives carrying deletions of the *HIS3* 3′ *UTR* were constructed using the plasmid YIp55-Sc3309 (Struhl, 1984) which contains the complete *HIS3* gene and flanking sequences. The *HIS3* gene is under the control of the Gal4 activator as a 365-bp fragment containing UAS_{GAL} has been introduced upsteam from the *HIS3* gene. Internal deletions of the 3′ *UTR* were constructed using existing restriction sites (Fig. 1). *Bam*HI-*Eag*I fragments carrying the wt *HIS3* gene and the 3′ *UTR* deletions were subcloned into the pRS316 vector (Sikorski and Hieter, 1989) which has the *URA3* gene as a selectable marker. The deletions ΔNE450, ΔKP60/ΔNE450, and ΔPE540 (see Fig. 1) were constructed using the plasmid pUC8-Sc2676 (Struhl, 1984). This plasmid carries a 1.8-kb *Bam*HI fragment containing the complete *HIS3* gene under its own promoter. The *HIS3* alleles containing the deletions were subcloned into the pRS314 vector (Sikorski and Hieter, 1989), which is similar to the pRS316 vector, but carries the *TRP1* gene as the selectable marker. Northern analysis was carried out by standard procedures (see Sambrook et al., 1989). RNA samples were prepared from transformants of the yeast strain KY320 [**a**, *ura3*-52, *lys2*-801, *ade2*-101, *trp1*-Δ1, *his3*Δ200, Gal^+] carrying the wt and mutant *HIS3* alleles. Transformants were grown in minimal medium containing 2% galactose or glucose, omitting uracil or tryptophan depending on the plasmid vector used. After electrophoresis on 1.8% agarose-2.2 M formaldehyde gels, RNA samples were transferred to Hybond N^+ nylon membrane (Amersham). The blots were probed with a *HIS3* specific probe obtained by ^{32}P labelling an *Msc*I-*Xho*I fragment containing the 3′ end of the *HIS3* gene.

mRNA levels, most likely because of the loss of 'upstream' sequences that direct 3′ end formation (Fig. 3B, lane 3). The complementary deletion that covers the region between the *Bst*1107I and the *Xho*I sites (ΔBX140), containing all the known *HIS3* processing sites, also resulted in loss of the *HIS3* mRNA (Fig. 3B, lane 4). Thus, though the sequences covered by the PB32 deletion are necessary, they are not sufficient for 3′ end formation.

Deletion of all sequences downstream from the *Nsi*I site (ΔNE450) did not result in loss of the *HIS3* message (Fig. 3C, lane 3). Even when the 60 bp upstream from the *Pst*I site were deleted (ΔKP60) in the ΔNE450 background, detectable levels of *HIS3* transcripts can be seen (Fig. 3C, lane 5). The reduction in the *HIS3* signal may be due to instability of the message as the deletion also covers *HIS3* coding sequences including the normal translation termination signal. In contrast, the mutant ΔPE540, which carries a deletion of all sequences downstream from the *Pst*I site, did not show detectable *HIS3* transcript (Fig. 3C, lane 4). Therefore, the 90-nt region between the *PstI* and *Nsi*I sites within the *HIS3* 3′ *UTR* appears to be necessary and sufficient for directing the formation of at least a subset of the *HIS3* 3′ end. However, the ability of this sequence to direct 3′ end formation outside the *HIS3* context is yet to be tested.

To test whether termination of transcription downstream from the *HIS3* 3′ processing site is related to the processing event, a dot-blot analysis was carried out using a downstream probe to detect transcription past the normal 3′ end in the different mutants. The dot blot was used since the pre-mRNA is likely to be unstable and even partially degraded pre-mRNA, undetected on the Northern blot (Fig. 3), could be detected. Higher levels of transcription of sequences downstream from the normal *HIS3* message were detected only in the ΔPN90 mutant when *lacZ* (present downstream from *HIS3* in the plasmid) was used as a probe (sample 5, Fig. 4). The simultaneous loss of the *HIS3* message and production of transcripts past the normal 3′ end support the idea that 3′ processing and transcription termination are coupled (Zaret and Sherman, 1982). The background level of *lacZ* transcription seen in the wt and

