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Foreign transcriptional enhancers in yeast. II. Interplay of the polyomavirus transcriptional enhancer and *Saccharomyces cerevisiae* promoter elements

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ABSTRACT

In this paper, to further analyze the function of the polyoma enhancer in Saccharomyces cerevisiae, we use as reporter-genes derivatives of the yeast HIS3 gene flanked by two types of partially deleted promoters: in one, UAS elements are removed by deletion of sequences upstream of nt -80 (pGM3181) in the second both TATA boxes and UAS elements are removed by deletion of sequences upstream of nt -35 (pGM2809). These constructs have been studied both as free plasmids and after integration at the TRP1 chromosomal locus. We find that in general the polyoma holoenhancer (A + B domains) elicits transcription from the physiological HIS3 RNA start sites when the native TATA boxes are present. In contrast, an altered enhancer B-domain from polyoma mutant Py-B78, although active when inserted downstream of the test-gene or when coupled to a pseudopromoter (Ciaramella et al, accompanying manuscript), does not work properly in concert with the native yeast TATA boxes. We describe experiments that suggest an important role for the foreign enhancer in RNA start-site selection in yeast.

INTRODUCTION

In Saccharomyces cerevisiae accurate transcription depends on the interaction of at least three distinct elements : the TATA box, the upstream element (UAS or URS) and the RNA start site/s (IR). According to current ideas, the TATA box , controlled by a distal promoter element , directs transcription initiation to a window within which preferred IR sites are activated.(1,2)

The wild type HIS3 promoter possesses two varieties of UAS elements, poly-dAT and TGACTC, and cognate TATA boxes, T^c and T^r: polydAT sequences activate basal-level transcription probably by increasing chromatin accessibility, and TGACTC sequences regulate HIS3 RNA synthesis by promoting, via DNA binding, contacts between the general aminoacid control element GCN4 and the polII transcriptional apparatus. Basal-level transcription initiates equally at nts +1 +12 and at a lower level at nt +22; transcripts induced by GCN4 stimulation initiate at nts +12 and +22 but not at nt+1. Initiation at +1 is subservient to TATA box Tc, induced initiation at +12 and +22 to TATA box Tr (3,4).

In Saccharomyces cerevisiae the polyoma enhancer, a mosaic of foreign UAS-like elements, dramatically raises the level of transcription of a linked test-gene served by a susceptible quiescent pseudopromoter (5; Ciaramella et al, accompanying ms.). The foreign element promotes transcripts starting at multiple sites within a window of variable size and position, dependent on where the enhancer is inserted. Not unexpectedly, this initiation win-

dow does not overlap the natural IR sites: in the test-plasmid used both native UAS and TATA elements were deleted and replaced by a pseudopromoter sequence. Probably transcript initiation is effected by interaction of the enhancer with multiple TATA box-like signals in the AT rich pseudopromoter. This interpretation is unproven because the functional elements of the pseudopromoter have not been characterized.

In this paper we further explore the relationship between enhancer dependent potentiation and the choice of RNA start sites by testing the ability of the enhancer to interact productively with some well characterized elements of the HIS promoter. We study these interactions by introducing the enhancer into test plasmids containing diminishing segments of the native HIS3 promoter. These plasmids are studied both in the free and in the integrated state. We define conditions for the initiation of enhancer driven transcripts from native IR sites.

