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# The yeast *PHO5* promoter: Phosphate-control elements and sequences mediating mRNA start-site selection

**(gene regulation/transcription/yeast genetics)**

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**ABSTRACT** **Transcription of *PHO5* is strongly regulated in response to the level of inorganic phosphate ($P_i$) present in the growth medium. We have identified elements required for *PHO5* expression by analyzing small deletions in the *PHO5* promoter on chromosome II. The results reveal three functionally different components of the *PHO5* promoter: (*i*) regulatory regions, (*ii*) a "TATA" element, and (*iii*) specific mRNA initiation sites. The regulatory regions contain related 19-base-pair (bp) dyad sequences acting as phosphate-controlled upstream activation sites ($UAS_P$s). These $UAS_P$s mediate the transcriptional activation of *PHO5* observed in low $P_i$ conditions. The unlinked but coordinately regulated *PHO11* promoter contains a single copy of an almost identical dyad sequence, suggesting that there is a common regulatory $UAS_P$ for both genes. A TATA element is absolutely required for detectable *PHO5* transcription. Specific purine–pyrimidine motifs (RRYRR) (R = purine and Y = pyrimidine) serve as *PHO5* mRNA initiation sites, but only if they lie 55–110 bp downstream of a functional TATA element. Such an "initiation window" is not found in higher eukaryotes and implies mechanistic differences in the transcription machineries between yeast and higher eukaryotes.**

Faithful *in vivo* transcription of most eukaryotic genes by RNA polymerase II requires at least two distinct promoter elements. In higher eukaryotes, the first element is a highly conserved TATA motif usually found at a fixed distance of ≈30 base pairs (bp) upstream from the transcription initiation site (1). The second essential promoter element typically lies >100 bp upstream of the cap site and includes enhancers and gene-specific modulators (2–5).

Transcription in yeast *Saccharomyces cerevisiae* depends on the same essential elements, but the TATA sequence is not in a strict positional relationship to the initiation site(s). Upstream elements can promote constitutive expression (6, 7) or act as regulatory sites, called upstream activation sites (UASs) (8), which interact with specific regulatory proteins.

A well-studied example of an UAS is the region between the divergently transcribed *GAL1* and *GAL10* genes, $UAS_G$, to which a regulatory protein (GAL4) binds at four dyad-symmetric sequences mediating the galactose induction of both genes (9–11). Genetic analysis of the regulatory circuits involved in the *GAL* system reveals a striking similarity to those controlling the expression of the multigene family of acid phosphatases (12).

This gene family is composed of at least three structural genes (*PHO3, PHO5, PHO11*), all coding for secreted acid phosphatases (13–17). The interplay of several regulatory proteins leads to differential expression of these genes with respect to the concentration of inorganic phosphate ($P_i$). The *PHO5* and *PHO11* genes are inducible by low $P_i$, whereas *PHO3* remains essentially unaffected or even decreases slightly under the same conditions (15). Coordinate transcriptional activation of *PHO5* and *PHO11* is thought to be executed by two positive factors, *PHO4* and *PHO2*, which act on both promoters in an as yet unknown fashion. The activation is repressed by negative factors, *PHO80* and *PHO85*, which presumably complex *PHO4* in the uninduced state (12).

This report presents a functional analysis of the *PHO5* promoter. Specifically, we describe promoter elements required for the phosphate control of *PHO5* and examine sequences involved in transcription initiation and mRNA start-site selection.

## METHODS

The mutants and their construction are presented in Fig. 1. Media were as described (16) but contained 3% glucose to avoid *PDC1* repression (18). *PHO5* induction was done for 21, 24, and 27 hr as reported (16). Crude total RNA, prepared from frozen cells as described (19), was purified by LiCl precipitation (16), DNase I, and proteinase K treatment. Typically, 100–200 μg of RNA was obtained from 25 ml of culture. RNA mapping and quantitation by the reverse transcriptase assay are described in Figs. 3 and 4. Radiolabeled primers were obtained by enzymatically elongating synthetic oligomers specific to either *PHO5* or *PDC1* on single-stranded *PHO5* or *PDC1* templates as described (20).