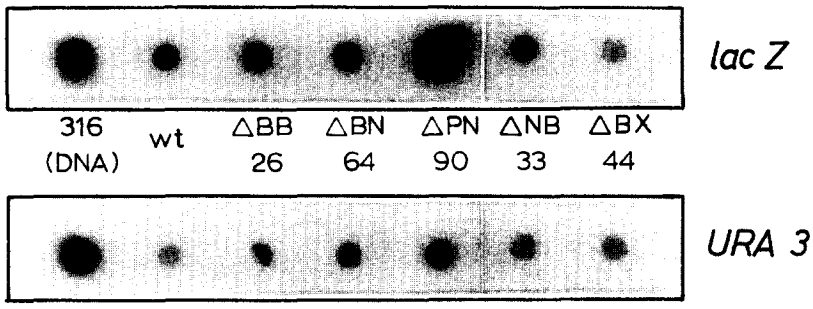

Fig. 4. Dot-blot analysis of total RNA from wt strains and strains containing deletions of *HIS3* 3′ end using *URA3* and *lacZ* probes. Deletions are described in Figs. 1 and 5. **Methods:** Blots were prepared using standard procedures (see Sambrook et al., 1989). After fixing the RNA samples on the membrane, the blots were probed with a radioactively labelled 0.6-kb *Eag*I-*Ssp*I fragment from the pRS316 vector, which contains the sequences immediately downstream from the *HIS3* gene, including a portion of the *lacZ* gene. A labelled 0.7-kb *Pst*I-*Alw*NI fragment contaning the *URA3* gene was used as a control probe for normalising RNA.

the other four mutants is likely to be due to transcription initiated at the *DED1* promoter. It is also interesting that the ΔBX44 deletion that results in reduction in *HIS3* signal does not cause appreciable readthrough into the downstream genes. The difference between the two mutants may be related to the degree of block in 3′ processing; the ΔPN90 deletion shows total loss of *HIS3* signal, whereas the ΔBX44 mutant shows detectable levels of *HIS3* transcripts.

2.3. *Mapping the 3′ ends of* HIS3 *mRNA in strains carrying deletion mutations*

The observation that the 3′ processing site is redundant in the wt suggests that multiple signals for polyadenylation are present at the 3′ *UTR* of the *HIS3* mRNA. This is also consistent with the observation that the various deletion mutants described above show *HIS3* mRNA of near normal size on Northern blots. To localise precisely the sites of polyadenylation in these deletion mutants, the 3′ end of the mRNA was amplified using RT-PCR as described earlier for the wt RNA. In each case, clones carrying the PCR products showed the presence of inserts of fairly homogeneous size on agarose gels. Two to four clones in each case were subjected to nt sequence analysis as described above. Sequence analysis of twelve individual clones obtained showed that the polyadenylation sites utilised in the four mutants characterised are the same as the ones found in wt, located outside the regions that have been deleted (Fig. 5). Therefore, though the polyadenylation sites are degenerate, there appears to be some specificity in the choice of the sites. However, since the number of PCR clones analysed is small, it cannot be categorically stated that processing at other sites does not occur in the case of the mutants.

2.4. *Computer analysis of RNA conformation*

To determine whether there is any correlation between processing site selection and mRNA secondary structure, the nt sequence of the *HIS3* 3′ *UTR* was analysed using the 'Fold' computer programme (Zuker and Steigler, 1981). The predicted RNA structure for the *HIS3* 3′ *UTR* showed several interesting features (Fig. 6). The 3′ *UTR* could fold to form two large hairpin structures separated by a hinge region, with a total free energy of −42.5 kcal. The most widely used polyadenylation site

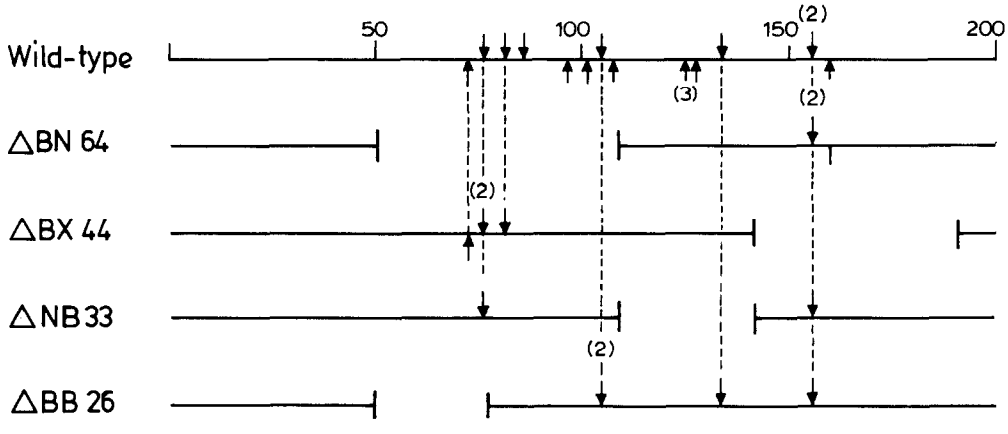

Fig. 5. Mapping of polyadenylation sites in mutants carrying internal deletions of the *HIS3* 3′ end. The methods used were similar to those used for mapping the 3′ sites of the wt *HIS3* gene (Fig. 2). The sequences deleted are indicated by gaps. Arrows are as in Fig. 2.