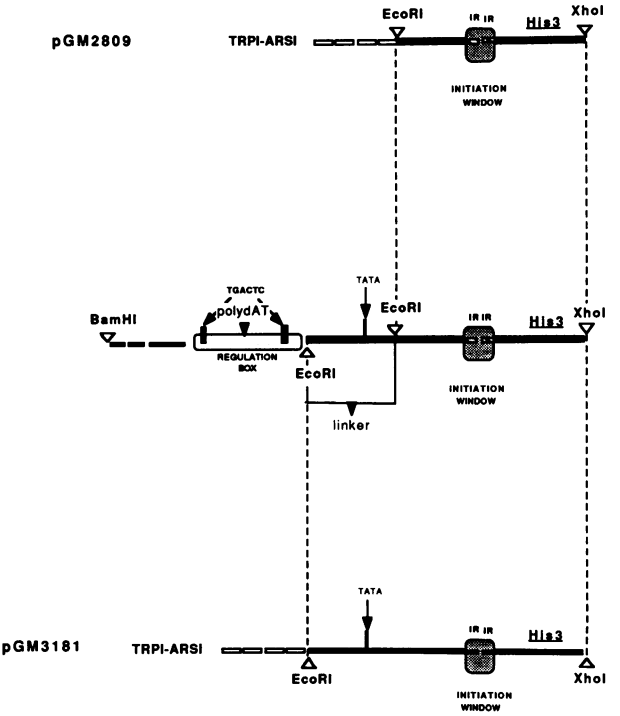


Figure 1. Construction of the recombinant test plasmids. The EcorI-XhoI fragment of pGM8 (Ciaramella et al, accompanying manuscript) is replaced with EcoRI-XhoI fragments excised from derivatives of the Saccharomyces cerevisiae HIS3 BamHI fragment (6), containing linker inserts at nt -80 (pGM3181) or at nt -35 (pGM2809).

The upstream flanking sequence of pGM3181 is:
GAATTCCGATTGCATTTATCACATAATGAATTATACATTATATAAAGTAATGTGATTTCTTCGAAGAATATA-
CAAAAAATGAGCAGGCAAGATAAACGAAGGCAAAGATGATGACAGA

The upstream flanking sequence of pGM2809 is:
GAATTCTGTGATTTCTTCGAAGAATATACTAAAAAATGAGCAGGCAAGATAAACGAAGGCAAAGATGATGACAGA

MATERIALS AND METHODS.

The test-plasmids. The test-plasmids (Fig. 1), derived from pAT011, are related to pGM8 (Ciaramella et al, accompanying ms) and carry two adjacent yeast sequences: a) a 1460 bp EcoRI fragment containing the TRP1-ARS1 sequences for plasmid selection and maintenance and b) BamHI restriction fragments comprising either the entire HIS3 gene and flanking sequences or deletion derivatives of the yeast HIS3 transcription unit (Fig. 1) in which we have deleted (Materials and Methods) either sequences upstream of nt -80 (pGM3181), removing the UAS elements, or sequences upstream of nt -35 (pGM2809), removing UAS elements and TATA boxes. These constructs were obtained by replacing the EcoRI-XhoI fragment of his-del4 from pGM8 (the test-plasmid described in Ciaramella et al, accompanying manuscript) with EcoRI- XhoI fragments of HIS3 wild-type derivatives into which EcoRI linkers had been introduced at nt -80 and -35 (a gift from K. Struhl)(6). Two different restriction sites in these test-plasmids were used for insertion of enhancers provided with appropriate linkers (Ciaramella et al, accompanying manuscript)): a 5′ EcoRI site at nt -80 in pGM3181 or at nt -35 in pGM2809 and a 3′ BamHI site at nt +1318 in both plasmids.

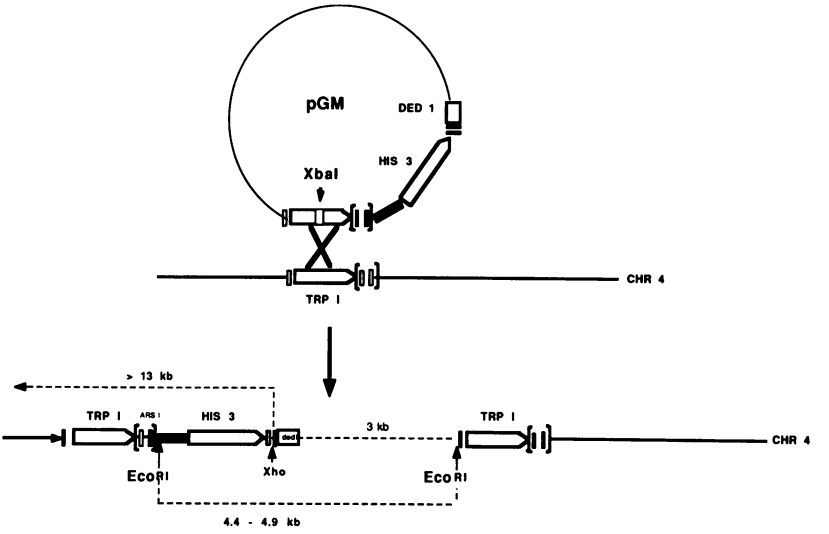


Figure 2. Integration of plasmids.
The top part of the figure illustrates the overall structure of the integrating test-plasmids and the TRP1 locus on chromosome IV (carrying the trp1-289 allele). The plasmids are linearized with the restriction enzyme XbaI, that cuts within the TRP1 sequence thus directing integration preferentially to the TRPI chromosomal locus.

