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Genetics

The yeast *his3* promoter contains at least two distinct elements

(eukaryotic gene expression/transcription/chromatin/RNA polymerase II/deletion mutants)

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ABSTRACT **Phenotypic analysis of 65 mutations indicates that the yeast *his3* promoter is composed of at least two separate regions of DNA. Each is necessary, but neither is sufficient for wild-type levels of *his3* expression. Deletion mutations that destroy either promoter element express *his3* poorly or not at all. The upstream element is located between 112 and 155 base pairs before the site of transcriptional initiation (nucleotides −112 to −155). A comparison of derivatives strongly suggests that the downstream element maps somewhere between nucleotides −32 and −52 and includes a sequence between nucleotides −45 and −52. This location coincides with sequences conserved before most eukaryotic genes (the TATA box region). By using derivatives in which *his3* sequences are replaced by a small fragment of coliphage M13 DNA, three properties of the *his3* promoter were established. First, *his3* TATA box deletions fail to express *his3* because they lack specific sequences and not because they disrupt spacing relationships between other sequences. Second, the TATA box region can be replaced functionally by the one orientation of the M13 DNA fragment that contains a TATA-like sequence. Third, the distance between the two elements (normally 90 base pairs) can be varied between 40 and 160 base pairs without markedly affecting promoter function. These results strongly suggest that yeast RNA polymerase II, unlike its *Escherichia coli* counterpart, does not bind simultaneously to both promoter elements, and they add further support to the view that the upstream element is not part of a transcriptionally competent binding site. This ability of the *his3* upstream promoter element to act at a long and variable distance is similar to properties of viral enhancer sequences and is reminiscent of position effects in yeast.**

Promoters are sites of DNA necessary for the transcription of structural genes (1). *Escherichia coli* promoters are composed of two noncontiguous sequences (reviewed in refs. 2 and 3). These are located approximately 32–37 base pairs (the −35 sequence) and 6–12 base pairs (the Pribnow box) upstream from the start of transcription. *E. coli* RNA polymerase interacts directly with each of these elements, thus leading to the conclusion that the prokaryotic promoters may be equated with RNA polymerase binding sites that are necessary and sufficient for transcription.

Transcription of eukaryotic genes by RNA polymerase II also requires DNA sequences flanking the 5′ end of the mRNA coding region (4–6). However, unlike the situation of *E. coli*, the phenotypes of mutant genes depend upon the assay. When phenotypes are determined *in vivo*, sequences more than 100 base pairs upstream from the mRNA coding region are implicated as promoter elements (6–8). On the contrary, when phenotypes are assayed by transcription *in vitro* of purified DNAs, these upstream sequences seem to play no role. Here, the critical sequence, the TATA box, is located much closer to the site of transcriptional initiation (9, 10). The TATA box was initially proposed as a promoter element because it is (*i*) highly conserved before most eukaryotic genes and (*ii*) homologous in DNA sequence to the *E. coli* Pribnow box (11). However, deleting the TATA box of the simian virus 40 (SV40) tumor antigen gene seems to have no effect on the expression level (6), and similar deletions of the sea urchin histone 2b gene (5) or the herpesvirus thymidine kinase gene (8) (assayed after microinjection into frog oocytes) decrease transcription by a factor of ⅕ at most, a relatively minor effect. In view of these apparently conflicting results, it is difficult to draw firm conclusions about the nature of a eukaryotic promoter.

This paper continues previous genetic analysis of the *his3* promoter of baker's yeast, *Saccharomyces cerevisiae* (4, 7). The key feature of this approach is that the phenotypes of all mutant genes are determined under true physiological conditions; i.e., cloned mutant DNAs are introduced back into genetically defined yeast strains such that each resulting cell contains one copy at the normal *his3* chromosomal location. Previously, I showed that the *his3* promoter is surprisingly large in that it includes a sequence located 112–155 base pairs before the start of transcription (7). Here, I describe 41 additional mutations that lead to the following conclusions: (*i*) The *his3* promoter contains at least two distinct elements. (*ii*) the *his3* TATA box region suffices for the downstream element. (*iii*) The distance between upstream and downstream elements is not a critical factor for promoter function.