## RESULTS

**Multiple Upstream Regions Regulate Transcription from the *PHO5* Promoter.** The first regulatory region, located ≈370 bp upstream of the ATG, is localized by the partially overlapping deletions Δ11 and Δ12. Both reduce transcriptional activation by a factor of 10. The flanking deletions Δ10 and Δ13, expressing essentially wild-type RNA levels, reduce the size of this element further to 31 bp (between positions −382 and −350).

Further analysis reveals another more complex regulatory region in the *PHO5* promoter. Removal of 82 bp, between −255 and −174 (Δ17), results in a reduction of total *PHO5* mRNA by a factor of 5. This region contains apparently two separate regulatory elements: one located close to the 5′ and the other near the 3′ border of Δ17. The approximate location of the 3′ element is evident from mutant Δ20, in which only 14 bp are removed reducing transcriptional activation by a factor of ≈3. Surprisingly, this defect can be restored progressively by increasing the size of deletion Δ20 in either direction (see Δ19, Δ18 and Δ21, Δ22; Fig. 1). Only the relatively large deletion Δ17 is again less inducible and

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Abbreviation: UAS, upstream activation site.

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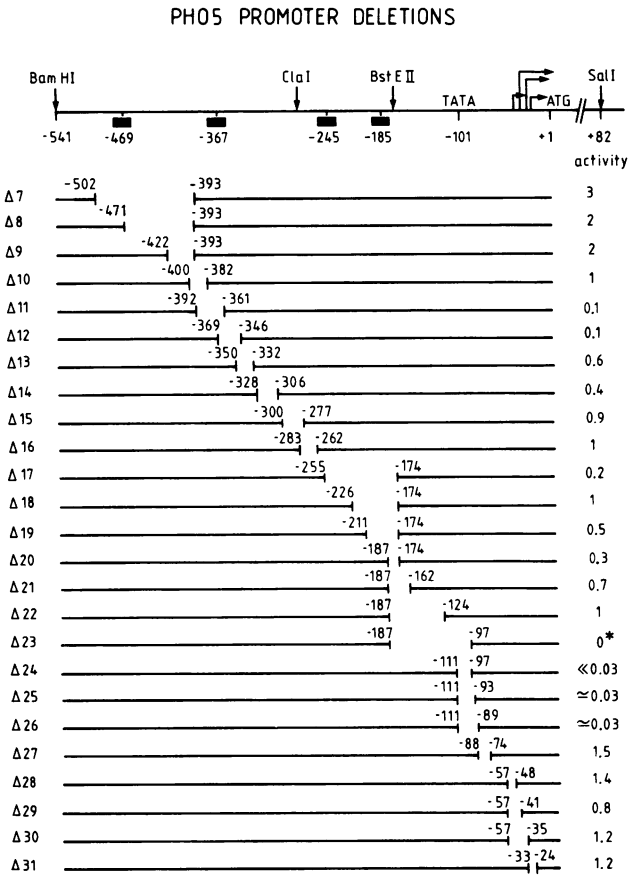


FIG. 1. The *PHO5* promoter and deletion derivatives. The restriction map shows 623 bp of the wild-type promoter. mRNA starts are indicated by arrows; TATA box and ATG are marked. Boxes represent the dyads discussed in *Results*. The deletion made in a particular mutant and the relative amounts of total PHO5 mRNA (wild type = 1, the asterisk marks a PHO5 mRNA level below our detection limits) measured as described in Figs. 3 and 4 are indicated. The deletions were made according to standard procedures (21). M13mp8 and M13mp9 derivatives (22) carrying the *Bam*HI/*Sal* I fragment shown were cut (by *Bam*HI, *Sal* I, or *Bst*EII), treated with BAL-31, ligated to *Eco*RI linkers, and recircularized. All deletions were sequenced (22). Internal deletions were made by ligating *Bam*HI/*Eco*RI and *Eco*RI/*Sal* I fragments obtained from the first series to the 7.9-kilobase *Bam*HI/*Sal* I fragment of YRp7-*PHO5* (23). Each construct was transplaced to the *pho5-URA3* locus of strain IH20 and verified by genomic DNA hybridizations as reported (23). The Ura3$^-$ cells were selected with 5-fluoroorotic acid (5-FOA; ref. 24) by incubating overnight $10^5$ cells from a pool of transformants in 1 ml of synthetic medium (25) without uracil but containing 1 mg of 5-FOA. Ten microliters of a uracil solution (2 g/liter) was added and incubation was continued for 2 or 3 days.