The lower part of the figure illustrates the overall structure of the integrants. A 4.4 to 4.9 kb (according to the plasmid) genomic EcoRI fragment homologous to a HIS3 specific probe is diagnostic for integration at TRP1. Integration in DED1 would generate a much larger (8-8.4 kb) EcoRI fragment.

Digestion of genomic DNA with restriction enzyme XhoI generates a single large fragment (>13 kb) if the DNA is extracted from single-copy integrants. In the case of multiple-copy integration, two XhoI fragments homologous to a HIS3 specific probe are produced, one >13 kb and one 5.8 to 6.3 kb (according to the plasmid) fragment. The ratio of the areas under densitometer tracings of autoradiographs of Southern blots of the fragments is an indication of the copy number of the integrant plasmid (cfr Materials and Methods)

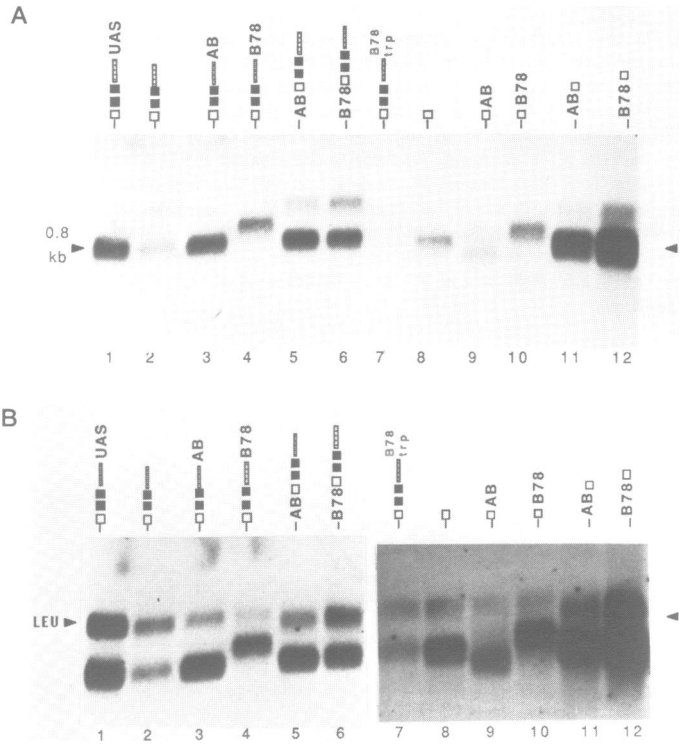


Figure 3. Enhancement of HIS3 transcripts by the polyoma enhancer.

A) Northern blots of total RNA hybridised to ^{32}P-labelled antisense single-stranded RNA probe transcribed in vitro with T7 RNA polymerase from a restriction fragment internal (nt +121 to +328) to the HIS3 gene, cloned into pGEM4™ (Promega, Biotec) (Ciaramella et al, accompanying ms). lane 1, pGM9, contains the wild-type promoter; lane 2, pGM3181, the test-plasmid in which sequences (including the UAS elements) upstream of nt -80 have been deleted from the wild-type HIS3 BamHI fragment in pGM9 and replaced with an EcoRI linker (Figure 1); lane 3, pGM3182-1 = pGM3181 + the polyoma holoenhancer inserted at the -80 EcoRI linker in early orientation. lane 4, pGM3182-2 = pGM3181 + the polyoma enhancer B-domain derivative from the PyFL78 mutant inserted at the -80 EcoRI linker in late orientation. lane 5, pGM3183-1 = pGM3181 + the polyoma holoenhancer inserted at the nt +1318 BamHI site (Figure 1). lane 6, pGM3183-2 = pGM3181 +the polyoma enhancer B-domain derivative from the PyFL78 mutant inserted at the nt +1318 BamHI site (Figure 1); lane 7, pGM3184-2 = pGM3181 +the polyoma enhancer B-domain derivative from the PyFL78 mutant inserted at the nt -1540 EcoRI site (Figure 1). lane 8, pGM2809, the test-plasmid in which sequences (including the UAS and TATA box elements) upstream of nt -35 have been deleted from the wild-type HIS3 BamHI fragment in pGM9 and replaced with an EcoRI linker (Figure 1); lane 9, pGM2810-1 = pGM2809 + the polyoma holoenhancer inserted at the -35 EcoRI linker in late orientation; lane 10, pGM2810-2 = pGM2809 + the polyoma enhancer B-domain derivative from the PyFL78 mutant inserted at the -35 EcoRI linker in late orientation; lane 11, pGM2811-1 = pGM2809 + the polyoma holoenhancer inserted at the nt +1318 BamHI site (Figure 1); lane 12, pGM2811-2 = pGM2809

+the polyoma enhancer B-domain derivative from the PyFL78 mutant inserted at the nt +1318 BamHI site.

