Many eukaryotic genes undergo alternative 3′-end poly(A)-site selection producing transcript isoforms with 3′-UTRs of different lengths and post-transcriptional fates. Gene loops are dynamic structures that juxtapose the 3′-ends of genes with their promoters. Several functions have been attributed to looping, including memory of recent transcriptional activity and polarity of transcription initiation. In this study, we investigated the relationship between gene loops and alternative poly(A)-site selection. Using the KYC1 gene of the yeast Kluyveromyces lactis, which includes a single promoter and two poly(A) sites separated by 394 nucleotides, we demonstrate in two yeast species the formation of alternative gene loops (L1 and L2) that juxtapose the KYC1 promoter with either proximal or distal 3′-end processing sites, resulting in the synthesis of short and long forms of KYC1 mRNA. Furthermore, synthesis of short and long mRNAs and formation of the L1 and L2 loops are growth phase-dependent. Chromatin immunoprecipitation experiments revealed that the Ssu72 RNA polymerase II carboxyl-terminal domain phosphatase, a critical determinant of looping, peaks in early log phase at the proximal poly(A) site, but as growth phase advances, it extends to the distal site. These results define a cause-and-effect relationship between gene loops and alternative poly(A)-site selection that responds to different physiological signals manifested by RNA polymerase II carboxyl-terminal domain phosphorylation status.

Transcription termination is coupled to 3′-end processing, which involves endonucleolytic cleavage of the nascent mRNA and addition of a poly(A) tail (1–3). Many genes exhibit two or more alternative 3′-end processing sites (APAs). In human cells, more than half of protein-encoding genes have alternative 3′-end processing sites (4). The cellular choice of 3′-end processing sites is regulated (4–6). Differential 3′-ends often occur within introns, thereby encoding proteins with markedly different functions (7–10). As many of the elements that modulate subsequent steps in mRNA fate are present within 3′-UTRs, mRNA length variation increases regulatory possibilities, including mRNA stability and translational efficiency (9). In humans, APA affects many cellular events, including inflammatory processes (4), and is regulated in a tissue-specific manner (11, 12).

Yeast also exhibits APA sites that have been correlated with distinct regulatory parameters (13–16). As a model gene for APA, we analyzed the CYC1 gene of the yeast Kluyveromyces lactis. KYC1 is transcribed as two mRNA isoforms, a consequence of two distinct 3′-end processing sites, generating short (S) and long (L) transcripts. The proximal site yields a transcript of 1.1-kb (S), whereas the distal site is 394 nucleotides downstream, producing a 1.5-kb transcript (L) (Fig. 1A) (15, 17). Alternative KYC1 processing is growth phase-dependent and is readily distinguished by changes in the ratio of short/long transcripts (15, 18). Components of Saccharomyces cerevisiae 3′-end processing complexes CF-1A and CPF affect proximal versus distal processing (17, 18).

CF-1A consists of Rna14, Rna15, Clp1, and Pcf11. CPF consists of an APT (Associated with Pta1) subcomplex that includes Pta1, Swd2, Syc1, and Ref2, two protein phosphatases, Glc7 and Ssu72, and the Pta1 protein, which appears to be a scaffold that bridges the APT subcomplex with core-CPF to form holoc-PF. These complexes and their subunits are conserved among yeast and mammalian cells (2, 19–21). CPF and CF-1A recognize specific RNA sequences in the nascent transcript. Subsequent to assembly of these two complexes, pre-mRNA is endonucleolytically cleaved, followed by addition of a poly(A) tail, catalyzed by poly(A) polymerase (2). 3′-End processing and transcription termination, defined by dissociation of the trimeric DNA-RNA-RNAP II complex, are coupled events, although the mechanism of termination and how it is coupled to 3′-end processing remain to be elucidated.