exhibits more functional damage than Δ20. This indicates a second regulatory element present within Δ17 just upstream of Δ18. Deletions Δ15 and Δ16 both allow full expression and mark the left border of the important sequences. We conclude from these data that the *PHO5* promoter contains in addition to the element mapped between deletions Δ10 and Δ13 two other regulatory elements: one in the region from −262 to −226 and the other around −187 to −174.

**The *PHO5* and *PHO11* Upstream Regions Contain Short Related Sequences with Dyad Symmetry.** A comparison between the promoter sequences of *PHO5* and *PHO11* including ≈350 bp upstream of the ATG has been published (26). We cloned the *PHO11* gene and extended the sequence analysis to about −500 bp (unpublished data). Both promoters contain a cluster of 29 nucleotides with nearly perfect homology (27 identical bases) flanked by nonhomologous sequences (Fig. 2*A*). This conserved region almost exactly coincides with the critical regulatory element identified between Δ10 and Δ13 by our deletions.

Nineteen bases of the cluster form a dyad centered at position −367 with five matching nucleotides. The symmetry becomes perfect when the actual DNA sequence is converted into a R/Y notation (R = purine and Y = pyrimidine) (Fig. 2*A*). In this respect it is interesting to note that the target sites of several restriction/modification enzymes contain a R/Y degeneracy at symmetric positions (for review, see ref. 27). These cases illustrate that at least some highly specific protein–DNA interactions require only a few conserved nucleotides and tolerate degenerate bases. The interaction between regulatory proteins and their target sites might follow similar rules. Therefore, we examined the *PHO5* promoter for copies of the symmetric R/Y motif to test whether they, if present at all, would share some conserved nucleotides.

Such copies were found at three other positions in the *PHO5* promoter by computer analysis. Two variants, at −245 and −185, lie within the Δ17 region, each close to one boundary. The third one is located at −469. The strong relationship of these five sequences becomes evident when all of the 10 individual dyad arms are examined for an underlying consensus sequence (Fig. 2*B*). Six nucleotides—three forming the core of the dyad, two in the middle, and one at the outside of an arm—are highly conserved. The other three positions show a strong bias to either a purine or a pyrimidine base.

Preliminary experiments using promoter fusions on plasmids indicate that each of these regions with dyad symmetry can confer phosphate-regulated expression. In these experiments, various fragments were inserted into the *GAPDH* promoter (at about 80 bp upstream of its presumptive "TATA" box; ref. 28) in both orientations and the hybrid promoters were tested for expression on high and low phosphate. In one set of experiments, the *Bam*HI/*Cla* I (or the *Cla* I/*Bst*EII) fragment of *PHO5* (see Fig. 1) and, in the other set, a synthetic oligomer containing the exact sequence from −382 to −350 were inserted into *GAPDH*. In all these constructions, the promoter fusions are strongly phosphate-regulated (unpublished data).

**A Single TATA Box Functions as Promoter Downstream Element.** Four deletions (Δ23–Δ26) show the most dramatic effects on transcription (see Figs. 1 and 4*B*). In strain Δ23, *PHO5* transcription is completely abolished, whereas Δ24 shows very low but still detectable amounts of *PHO5* mRNA. This residual level is significantly increased in Δ25 and Δ26, which exhibit reduction by a factor of ≈30 in transcriptional activity as compared to wild type.

The critical element responsible for the effects observed should lie between −124 and −88, since neighboring deletions (Δ22 and Δ27) exhibit wild-type activity. This 35-bp region includes a TATATAA motif at −101, in perfect agreement to the canonical sequence (1). A cryptic TATA-like sequence, possibly the CATATA motif at −154, might be responsible for the residual expression in Δ24, Δ25, and Δ26. Removal of both TATA boxes (Δ23) eliminates *PHO5* transcription; a deletion of the cryptic element, however, has no detectable effect (Δ22).