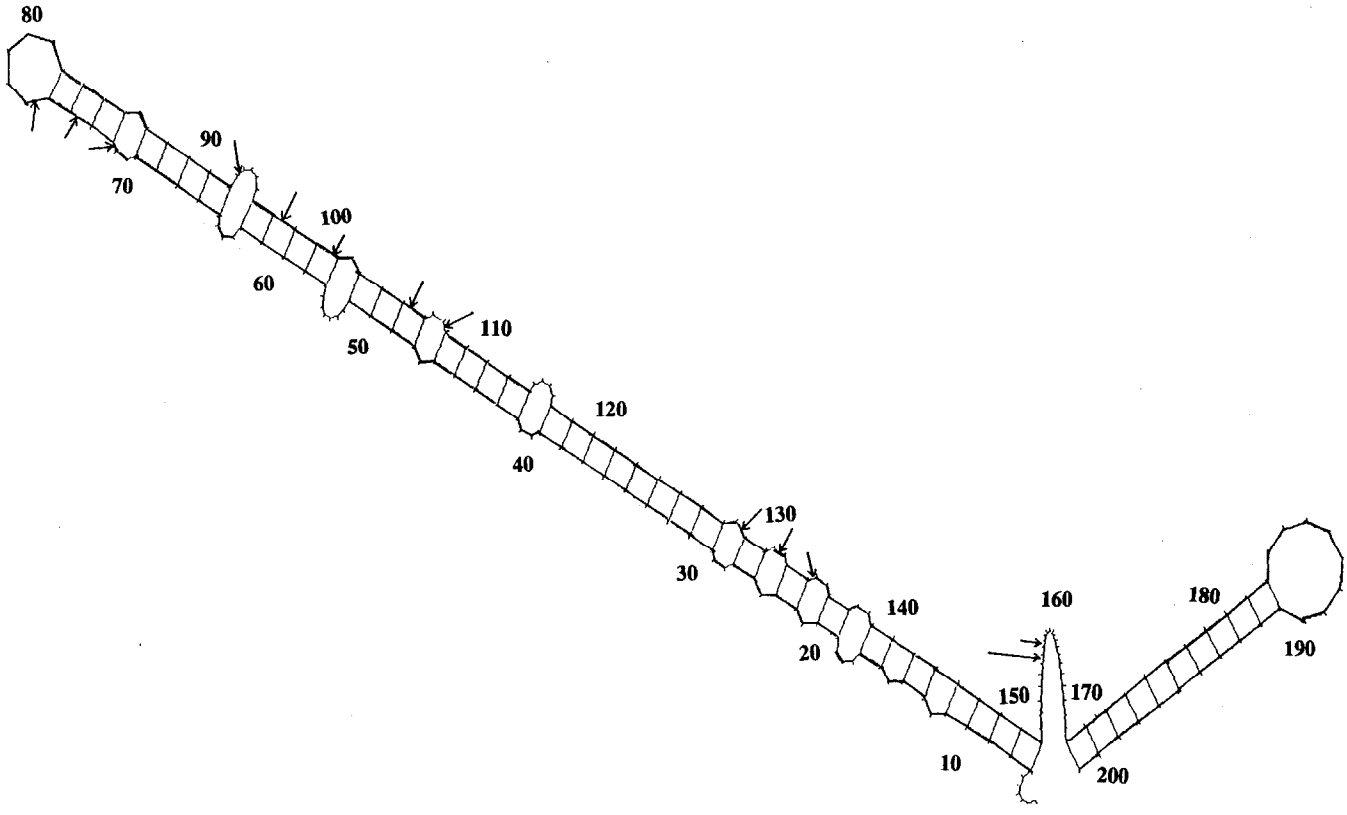

Fig. 6. Potential secondary structure of the *HIS3* 3′ end. Secondary structure of the 3′ *UTR* of the *HIS3* mRNA was analysed using the 'Fold' programme (Zuker and Steigler, 1981). The sequence analysed for secondary structure corresponded to 201 bp starting from +693 to +894 of the published *HIS3* sequence (Struhl, 1985; GenBank Accession No. X03245). Polyadenylation sites are indicated by arrows.

is located within the ss hinge region. Other polyadenylation sites detected in the wt are predominantly distributed at ss regions of the putative secondary structure. A possible involvement of mRNA secondary structure in processing site selection may explain the extreme heterogeneity and degeneracy seen in the processing sites. (The first hairpin structure is large enough to absorb changes brought about by the smaller internal deletions. In the case of the large deletion ΔBN64, the restriction sites are located on the opposite faces of the stem, halfway on the hairpin such that the deletion will result in a shorter structure compared to the wt. Similarly, in the presence of the deletion ΔNB33, the 3′ *UTR* can assume a structure with the region complementary to the deleted segment assuming an independent hairpin configuration. Therefore the deletions do not alter the predicted mRNA structure appreciably.)