To address the factors that dictate proximal versus distal 3′-end processing events, we considered the potential effect of gene loops on APA selection. Gene loops juxtapose promoter-terminator regions and have been shown to facilitate the kinetics of transcription reinitiation (22–24) and promoter directionality (25). These effects are likely to be a consequence of RNAP II “hand-off,” resulting in recycling of RNAP II from...
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TABLE 1
DNA primers used in this study

| Name                          | Sequence                      | Position |
|-------------------------------|-------------------------------|----------|
| **F1**                        | 5’ ATCGATTTAAGGATAGCTCT    | 3’ P     |
| **F2**                        | 5’ CCGGTGTTTCGACCAAGG         | 3’ ORF   |
| **F3**                        | 5’ CTTTCACGTACACGTCACT        | 3’ ORF   |
| **F4**                        | 5’ GAACTCATTTTTGTATGAGAC     | 3’ T     |
| **F5**                        | 5’ CCTTTAATACCCATCGAACTATT   | 3’ T     |
| **F6**                        | 5’ CCCTTCACGTACACGTCACT      | 3’ ORF   |
| **R**                         | 5’ CAACTCATTCTGCTGATG        | 3’ T     |
| **R2**                        | 5’ ACTTAAAGAAGACCTTGAACAC     | 3’ ORF   |
| **R3**                        | 5’ TTTTAAATACCCATCGAACTATT   | 3’ T     |
| **R4**                        | 5’ TTTAAATACCCATCGAACTATT    | 3’ ORF   |
| **ACTF**                      | 5’ ATCGACACTCTAAGAATGA       | 3’ ORF   |
| **ACTR**                      | 5’ TCGACGCGAGGAAATGAGA       | 3’ ORF   |

**Table 1**

- **Primers for DNA-looping experiments**
- **Primers for ChIP experiments**
- **Tableau 1**: DNA primers used in this study.
- **Tableau 2**: Primers for ChIP experiments.

Materials and Methods

**Strains, Media, and Growth Conditions**—The K. lactis yeast strain NRR-L-Y1140, which expresses the wild type KICYCI gene encoding cytochrome c, was used in this study. The strain ZW13 was used to study KICYCI DNA loops in S. cerevisiae (32). The S. cerevisiae isogenic strain pair XH6 (WT) and POC8-23d (pta1-1), described previously (17), was used to analyze the effects of Pta1 on alternative gene loops. Cells were grown in YPD medium (33) to early logarithmic phase (A_600 = 0.6) or to mid-logarithmic phase (A_600 = 1.5), denoted E or M, respectively, in all experiments.

**RT-PCR**—Total RNA was isolated using the RNeasy Midi RNA isolation procedure (Qiagen) using cell pellets obtained from 50 ml cultures, grown in parallel with the cultures used for 3C analysis. The F3-R2 primer pairs detected both transcripts (L and S); R3-R4 was specific for the long form. Two actin gene (ACT1) primers were used as a PCR template control. PCR products were analyzed as described for 3C.

**3C Assay**—Gene loops were assayed as described previously (31), with the following modifications. Transient protein-protein and protein-DNA interactions were fixed by incubation of cells in 2% formaldehyde for 30 min at room temperature with gentle shaking. Cross-links were released by incubation in 0.25 mM glycerol for 10 min. Cells were washed in TBS buffer containing 1% Triton X-100 and resuspended in FA lysis buffer (50 mM HEPES-KOH, pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate). The chromatin pellet was collected by centrifugation and resuspended thoroughly in 100–120 μl of FA lysis buffer, followed by digestion overnight with the restriction enzyme MspI. The MspI digestion sites within KICYCI enable resolution of the promoter, ORF, two fragments of the 3′-UTR, and the two polyadenylation sites, denoted Py(A)-1 and Py(A)-2 (Fig. 1A). MspI digestion fragments were subsequently ligated in dilute solution to maximize intramolecular reaction products (31). Ligation products were assayed by PCR using tandem primers, F1 to F5, that anneal across the length of the KICYCI gene (Fig. 1B; Table 1). Primer pair efficiency control PCRs were performed using the KICYCI plasmid pCT2 as template DNA (15). pCT2 was digested by MspI and ligated in a 10-μl volume followed by PCR amplification using the same set of primers (Fig. 1B). PCR products were resolved in 1.5% agarose gels containing ethidium bromide. Band intensities were quantified using an AlphaImager 2200 (Alpha Innotech). Quantification was normalized by the ratio of the looping signal (SL) (for L1 or L2) to the control-loading signal obtained from the convergent primers F + R (SL/SS + SR).