**Preferred Initiator Sequences Downstream of the TATA Box Determine the mRNA Start Sites.** In wild type, *PHO5* mRNA synthesis starts at four specific sites within a 20-bp-long initiation region located 53–72 nucleotides downstream of the TATA box. This was established by two independent RNA mapping procedures—S1 nuclease analysis (17) and primer extension (Figs. 3 and 4). Two equally abundant transcripts initiate at the major sites (−37 and −43) and comprise together ≈75% of total *PHO5* mRNA (Fig. 4). Two minor sites (at −49 and −34) flanking the major starts produce ≈25% of total *PHO5* mRNA with a slight preference for the shorter transcript.

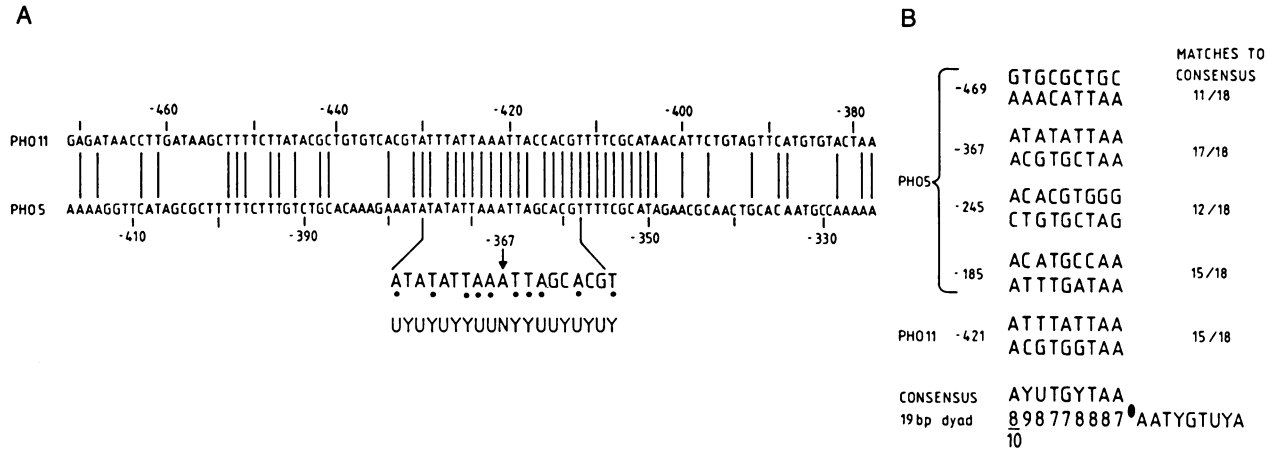


FIG. 2. Dyad-symmetric sequences present at *PHO5* and *PHO11*. (*A*) *PHO5* and *PHO11* 5′ flanking sequences. Numbers mark the distance from each ATG. The 19-bp dyad at position −367 in *PHO5* is displayed in a purine (U)–pyrimidine (Y) notation and dots mark the matching nucleotides. N, core of the dyad. (*B*) Five dyads found in both promoters and their positions. The resulting consensus is shown at the bottom, and the fitting of each motif to this sequence is indicated.

Five mutants with altered initiation of *PHO5* mRNA were obtained (Fig. 4). Shortening the distance between TATA and the initiation region by 7 bp (Δ27) results in increased expression from sites distal to the TATA element and concomitant repression of the proximal starts—a phenomenon similar to that observed with other yeast genes (30–32). Two mutants, Δ28 and Δ31, keep the sequence of the initiation region and its distance to the TATA box essentially intact but carry the 8-bp linker very close to the 5′ or the 3′-side of the initiation region. In Δ28, very little RNA is initiated at the site representing the first major start in wild type, although this nucleotide is only shifted two bases toward the TATA box. Mutant Δ31 shows faithful initiation at the sites proximal to TATA, but the frequency of initiation at the other sites differs clearly from wild type. The mutants Δ29 and Δ30, in which either one or both major starts are deleted, illustrate that new but specific sites can efficiently substitute for wild-type initiation sequences.