3. Conclusions

(1) A major aspect of polyadenylation is the redundancy of poly(A) sites in many yeast genes that have been characterised. Sites of polyadenylation can be as many as three to twelve (Heidmann et al., 1992). The results reported here show that the *HIS3* mRNA is polyadenylated at least at 13 distinct sites. The striking feature of these sites is their heterogeneity. The only common feature among the different sites is that polyadenylation occurs preferably next to a pyrimidine, preceding an A residue. The sequence YAAA has been proposed as a consensus poly(A) site based on analysis of the poly(A) sites of the *ADH1* gene of *S. cerevisiae* (Heidmann et al., 1992). Though most of the sites seen in the *HIS3* mRNA have the sequence YA, none of them corresponds to the consensus YAAA. The most prominent site seen in six out of the 13 sites characterised has the sequence CTTT. Therefore the processing sites are degenerate in the different mRNAs examined. It is not clear why the sites are redundant and degenerate in yeast, unlike the higher eukaryotes where the processing sites are distinct. One possibility is that the processing reaction is intrinsically weak and the large number of sites present increase the probability of processing at least at one of the sites. Alternatively, the large number of processing sites may have evolved to assure complete 3′ processing and termination since intergenic regions are small in yeast and readthrough of transcription into downstream genes can be detrimental.

(2) An important characteristic of many upstream

sequences directing 3′ end formation is that they are necessary, but not sufficient (Heidmann et al., 1994). This suggests that there are additional sequences involved in 3′ processing. The deletion analysis of the *HIS3* 3′ *UTR* shows that the region between the *Pst*I and *Nsi*I sites is necessary and sufficient for 3′ processing: deletion of this sequence abolishes 3′ processing whereas deletion of sequences downstream from the *Nsi*I site and upstream from the *Pst*I site does not eliminate *HIS3* mRNA. By defining a sequence that appears to be necessary and sufficient for 3′ processing, characterisation of all the different signals located in the region will be possible. The 90-nt sequence contains a known 'upstream' element involved in directing 3′ end formation and a subset of processing sites. This region does not contain the 'downstream' elements TTAAGAAC and AAGAA observed in the *CYC1* 3′ *UTR* (Russo et al., 1993).

(3) The deletion analysis shows that the distance between the 'upstream' signal and the site of polyadenylation is variable. The ΔNB33 deletion that removes 30 nt does not affect processing at site 157 (Fig. 5). Similarly, the ΔBB26 and ΔBN64 deletions, which differ by 38 nt, do not affect processing at the same distal site. Since processing in the two mutants is directed by the same sequences upstream from the first *Bst*1107I site, a difference of 38 nt is tolerated by the machinery. However, these experiments do not rule out the possibility that the efficiency of processing at these sites is diferent in the mutants since the number of clones examined is small.

(4) Another interesting feature of the deletion analysis is that the processing sites seen in the mutants are the same as those seen in wt. This result suggests that the sites have some specificity which is not immediately apparent.

(5) Many upstream processing sites can function bidirectionally. One of the proposals that can explain this observation is that in addition to *cis*-acting sequences, mRNA secondary structure may be involved in directing 3′ processing (Irniger et al., 1991; Sadhale and Platt, 1992; Humphrey et al., 1991). Analysis of potential secondary structure of the 3′ end of the *HIS3* mRNA using a standard programme shows that the 3′ end has the potential to fold into two large hairpins with a hinge region (Fig. 6). In addition, the preferred processing site falls within the ss hinge region. Other processing sites observed are also located predominantly at ss regions. In the absence of a clear consensus for the 3′ processing site, mRNA structure, in combination with upstream determinants, offers an additional factor for processing-site selection. However, at this point, any role for mRNA secondary structure in 3′ processing remains purely speculative.

(6) The presence of degenerate signals for 3′ processing suggests the possibility that the factors involved in 3′ processing are also redundant. Alternatively, the same *trans*-acting factor may recognise different signals under different contexts. Elucidation of the precise mechanism of 3′ processing may have to await the biochemical characterisation of the processing reaction.

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