MATERIALS AND METHODS

The wild-type *his3* gene used in these experiments is a 6.1-kilobase-pair (kb) fragment (Sc2605) that contains the entire gene as well as 2.5 kb flanking the 5′ end and 2.8 kb flanking the 3′ end (12). All the deletion mutations described here derive from Sc2605. The schemes for generating mutants are sketched in the text and in Fig. 2; the details concerning isolation, characterization, and DNA sequence analysis will be published elsewhere.

The procedures used to assay the phenotypes of mutant and wild-type genes have been published (7, 13). Briefly, *his3* DNA fragments cloned in the *ura3*$^+$ vector YRp14 (4) are introduced into a *ura3*$^-$ *his3*$^-$ yeast strain. Uracil-independent transformants are selected, and those containing one copy of the transforming DNA integrated at the *his3* locus are analyzed for *his3* expression. The phenotypic test is the ability of the relevant transformants to grow in the absence of histidine. Those that grow (His$^+$) express *his3*, whereas those failing to grow (His$^-$) do not express the gene. For some mutations, the level of gene expression is quantitated by measuring the enzyme activity of

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Abbreviations: kb, kilobase pair(s); His$^+$ and His$^-$, histidine independence and dependence; SV40, simian virus 40.

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the *his3* gene product (imidazoleglycerol-phosphate dehydratase).

RESULTS

The *his3* Promoter Contains at Least Two Elements. Previously, I described 24 deletion mutations that successively encroach upon the 5′ end of the *his3* gene (7). These derivatives fall into three distinct phenotypic classes. Derivatives that retain more than 155 base pairs upstream from the start of *his3* transcriptional initiation (class I) are phenotypically indistinguishable from the wild-type allele. Class II derivatives retain between 60 and 113 upstream nucleotides and express *his3* poorly, while class III derivatives, which contain less than 45 upstream base pairs, fail to express *his3*.

From these results, two strong conclusions can be drawn. First, the entire *his3* promoter is located within the 155-base-pair region adjacent to the 5′ end of the structural gene. Second, the most upstream promoter element includes a sequence between −113 and −155. In addition, the distinction between derivatives that express *his3* partially or not at all suggests that the promoter may contain a second element.

To prove that the *his3* promoter contains two distinct elements, it is necessary to show that the upstream element is not sufficient for wild-type levels of gene expression. Nine deletion mutations retain the entire upstream element but nevertheless fail to express the gene (see Fig. 1). Thus, it is possible to delete either of two separate regions of DNA and greatly reduce *his3* promoter function. This indicates that the promoter contains at least two elements, both of which are necessary but neither of which is individually sufficient.

The TATA Box Region Can Serve as the Downstream Promoter Element. The extent of *his3*-Δ38, the smallest deletion that leaves the upstream element but eliminates promoter function, indicates that the downstream element includes a sequence located 32–80 base pairs upstream from the mRNA coding region (nucleotides −32 to −80). The region between nucleotides −37 and −51 includes sequences conserved among essentially all eukaryotic genes, the TATA box. In this section, I show that the *his3* TATA box region is sufficient to constitute the downstream promoter element.

The experimental design is as follows. The promoter region is disrupted at various locations by insertion of the octadeoxynucleotide 5′-G-G-A-A-T-T-C-C-3′ (*Eco*RI linker) (experimental details to be published elsewhere). The promoter is then divided into upstream and downstream "halves" by cleavage with *Eco*RI endonuclease. New *his3* derivatives are constructed by pairwise ligation of upstream and downstream promoter fragments. Thus, a matrix of deletion mutations is generated (Fig. 1). The downstream deletion end points map at −8, −20, −32, −53, and −73. The upstream deletion end points map at −32, −80, −106, −112, and −119.