## DISCUSSION

**Phosphate-Control Elements.** Our deletion analysis reveals multiple regulatory regions in the *PHO5* promoter, presumably interacting with the regulatory proteins (PHO4 and PHO2), defined by genetic and molecular analysis (12, 33). For these protein–DNA interactions, two distinct mechanisms can be envisaged: In one model, the regulatory regions found represent two different classes of binding sites with one binding exclusively *PHO4* and the other binding solely *PHO2*. An alternative model postulates that only one of the regulatory proteins (or a complex of both) binds at several essentially identical sites to the *PHO5* promoter. The former model is analogous to *CYC1* (34); the latter is analogous to the *GAL* system.

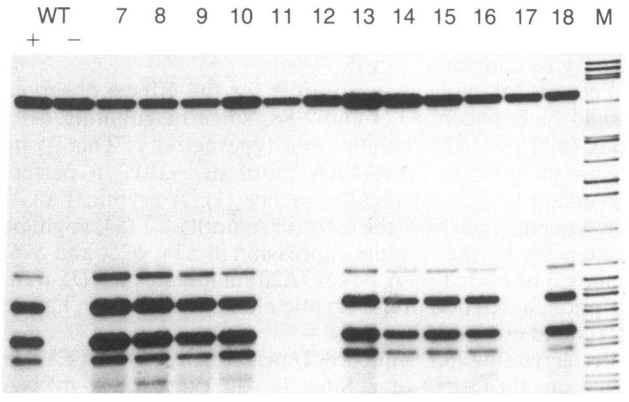


FIG. 3. Quantitative RNA mapping. Twenty micrograms of total RNA was precipitated together with *PHO5* and *PDC1* primer (0.04 pmol each, sufficient to detect quantitatively PHO5 and PDC1 mRNA as verified in test experiments; data not shown). PHO5 cDNA, resulting from reverse transcriptase reaction without actinomycin D (29), was alkali-treated (21) and analyzed by gel electrophoresis. For each strain, RNA from all three induction periods was assayed (only 24-hr samples shown). Two lanes (WT) show wild-type RNA assayed with (+) or without (−) *PHO5* primer; other lanes represent the mutants Δ7–Δ18.

Several observations suggest the second model. First, the *PHO11* promoter, which is also phosphate-controlled (33), contains a highly conserved copy of the putative binding site (at −421; see Fig. 2*A*) in a background of nonhomologous sequences. These findings and preliminary data from the promoter fusion experiments strongly suggest a functional role for this dyad symmetry element and imply that one regulatory region is sufficient to mediate phosphate control.

Second, we have shown that the regulatory regions of the *PHO5* promoter contain dyad-symmetric sequences strongly related to a common consensus dyad. In our single binding site model, variants of this sequence should function as binding sites, each with a differential affinity to the regulatory protein (complex). Indeed, we observe that the functional damage due to deletions of the individual regulatory sites reflects their departure from the consensus.

Finally, transcriptional activation of *PHO8* (a vacuolar alkaline phosphatase) does not depend on *PHO2* but requires *PHO4* (35), which might therefore be the crucial DNA-binding protein. Moreover, the possibility that one of the positive factors acts posttranscriptionally during *PHO5* induction has not been excluded.

In summary, we conclude that transcriptional control of the *PHO5* promoter is coordinately mediated by repetitive units (at −367, −245, and −185; see Fig. 1), each representing a functional phosphate-controlled $UAS_P$. The results with the promoter fusions suggest that both states of the *PHO5* promoter—activation and repression—are mediated through the same DNA sequences, the $UAS_P$s. They work in both orientations and confer their specific regulation on unrelated yeast promoters, as shown for several other UAS regions (36–38). Repetitive structures have been proposed for the upstream regions of several viral and cellular genes (for review, see ref. 39). In yeast, functional redundancy is seen with a short sequence found nontandemly repeated upstream of genes subjected to the general amino acid control (40). Similarly, the $UAS_G$ region shows within 120 bp four *GAL4*