**Chromatin Immunoprecipitation**—Yeast cells were grown under identical conditions as described for 3C. ChIP experiments were performed as a described (28), with the following modifications. Briefly, cells were lysed by vigorous shaking in a minibur beater (MiniBeadBeater-16, Model 607) for four cycles of 1 min each at 4 °C, and chromatin sonication times were increased to 12 pulses at 15 s each with 30-s intervals in ice-cold FA lysis buffer. The following antibodies were used to immunoprecipitate sonicated chromatin: α-Rpb3 (Neocline), spe-

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specific for the Rpb3 subunit of RNAPII; 8WG16 (Covance; ChIP grade), specific for hypophosphorylated Ser-2 of the CTD; and polyclonal H9251-Ssu72, specific for the Ssu72 CTD phosphatase (27, 28). These antibodies are used routinely in ChIP experiments using S. cerevisiae (28, 34, 35). Here, we demonstrate that they are equally effective and specific for ChIP experiments using K. lactis.

The primer pairs used for PCR amplification are listed in Table 1. PCR products were fractionated and detected as described above for 3C. The data were normalized by determining the IP_x/input ratio of each probed region (x) to the same IP/input ratio of the K. lactis Klla0F intergenic region (I) located 1330 bp from KICYC1 gene termination codon: ((IP_x/input_x)/(IP/input)). Data were quantified in all experiments as the mean of three independent experiments, each done in triplicate, as described (28). Values represent the mean; error bars were calculated as standard deviation of the mean. The statistical significance of means was determined by analysis of variance using SPSS software (IBM) (Table 2).

**Results**

**Experimental Strategy**—Transcription of the endogenous KICYC1 gene of K. lactis yields two distinct transcripts that differ in the lengths of their 3'-UTRs (15). 3'-End processing at the promoter-proximal poly(A) site produces a 1.1-kb transcript (S), whereas processing at the downstream sites yields a clearly discernable 1.5-kb transcript (L) (Fig. 1A). The poly(A) sites producing these transcripts are schematically depicted in Fig. 1B. Synthesis of the S and L forms is differentially regulated in response to cell culture growth phase, i.e. growth phase affects KICYC1 3'-end formation. The mechanism by which the 3'-end processing machinery differentially selects the poly(A)-1 versus poly(A)-2 site to generate the S versus L forms is related to the interplay between the proximal AU-rich element and the 3'-end processing machinery (17). RT-PCR analyses of the transcript isoforms under different regulatory conditions are shown in Fig. 1C. As expected, during early log phase growth (A_600 = 0.6) the S form is predominant, with no detectable synthesis of the L form, and in mid-log phase growth (A_600 = 1.5), synthesis of the L form is readily apparent. Note that in M phase, the F3-F2 signal (Fig. 1C, lane S) corresponds to the two transcripts, whereas R3-R4 (lane 6) is specific to the L form. Whether poly(A) site selection is associated with the establishment of two different DNA loops is unknown. Mechanistically, this is an important issue to understand co-transcriptional RNA processing. These results guided our experimental strategy to investigate the relationship between formation of DNA loops and APA.

**Gene Loops Correlate with Formation of the S and L Forms of the KICYC1 Transcript**—Juxtaposition of promoter-terminator regions to form gene loops raises the question of whether alternative poly(A) site selection affects differential loop formation. To address this issue, we first assayed gene loops at the KICYC1 locus in K. lactis. Results are presented in Fig. 2A. Cells harvested in early log phase growth (Fig. 2A, panel E) displayed the most intense 3C signal using primer pair F1 and F4, which anneals to the DNA fragment encompassing poly(A)-2, F1 yielded a PCR fragment, corresponding to gene loop L2, but at only ~24% of the intensity of the F1–F4 fragment (Fig. 2A, lane 4). By contrast, cells harvested in mid-log phase (subpanel M) yielded F1–F4 and F1–F5 PCR bands of approximately equal intensity (loops L1 and L2). To establish that these PCR products were specific to promoter-terminator regions, we demonstrated the absence of PCR products among the other primer pairs that anneal along the length of KICYC1 (Fig. 2B).