The phenotypes conferred by these deletion mutations fall into a clear pattern. All 12 derivatives that lack the region between nucleotides −32 and −52 fail to express *his3*, whereas the 9 mutations that retain this region all express the gene at some level. Furthermore, deletion 36 (which removes nucleotides −31 to −8) and deletion 28 (which removes nucleotides −106 to −53) express *his3* at the wild-type level. Thus, the region between nucleotides −32 and −52 suffices as the downstream promoter element. Because this location completely includes and almost exactly coincides with the *his3* TATA box region, the downstream element will be called the TATA box element.

As stated in the previous section, the existence of the downstream promoter element was initially inferred from the distinction between class II and class III derivatives. From positions of the relevant deletion break points, this element was proposed to include a sequence between nucleotides −45 and −60. Thus, the two methods used to localize the downstream element (sequential deletions from the 5′ end and a matrix of internal deletions) give overlapping answers. The combined results indicate that the downstream element includes a sequence between nucleotides −45 and −52 and it may include sequences as far downstream as −32. This provides strong support for the idea that deletion of the upstream element (such as in class II mutations) reduces promoter function significantly, while deletion of both elements (class III derivatives) or the downstream element alone eliminates the promoter altogether.

The *his3* TATA box region (nucleotides −37 to −51) includes three regions that closely resemble T-A-T-A-A-A, the canonical sequence (11, 14). These are T-A-T-A-C-A (between −51 and −46), T-A-T-A-T-A (between −44 and −39), and T-A-T-A-A-A (between −42 and −37). It is too early to tell which, if any, is the key sequence. However, the two most downstream TATA boxes are unlikely individually to suffice because *his3*-Δ8, which removes all sequences further upstream from −44, eliminates promoter function (7, 14).

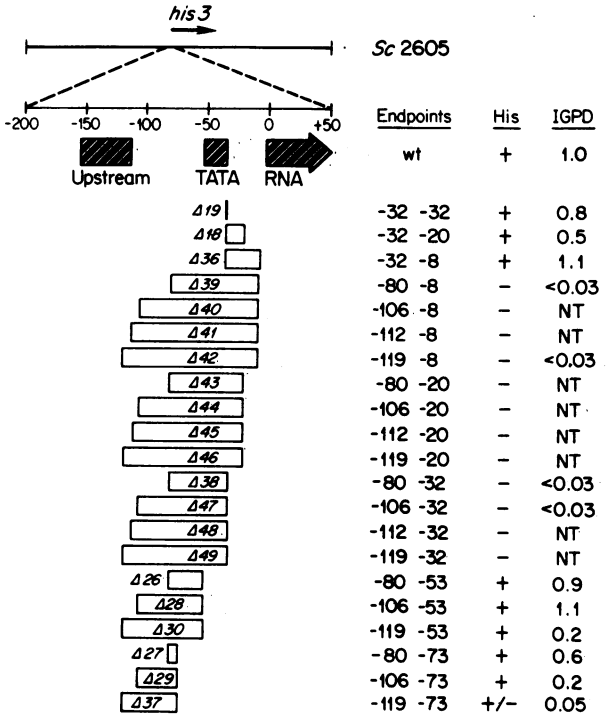
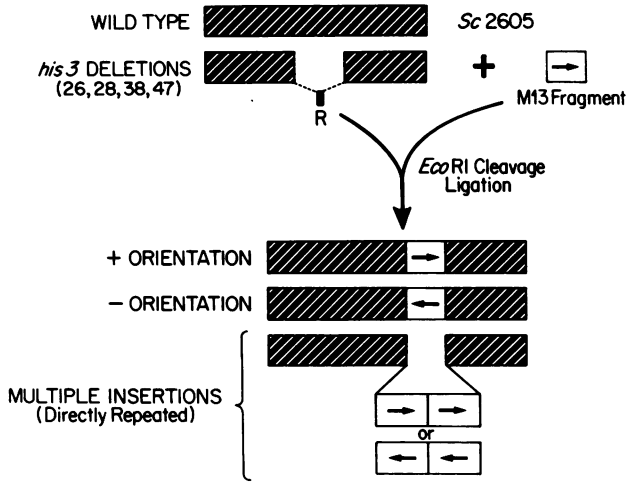