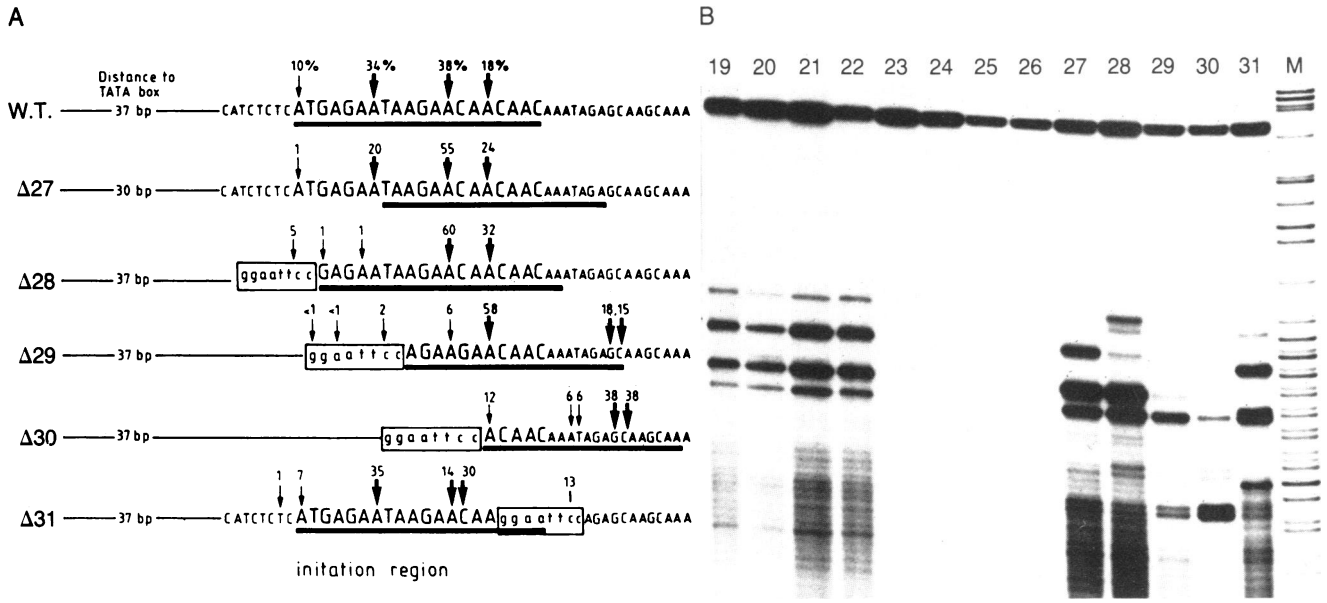


FIG. 4. Normal and altered PHO5 mRNA initiation. For RNA quantitation performed with all 26 strains, the cDNA bands and adjacent background slices were cut out from the gels and scintillation counted. Total PHO5 mRNA was calculated as the sum of the corrected values for the individual transcripts divided by the *PDC1* value. Individual transcripts were calculated as percentage of total PHO5 mRNA. Most strains, except Δ27–Δ31 and TATA deletion mutants (only total PHO5 RNA determined), produced the transcripts in essentially the same ratio as wild type (data not shown). Arrows mark the 5′ end of a transcript and give its amount relative to total PHO5 RNA as found in mutants Δ27–Δ31 and wild type. Nucleotides present in the 20-bp wild-type initiation region are displayed in capital letters. The corresponding region in each mutant (53–72 bp downstream of TATA) is indicated by a solid bar. The synthetic linker sequence is boxed. (*B*) Reverse transcriptase assay for mutants Δ19–Δ31 performed as described in Fig. 3.

binding sites (11). The $UAS_{PS}$ of the *PHO5* promoter lie within a 200-bp region (Fig. 1). The results with two sets of nested deletions (Δ18–Δ20 and Δ20–Δ22) suggest that the distance between UASs and TATA can influence the expression level. Formally, the removal of negative elements present on both sides of Δ20 could also explain the expression observed in these mutants, which is increased relative to Δ20. However, none of the strains showed increased phosphatase activity on high $P_i$ medium as compared to wild type (data not shown).