These sets of primer pairs indicate that KICYC1 loop specifically juxtaposes the promoter and polyadenylation sites and...
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To determine the distribution and phosphorylation status of RNAP II at the KlCYC1 locus, we performed ChIP experiments, using the 8WG16 antibody, which immunoprecipitates RNAP II that is hypophosphorylated at Ser-2 of the CTD, and using

excludes physical interactions among other regions of the gene. These results are not a consequence of different primer pair efficiencies, as a control experiment established that all primer pairs yielded PCR products of comparable intensities using KlCYC1 template DNA that had been digested to completion with MspI and subsequently ligated (see under “Materials and Methods”; results using primer F2 are an exception but do not interfere with interpretation of our results.) As additional controls, we also demonstrated that detection of the L1 and L2 gene loops by 3C are dependent upon both formaldehyde cross-linking and ligation subsequent to MspI digestion (Fig. 2C). Thus, gene loops L1 and L2 correlate specifically with formation of KlCYC1 mRNA termination at the proximal poly(A)-1 site and distal poly(A)-2 site, respectively.

Distribution of RNAP II Along the KlCYC1 Gene during Early and Mid-log Phase Growth—RNAP II occupancy was assayed by immunoprecipitation of the Rpb3 subunit, as described previously; indeed, immunoprecipitation of Rpb3 is universally used to detect occupancy of RNAP II complexes, irrespective of phosphorylation status (36). We studied the presence of Rpb3 at the two growth points of the logarithmic phase and at the two UTR processing regions. Results are shown in Fig. 3, B and C. Gene loops have been proposed to facilitate translocation of RNAP II from the terminator to the promoter for subsequent rounds of transcription (22, 23). Indeed, RNAP II competition experiment indicates that RNAP II is recycled from terminator to promoter in a manner dependent upon looping. Recycling, whether directly from the terminator to the promoter of the same gene or from the nuclear pool of RNAP II, requires dephosphorylation of the RNAP II CTD.

FIGURE 2. Alternative polyadenylation of KlCYC1 correlates with changes in gene loops. A, PCR products generated by 3C analysis of the KlCYC1 gene are depicted for cells in early log (E) and mid-log (M) phase growth. Primer pairs (F1–F5) are depicted above each lane and shown schematically in Fig. 1B. The F1–F4 and F1–F5 PCR products correspond to looping between the KlCYC1 promoter and the poly(A)-1 (L1) and the poly(A)-2 sites (L2), respectively. Results are shown for a single experiment. The mean value of three independent experiments was determined (see under “Materials and Methods”), B, primer pair efficiencies. PCR products were generated with the indicated primer pairs. C, 3C PCR products are dependent upon formaldehyde cross-linking. 3C experiments were performed as in A using early log phase cells and the indicated primer pairs. PCR results from cross-linked DNA are shown in lanes 2 and 6 and represent the L1 loop, comparable with A, lanes 3 and 4. Omission of formaldehyde from the same experiment yielded the data shown in lanes 3 and 7 (−F); results from omission of DNA ligase subsequent to MspI digestion of cross-linked chromatin are shown in lanes 4 and 8 (−L). The absence of PCR products from the F2-R and F3-R2 primer pair (lanes 10 and 11, −L) demonstrates the efficiency of MspI digestion. The 3C positive control experiments are depicted in lanes 12 and 13. Molecular markers (m) confirm PCR products of the anticipated length (lanes 1, 5, and 9).
antibody to the Rpb3 subunit of RNAPII, which immunoprecipitates RNAP II irrespective of CTD phosphorylation status. Results are presented in Fig. 3. Fig. 3A depicts a schematic of the KICYC1 locus indicating the regions probed by PCR primers. P1 and P2 are upstream of the promoter; P3 encompasses the promoter; OR and OT1, probe the early ORF and immediate 3′-UTR, respectively; T1 and T2 probe DNA encoding the indicated 3′-end processing sites; I probes the region immediately downstream of the poly(A)-2 site; and C serves as a control, probing a non-transcribed region located upstream of KICYC1. B, ChIP results for cells harvested in early log (E) and mid-log (M) phase using the 8WG16 or Rpb3 antibodies. C, quantification of Rpb1 and Rpb3 ChIP analysis comparing the growth phase (E and M). D, quantification of ChIP analysis comparing results using the Rpb1 and Rpb3 antibodies. All data are from three independent experiments equivalent to those shown in B. E, ChIP results from cells harvested in early log (E) and mid-log (M). Location of Ssu72 along KICYC1 and its dependence on the growth phase, using the Ssu72 antibody, are shown. Error bars represent the standard error of the mean of three independent experiments.