FIG. 1. Structures and phenotypes of *his3* deletion mutations. The top line represents a 6.1-kb fragment of wild-type (wt) yeast *his3* DNA (Sc2605), from which the deletion mutations described here were derived. The second line is an expanded view of the *his3* promoter region. The scale is in nucleotides and position zero marks the boundary between transcribed (+) nucleotides and nontranscribed (−) nucleotides. The nucleotide sequence for the promoter region between nucleotides −160 and +10 (probable initiation site underlined) is 5′-TAGTACACTCTATATTTTTTTATGCCTCGGTAATGATTTTCATTTTTTTTTTTCCACTAGCGGATGACTCTTTTTTTTTTCTTAGCGATTGGCATTATCACATAATGAATTATACATTATATAAAGTAATGTGATTTCTTCGAAGAATATACTAAAAAATGAGCAGGCAAG-3′ (4, 7, 14). The locations of the 5′ end of the *his3* transcript (14, 15), the TATA box, and the upstream promoter element are indicated. The open boxes below this line indicate the extents of the deletions (determined by DNA sequence analysis). All mutations have the sequence 5′-G-G-A-A-T-T-C-C-3′ at the site of deletion. The His phenotype (+ means growth at the wild-type rate, +/− indicates slow growth, − indicates no growth) and for some derivatives the levels of imidazoleglycerol-phosphate dehydratase (IGPD) activity are shown. NT, not tested.

Three other points are worthy of note. First, the fact that *his3*-Δ28 (deleted between −106 and −53) expresses *his3* at the wild-type level is consistent with previous evidence suggesting that the upstream element is completely included between nucleotides −112 and −155. Second, the fact that deletions with upstream end points at −119 express *his3* at lower levels than analogous deletions with end points at −106 suggests that the region between −106 and −119 may be part of the upstream element. Third, deletions with downstream endpoints at −52 (Δ26, Δ28, Δ30) express *his3* better than analogous deletions with endpoints at −72 (Δ27, Δ29, Δ37). Though this is a minor quantitative effect, it is mysterious that more extensive deletions result in less functional damage.

The TATA Box Element Requires Specific Sequences. Deletion of the TATA promoter element could eliminate *his3* expression for either of two reasons. The usual explanation is that the mutations delete specific sequences that are critical for function. An alternative explanation is that the mutations destroy spacing relationships between other sequences. In formulating molecular mechanisms that explain how a eukaryotic promoter works, it is essential to distinguish between these two possibilities.

The experimental design is to replace *his3* sequences deleted by various mutations with sequences from coliphage M13 DNA (see Fig. 2). By virtue of their construction, the deletion mutants described in the previous section all have an *Eco*RI site at the deletion breakpoints. Thus, it is easy to insert a 31-base-pair M13 DNA fragment generated by *Eco*RI endonuclease (or multiple tandem copies of it) into the "space" of the deletion. The M13 DNA fragment was inserted into four deletion mutants. Two of these (Δ38 and Δ47) lack the TATA promoter element, whereas the analogous pair (Δ26 and Δ28) contain this region.