Recently, Bergman *et al.* (41) presented a deletion analysis of the *PHO5* promoter and identified sites for phosphate regulation that are different from those defined here. They conclude that a sequence (CTGCACAA$^{A}_{T}$G) apparently found twice in the *PHO5* promoter (from −391 to −382 and from −341 to −332) acts as a regulatory element in phosphate control. Our data show that these repeat regions contribute only weakly, if at all, to *PHO5* expression (see Δ10 and Δ13). Based on our analysis of integrated constructs, we have assigned regulatory functions to different promoter regions (from −382 to −350, from −262 to −226, and from −187 to −174). One explanation for the difference between our results and those of Bergman *et al.* (41) is that our constructs were all examined at their normal chromosomal location without flanking vector sequences, whereas those of Bergman *et al.* (41) were tested on high-copy plasmids. It is possible that flanking vector sequences, high-copy number, and/or location on a circular plasmid influence the expression of their constructs.

**mRNA Start-Site Selection.** Studies with *CYC1* (30), *HIS3-DED1* (31), and *HIS4* (32) suggest that yeast RNA polymerase initiates mRNA synthesis at specific sites within an initiation window from 40 to 120 bp downstream of a functional TATA element. Functional analysis of initiation sites at *CYC1* indicated that the sequence TC$^{G}_{A}$A was a strong initiation site and comparisons of initiation sites in published sequences showed 17% had this motif and 38% had a different sequence (RRYRR; see ref. 30).

Transcription initiation at *PHO5* has many similarities to mRNA synthesis from other yeast promoters. The *PHO5* promoter contains a TATATAA sequence at −101 and the initiation sites are all within 53–72 bp downstream of this sequence. Deletions of TATA (Δ24–Δ26) lead to low levels of PHO5 mRNA, which is initiated at the normal sites. This result indicates that the TATA element affects primarily the frequency and not the position of an initiation event. The inability of the most proximal site to initiate mRNA in Δ27 reflects the general observation that a minimal distance from the TATA element is required. Furthermore, as in other yeast genes, deletion of normal initiation sites changes the position of transcript initiation but does not lower the total expression. Three of the four initiation sites at *PHO5* contain the RRYRR motif seen in a number of other yeast genes (30).

Our mutants offer some interesting insights into the utilization of mRNA start sites. The suggestion that the RRYRR motif represents a strong site for RNA initiation is substantiated by our analysis of Δ29 and Δ30, which lack some of the normal initiation sites and bring new sequences closer to TATA. In these deletion mutants two new initiation sites appear and both contain the RRYRR sequence. In fact, in Δ30 ≈75% of all transcripts initiate from these new RRYRR sites. Since these strong initiation sites lie within the initiation window, it is curious that they are not used in wild type and only appear in the deletion strains. Furthermore, initiation from these cryptic sites is much more efficient in Δ30 as compared to Δ29, even though in both deletions these sites are at similar positions relative to the TATA element and lie well within the initiation window. Therefore, we conclude that it is the stepwise removal of the proximal initiation sites and not the slightly reduced distance from TATA that accounts for the gradual activation of the cryptic sites.

In this view, the initiation window (55–110 bp downstream of TATA) contains multiple sites competent for mRNA synthesis. We assume that the actual starts within this

window are selected by a mechanism that prefers sites proximal to TATA. Consequently, since RNA synthesis is initiated at competent sites, efficient proximal starts prevent the use of potential sites located downstream in the window. Correspondingly, the loss of some proximal starts shifts initiation toward distal sites. Our experimental data strongly support this view. The observed sequence specificity (RRYRR and other motifs; see ref. 30) could either reside in the RNA polymerase (subunits) or be mediated by initiator protein(s). That other factors such as neighboring sequences can influence the selection of start sites is shown by the phenotypes of mutants Δ28 and Δ31. The availability of an *in vitro* transcription system would clarify the mechanism underlying this directive mode of mRNA start-site selection.

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