A second observation from this experiment is that occupancy of RNAP II associated with KICYC1 is higher at the termination region than along the length of the gene, an observation that has been seen for RNAP II at other loci (20, 37–39) presumably reflecting stalled RNAP II at poly(A) sites as part of the mechanism of 3′-end processing and transcription termination (Fig. 3, B and C). This ChIP result was substantiated and quantified using anti-Rpb3 antibody (Fig. 3D).
We note the difference at mid-log phase between RNAP II occupancy at T1 and T2; a significant difference ($p$ value = 0.030; Table 2, row 10) at the end of the KICYC1 ORF was also observed. The Rpb3 antibody signal reveals a significantly higher occupancy of phosphorylated RNAP II at T2 in cells harvested at mid-log phase (Fig. 3B, compare OT$^*$ in Rpb1 (8WG16) and Rpb3 in E and M). We interpret this difference to elongating RNAP II complexes reaching the distal processing position at mid-log phase. This result is consistent with an increase of 3’-end processing at the distal site (Fig. 1C) and also with formation of the L2 loop at M phase (Fig. 2A).

Alternative Polyadenylation and Ssu72 Occupancy of KICYC1—Ssu72 is a CTD phosphatase specific for Ser(P)-5 and Ser(P)-7 and an integral component of the CPF 3’-end processing complex (27, 40). Accordingly, Ssu72 is a reliable marker for assembly of CPF at the 3’-ends of genes. We determined the profile of Ssu72 association with KICYC1 by ChIP. Results are shown in Fig. 3E. In early log phase, Ssu72 is weakly associated with the promoter (Fig. 3E, P3, lane 4), and its occupancy increases substantially at the 3’-end of the gene (Fig. 3E, cf. OT, T1, and T2, lanes 6–8). In mid-log phase, Ssu72 exhibits a 2-fold increase in occupancy of the termination regions OT and T1 ($p$ value = 0.045, Table 2, row 5), coincident with enhanced synthesis of the S form of KICYC1 mRNA (Fig. 1), and a 4-fold increase in formation of the F1–F4 gene loop relative to the F1–F5 loop (Fig. 2). In contrast, we found no association of Ssu72 with the promoter (P3) in mid-log phase. As RNAP II processed along the gene, Ssu72 occupancy increased, but the relative amounts of Ssu72 are much lower at OT and T1, as expected for processive elongation of RNAP II beyond region T1 into region T2 (Fig. 3E, lanes 6–8). There was no significant difference, however, in Ssu72 association at T2 in early and mid-log phase cells, consistent with enhanced synthesis of the L form of KICYC1 mRNA and formation of the F1–F5 gene loop at this phase of cell growth (Fig. 2A). Therefore, there is a significant increase of Ssu72 association with the 3’-UTR regions where 3’-end processing and DNA-loop formation are predominant.

Gene Loops Direct Formation of the S and L Forms of the KICYC1 Transcript Expressed in S. cerevisiae—To determine whether the correlation between alternative polyadenylation of KICYC1 transcripts might be a general effect rather than idiosyncratic to K. lactis, we expressed plasmid-borne KICYC1 in S. cerevisiae and determined gene loops as described above. We observed the same result; in early log phase (OD = 0.6), looping occurs between the promoter and proximal poly(A) site (F1–F4 primer pairs) and is diminished between the promoter and distal poly(A) site (F1–F5 primer pairs) (Fig. 4A). These data are comparable with that observed for KICYC1 gene loop formation in K. lactis (Fig. 2A cf. Fig. 5). We did not assay potential changes in looping when cells entered mid-log phase growth (OD = 1.5) as no difference was observed between synthesis of the S and L forms of KICYC1 expressed in S. cerevisiae during this growth transition.