(+) 5'-AATTCCGGACGTTGTAAAACGACGGCCAGTG-3'
(−) 5'-AATTCACTGGCCGTCGTTTTACAACGTCCGG-3'

FIG. 2. Replacement of *his3* sequences by M13 DNA (mutant construction). A sample original *his3* deletion allele is indicated by hatched bars (wild-type sequences), empty space (deleted DNA), and the *Eco*RI site (the sequence 5'-G-G-A-A-T-T-C-C-3') at the site of deletion. Ligation of *Eco*RI-cleaved *his3* mutant DNA and the M13 fragment (16) (also generated by *Eco*RI cleavage and obtained from Steve Anderson) results in the structures depicted beneath the arrow. The M13 fragment is drawn as an open box with an arrow [pointing rightward for the (+) orientation and leftward for the (−) orientation]. Many derivatives have tandem multiple insertions. In any given mutant, the multiple copies are all oriented in the same direction. It is likely that *E. coli* cells are unable to stably maintain molecules with perfect inverted repeats. The sequences of the sense strand of the (+) and (−) orientations of the M13 fragment (16) are shown, and a possible TATA box sequence is underlined.

Table 1. His phenotypes resulting from replacement of *his3* sequences by phage M13 DNA

Original allele	End points	Inserts	New allele	Change, bp	Element spacing, bp	His
wt				0	90	+
Δ26	−80, −53	0	26	−20	70	+
		1+	50	+11	(101)	+
		1−	51	+11	101	+
		2+	52	+42	(132)	+
		3−	53	+73	163	+
		5+	54	+135	(225)	+
Δ38	−80, −32	0	38	−41		−
		1+	55	−10		+
		1−	56	−10		−
		2+	57	+21		+
		2−	58	+21		−
		3+	59	+52		+
		3−	60	+52		−
		4+	61	+83		+
Δ28	−106, −53	0	28	−46	44	+
		1+	62	−15	(75)	+
		1−	63	+16	75	+
		2+	64	+47	(106)	+
		3+	65	+78	(137)	+
Δ47	−106, −32	0	47	−67		−
		1+	66	−36		+
		1−	67	−36		−
		2+	68	−5		+

The principle by which these mutations were constructed is diagrammed in Fig. 2. A 31-base-pair fragment of M13 DNA (or multiple tandem copies of it) was inserted in either possible orientation at the *Eco*RI site of four original alleles (deletion end points indicated). The amount of inserted DNA is indicated by the number of copies and the orientation [see text for definition of (+) and (−)] of the M13 DNA fragment (e.g., 3+). The following information is presented for each new allele. The "change" represents the total number of base pairs (bp) deleted (−) or inserted (+) when compared to the wild-type gene. The spacing between upstream and downstream promoter elements (see text) is given only for derivatives that contain both. Entries in parentheses indicate that these derivatives contain at least one additional downstream element [the (+) orientation of the M13 fragment]. All His^+ derivatives listed here allow cells to grow at the wild-type rate; this indicates that the amount of imidazoleglycerol-phosphate dehydratase is at least 20% of the wild-type level. His^- derivatives fail to confer any detectable growth, indicating imidazoleglycerol-phosphate dehydratase levels less than 3% of the wild-type values.

The phenotypes of the resulting derivatives (Table 1) fall into consistent patterns and are summarized below. All derivatives containing the M13 fragment in the (+) orientation express *his3*. Of derivatives containing the M13 fragment in the (−) orientation, only those with the *his3* TATA box region can express the gene. Thus, the orientation of the M13 fragment with respect to the *his3* gene has important qualitative effects on promoter function. In the (+) orientation, the M13 fragment can serve as the downstream promoter element. On the other hand, the (−) orientation behaves as neutral DNA. It cannot act as a promoter element, but it does not eliminate promoter activity from derivatives that contain the *his3* TATA box.

These results demonstrate that deletions without the *his3* TATA box promoter element fail to express the gene because

they lack specific sequences. The derivatives that differ only by the orientation of the M13 fragment have identical spacing relationships but opposite phenotypes (compare deletions 55 and 56, 57 and 58, 59 and 60, or 66 and 67). The (+) orientation of the M13 fragment has the sequence T-G-T-A-A-A, which strongly resembles the canonical TATA box (T-A-T-A-A-A), whereas the (−) orientation does not have such a sequence. Thus the absolute requirement for the downstream promoter element can be satisfied by the *his3* TATA box region or by a sequence that strongly resembles it.

The Spacing Between Promoter Elements Is Not Critical for Function. The 41 *his3* derivatives described here and elsewhere are very different, yet there is an absolute correlation between structure and function. The presence of two distinct regions is necessary and sufficient for promoter function; mutations that retain only one such region result in poor or no detectable function. There is no correlation between *his3* expression and the relative positions of any two regions of DNA. This strongly suggests that the spacing between promoter elements is apparently unimportant for function.

The two elements defined for the wild-type gene map somewhere between 112–155 and 32–52 nucleotides before the start of the structural gene. The distance between the midpoints of these regions is approximately 90 base pairs. Of the 13 derivatives that contain both promoter elements and hence express *his3*, the center-to-center distance ranges from about 40 base pairs (Δ28) to 160 base pairs (Δ53). The proposition that element spacing is unimportant is best supported by mutations in which the normal spacing is increased by the insertion of apparently neutral DNA [the (−) orientation of the M13 fragment] between the *his3* elements. The statement also holds for cases in which the normal spacing is decreased by deletion, although here it could be argued that sequences further upstream than nucleotide −155, which are not normally required, serve as a correctly spaced upstream element.

DISCUSSION

Promoters are defined as regions of DNA needed for gene expression (1). A prokaryotic promoter can be equated with a transcriptionally competent RNA polymerase binding site (reviewed in refs. 2 and 3). The two regions defined by genetic means as promoter elements are required for polymerase binding *in vitro* and are in close physical contact with the enzyme. It is generally assumed that the promoters for eukaryotic genes transcribed by RNA polymerase II must involve specific binding to the enzyme, although there is no direct evidence for this.

Previous sections of this paper have described genetic experiments that formally define two distinct *his3* promoter elements and some of their properties. In this section, I consider the results in molecular terms. The ensuing discussion is based on the assumption that measurements of gene expression *in vivo* and IGP dehydratase activity *in vitro* reflect *his3* transcription. This assumption is strongly supported by the following observations: (*i*) All the mutations examined map outside the mRNA coding region. (*ii*) There is no evidence for the processing of *his3* RNA (15). (*iii*) Analysis of 7 *his3* promoter mutations, including deletion 38, indicates that they fail to make RNA (ref. 14; unpublished results). (*iv*) The increases in imidazoleglycerol-phosphate dehydratase activity that occur under appropriate physiological conditions are accompanied by increases in transcription (14). Nevertheless, this assumption will be difficult to prove, especially because nontranslated messenger RNAs are degraded very rapidly in yeast cells (17, 18).

TATA Box Element. It is frequently suggested that the TATA box is a site of DNA that is recognized specifically by eukaryotic RNA polymerase II (5, 9–11, 19–22). This suggestion was originally based on its ubiquitous presence in front of eukaryotic genes, its relatively constant distance from the start of transcription, and its sequence homology with the Pribnow box, the major determinant for *E. coli* RNA polymerase binding (ref. 11, reviewed in ref. 19). Various functional tests also support this view. Deletion and point mutation analyses indicate that the TATA box is the primary ingredient for transcription *in vitro* (9, 10, 20). Furthermore, deletion of DNA between the TATA box and the normal start of transcription changes the site of initiation such that it remains the usual constant distance from the TATA box (21).

A problem with the hypothesis has been the inability to demonstrate *in vivo* a qualitative necessity for the TATA box. If polymerase binding is a key feature of a eukaryotic promoter and if the TATA box is the key binding site, deletion of this region should abolish gene expression. However, deletions and point mutations of the TATA box generally have no or minor quantitative effects *in vivo* (5, 6, 8, 10, 22). This is to be contrasted with mutations that delete upstream regions; these have major effects *in vivo* but minor if any effects *in vitro* (6). There are many explanations for these apparently conflicting results, but these will not be discussed here.