To address the cause-and-effect relationship between gene looping and alternative polyadenylation, we determined the consequences of a defective form of the CPF 3’-end processing complex on poly(A) site selection. This experiment was done using a pta1-1 mutant, shown previously to adversely affect KICYC1 APA (Seoane et al. (17)). As shown in Fig. 4B, the pta1-1 mutant blocks formation of the L1 loop, resulting in elimination of the S form of KICYC1 mRNA. Thus, Pta1 is essential for alternative DNA loop formation in S. cerevisiae. Furthermore, the coordinated formation of alternative DNA loops, associated with APA, is conserved between K. lactis and S. cerevisiae. These results define a novel function for gene loops, direction of alternative poly(A) site selection. Given the phylogenetic conservation of eukaryotic 3’-end processing complexes (2), we conclude that gene looping plays a general role in alternative poly(A) site selection.

Discussion

In this study, we demonstrate formation of alternative gene loops (L1 and L2) that juxtapose the KICYC1 promoter with either the proximal or distal 3’-end processing sites, correlating with formation of KICYC1 mRNAs that include short (S) and long (L) 3’-UTR regions. To our knowledge, this is the first report establishing a relationship between looping and alternative polyadenylation, suggesting a cause-and-effect relationship between these two events. It is also the first report showing formation of DNA loops in the yeast K. lactis. Moreover, looping and poly(A) site selection are physiologically regulated in response to cellular growth phase, in this case affected by early and mid-log phase growth.

What is the physiological relevance of the S versus L forms of KICYC1? These isoforms of KICYC1 mRNA exhibit different fates in response to different physiological conditions, including growth phase, oxygen availability, and the presence or absence of heme (18, 41). As cells progress through log phase growth, the S form is degraded, and the L form increases. The enhanced stability of the L form of KICYC1 ensures production of cytochrome c in the respiratory phase of growth. This effect is not specific to respiration but also occurs for transcripts encoding proteins of other metabolic processes (17). Accordingly, alternative 3’-UTR processing can be essential for many specific cellular functions.
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What is the relationship between synthesis of the L form of *KICYC1* mRNA and the L2 gene loop? *De novo* recruitment of RNAP II proceeds by assembly of the general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIF, and RNAP II) in a stepwise manner at the DNA promoter to form a transcription PIC. Subsequent to transcription initiation and promoter clearance, some of the general transcription factors dissociate from the promoter, whereas others remain behind, forming a scaffold-like structure (42). A pioneer round of transcription is then required to recruit the 3'-end processing complexes. In yeasts, CPF and CF-1A, which are analogous to the cleavage and polyadenylation-specific factor and cleavage stimulation factor complexes in mammalian cells (2), serve well defined functions in 3'-end processing that link the termination region back to the promoter scaffold.3 Accordingly, transcription reinitiation occurs by a pathway different from *de novo* PIC assembly. Subsequent to dephosphorylation of the RNAP II CTD, hypo-phosphorylated RNAP II is recycled from the terminator to the promoter. Given that the rate of transcription elongation by RNAP II is estimated at ~2000 nucleotides/min, yet PIC assembly and recruitment of the 3'-end processing machinery are relatively slow, requiring ~1 min for functional assembly (43), formation of the initiation and/or termination complexes, rather than transcription itself, is likely to be rate-limiting for gene expression, at least in relatively compact yeast genomes. Accordingly, formation and retention of gene loops that enable recycling of RNAP II would be a novel mechanism to enhance regulatory parameters. This conclusion is consistent with the looping-dependent, rapid kinetics of reactivation of the GAL10 and *HXX1* gene following a cycle of activation and repression (24, 39).

Differential association of Ssu72 with the 3'-UTR, as well as weaker but substantive association with the promoter (Fig. 3), has been reported previously for Ssu72 in *S. cerevisiae* (22, 23, 29, 30, 37). As Ssu72 is essential for loop formation (22, 23), this pattern of Ssu72 occupancy has been interpreted as a mechanism to enable loop formation and recycling of RNAP II from the terminator to promoter (44). A novel and significant observation from our studies is that as cells progress through the growth cycle, Ssu72 is displaced, presumably resulting in retention of the RNAP II CTD Ser5-P mark, allowing RNAP II to proceed past the poly(A)-1 site and progress to the poly(A)-2 sites located ~400 bp downstream (Fig. 3). Accordingly, the Ssu72 CTD phosphatase activity acts as a switch that either enhances or inhibits recognition of the proximal poly(A)-1 site; if inhibited, RNAP II would bypass poly(A)-1, resulting in enhanced processing at the distal poly(A)-2 site, formation of the L2 loop, and release of the L form of *KICYC1* mRNA.