In this regard however, the results presented here indicate that *in vivo* the downstream *his3* promoter element (*i*) is *absolutely* required for gene expression, (*ii*) requires orientation-specific sequences, and (*iii*) can be defined by the *his3* TATA box region or a sequence resembling it. Taken together, these observations provide additional, although indirect, support for the view that the TATA box encodes (part of) a binding site for RNA polymerase II.

Upstream Promoter Element. On the basis of the observation that the upstream element maps relatively far (at least 113 base pairs) from the transcriptional initiation site, I suggested previously that the upstream element is not a simple site of interaction between yeast RNA polymerase II and DNA (7). *E. coli* RNA polymerase, which is roughly the same size as the yeast enzyme (23), interacts only with a 50-base-pair region (3).

Strong support for this notion comes from the striking observation that the spacing between *his3* elements is not critical for gene expression. In contrast, the few relevant experiments on *E. coli* promoters indicate that the distance between the two prokaryotic elements is important. Deletion or insertion of 1 or 2 base pairs between the elements has major effects on promoter function (24, 25). This is expected because bound *E. coli* RNA polymerase interacts directly with the Pribnow box and the −35 sequence. On the other hand, it is extremely difficult to imagine how any protein could interact simultaneously with two regions of DNA that have no fixed positional relationship to each other. Thus, it seems likely that the two *his3* elements interact with different proteins (or different conformations of the same protein). If the TATA box is an RNA polymerase binding site analogous to the *E. coli* Pribnow box, then it follows that the upstream element is not part of this site.

There are two classes of models to explain the variable spacing relationship between promoter elements. One possibility is that the upstream element acts as a RNA polymerase entry site from which the enzyme can move to its transcriptionally competent binding site. The other possibility is that the upstream region interacts with protein(s) that allow RNA polymerase to bind to the TATA box. For example, such proteins may create an open domain of chromatin. Either model explains how the upstream element can act at a long and variable distance from the TATA box. Currently, there is little evidence to distinguish between these models.

Upstream Promoter Elements, Enhancer Sequences, and Position Effects. Compared to prokaryotic promoters, the un-

usual feature of the yeast *his3* promoter is that the upstream element can be moved with respect to the downstream one with little effect on function. Two other lines of evidence suggest that this may be a general property of eukaryotic promoters.

Sequential deletion analysis of the SV40 tumor antigen gene has demonstrated the existence of an upstream element (often called the 72-base pair-repeat) (6). This same region has also been termed an "enhancer" because its presence at various locations in a molecule increases the expression of certain genes (26, 27). Other properties of the SV40 sequence have not been tested for the *his3* upstream element—its functionality in both orientations, its ability to act at distances greater than 1 kb, and its apparent ability to function at the 5′ or 3′ end of the gene (this last property may be an artifact due to the circular molecules used in the viral experiments). However, the features that are striking and common to the *his3* upstream element and the SV40 and polyoma virus enhancers are the abilities to act at a long and variable distance.

The ability to act at a distance is also reminiscent of position effects in yeast. I showed previously that sequences at least 300–400 base pairs upstream from some genes can influence their expression (28). In these cases, the relevant alleles probably lack the normal upstream element. Thus, the position effects could result from a new upstream element located far from the gene. This explanation is unlikely to account for position effects on yeast mating type genes (29); in this case, there are special gene products that are probably important.

Thus, it is possible to relate enhancers and position effects to the properties of the *his3* upstream promoter element. It will be interesting to see whether this relationship has any mechanistic basis.

Note Added in Proof. McKnight and Kingsbury (30) have demonstrated that the herpesvirus thymidine kinase promoter contains multiple elements and that the TATA box is critical for transcription in live *Xenopus* oocytes.

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