Along the length of the *KICYC1* 3’-UTR, there are multiple putative consensus sequences for mRNA processing (15). These discrete 3’-UTR regions were also functional as separate processing units when expressed in *S. cerevisiae* (18). Indeed, multiple consensus 3'-end processing sites are a common feature of *S. cerevisiae* genes encoding multiple transcripts (45). Our results shed light on the mechanism of APA selection among consensus processing elements. ChIP analysis of RNAP II and the Ssu72 CTD along the length of *KICYC1* during two different stages of logarithmic cell growth demonstrated that at early log phase, RNAP II accumulates at the poly(A)-1 site and that this form of RNAP II is Ser-5 hypophosphorylated, resulting in processing at poly(A)-1 and formation of the L1 gene loop. By contrast, at mid-log phase, we observed an increase in RNAP II occupancy at the distal poly(A)-2 site that was not coincident with the hypo-phosphorylated form of RNAP II. Accordingly, differential phosphorylation/dephosphorylation of the CTD at Ser-5 and/or Ser-7 is the switch that determines whether RNAP II terminates at the proximal poly(A)-1 site or progresses further downstream to the distal poly(A)-2 site. These observations are not likely to be idiosyncratic to yeast, as recent studies in human cells have identified 3'-end processing proteins that are involved in alternative poly(A) site selection and can be attributed to different physiological conditions (46).

Alternative polyadenylation has been described in all eukaryotes (8). The relative abundance of processing factors affects APA site recognition. Here, we have shown that the AU-rich element at *KICYC1*, expressed either endogenously in *K. lactis*, or ectopically in *S. cerevisiae*, is the principal determinant of poly(A) site selection. Specifically for *KICYC1* expressed in *S. cerevisiae*, the AU-rich element at the proximal 3’-UTR determines APA selection, irrespective of growth phase, based on interaction of the RNA-processing factor Pta1. In this case, RNA processing is not dependent upon *PTA1* abundance (17). Our ChIP results demonstrate that Ssu72 occupancy at the *KICYC1* locus involves a change in UTR occupancy regulated by the growth phase that correlates with the predominant usage of the distal poly(A) site. Pta1 is very important in APA selection. The formation of the L2 loop also correlates with the predominant processing position. Ssu72 along with Pta1 are principal components of the CPF complex necessary to establish DNA loops (22, 28). Both factors have homologs in the human cleavage and polyadenylation-specific factor complex, which is analogous to yeast CPF (2, 47, 48). In Fig. 5 we present a model that correlates alternative polyadenylation with alternative gene loops, affected by the status of differentially phosphorylated RNAP II CTD phosphorylation. Moreover, changes in these parameters are affected by the physiological status of cell culture, with late log phase correlating with processing at the distal poly(A) site (poly(A)-2) and differentially phosphorylated RNAP II. We suggest that differential CTD phosphorylation is the driving force that distinguishes between poly(A)-1 and the L1 loop *versus* poly(A)-2 and L2 loop, facilitating an increase in the Py(A)-2 and L2 formation.

These results are likely to be of general significance, not specific to yeast, given the conservation of 3’-end processing components among eukaryotic organisms. As APA has been observed in all eukaryotic organisms, albeit to differing extents depending upon organism complexity, the basic mechanism(s) are likely to be universal. The interplays between APA, alternative gene loop formation, and modulation of RNAP II CTD phosphorylation are critical paradigms in the production of functionally distinct mRNA species encoded by a single gene. Moreover, transcriptional memory, maintained by gene loops, is likely to account for maintenance of functionally distinct mRNA species.
Author Contributions—M. A. F.-P. conceived and coordinated the study and wrote the initial draft of the manuscript. M. L. M. performed all of the experiments. The 3C and ChIP experiments (Fig. 3) were carried out under the guidance of B. N. S. while M. L. M. was working in the M. H. laboratory. M. H. worked with M. A. F.-P. to write the final draft of the manuscript. All four authors read and approved the content of the article.

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