B) The same filter as in A normalized by rehybridization to a ^{32}P-labelled antisense LEU2 riboprobe (Ciaramella et al, accompanying ms). Panels on the left and right are taken from different exposures of the same filter.

Symbols: UAS represents the native upstream activator; closed boxes are the native TATA-boxes; open boxes are TATA-like sequences; bars represent the "spacer" sequences interposed between the control elements under consideration. A and B mean respectively the A and B domains of the polyoma enhancer. B78 is the B-domain from the polyoma FL78 mutant.

These ARSI test plasmids are not stabilized by an additional CEN containing fragment. In any given experiment only 20%-30% of the cells carry the plasmid in multiple copies (because plasmid-bearing cells make a large excess of TRP1 product, cells that lose the plasmid continue dividing for a few generations even under selection). This situation, although un-natural has the advantage of reproducing conditions for transient expression assays in mammalian cells following calcium phosphate transformation.

Enhancer activity in cells bearing a single integrated copy of the test plasmid are also explored.

Enhancer fragments. The structure of the polyoma enhancer is described in detail in the accompanying paper. It will suffice here to recall that the wild-type holoenhancer is composed of two domains A and B; the B domain alone has little if any function in mouse fibroblasts or in yeast cells; a mutant derivative of the B domain (B78) is functional in mouse fibroblasts and in yeast cells.

Genetic methods and strains. As described in (Ciaramella et al, accompanying manuscript).

Two different Saccharomyces cerevisiae strains were used for studies on expression in free or integrated plasmids:

Sc117 (for description see accompanying manuscript) was used for studies on free plasmids. In this strain chromosomal HIS3 and TRP1 sequences have been completely deleted. The only region of homology to the test plasmids we use consists of the approximately 600 bp of DED-I gene sequence contained in the BamHI HIS3 fragment.

Sc104-17 (g, ade2-101, trp1-289,his3-del200) was used for studies that required integration of the test plasmid at the TRP1 locus on chromosome IV. This strain was constructed by standard genetic procedures by replacing the trp1-del1 allele of Sc117 (a complete deletion of the TRP1 gene; 7) with a point-mutant (trp1-289). The identity of the his-del200 allele (which completely deletes the HIS3 gene) was checked by hybridization of genomic digests of Sc104-17 to an appropriate probe.

Plasmid integration. Integration of recombinant plasmids was directed (8) to the TRP1 locus by cutting with restriction endonuclease XbaI that cleaves our test plasmids once within the plasmid-borne TRP1 gene (Fig. 2A). This procedure was used to reduce the chances of integration into DED-I. Integration at this locus would displace by recombination enhancers cloned at the BamHI or SalI sites flanking the 3′ end (nt +1318) of the HIS3 BamHI test-fragment inserted into the vector polylinker (Fig. 1, Ciaramella et al., accompanying ms.). The site of integration and copy number was decided from restriction digests of genomic DNA cut with appropriate enzymes (Fig. 2B). If integration occured at TRP1 EcoRI digestion of integrant DNA would result in a 4.4 to 4.9 kb fragment hybridizing to a HIS3-specific probe. Restriction enzyme XhoI digestion of integrant DNA results in a single >13 kb fragment hybridizing to the HIS3 probe if integration occures in single copy; if multiple copies have integrated, XhoI digestion results in an additional fragment corresponding to the size of the test-plasmid (about 6kb). We have used the ratio of densitometer tracings of the two bands to determine copy number.

RNA and DNA biochemistry Were as described in Ciaramella et al (accompanying manuscript).

5′ end mapping. 5′ ends were determined by RNA'ase mapping using the same probes and procedures described in the accompanying manuscript.

Low resolution RNAase mapping was used in preliminary characterization of transcription from the various constructs. RNA (10μg) was hybridized to a cold antisense-RNA probe transcribed in vitro with T7 RNA polymerase from the nt -257 to nt +328 fragment of his-del4 (Ciaramella et al, accompanying manuscript). Hybridization and RNAase digestion were as described (9); the hybrids were run on 1.2% agarose/formaldehyde gels (Ciaramella et al accompanying manuscript), blotted onto a nitrocellulose membrane and hybridized with a uniformally labelled Hind3-Sau3A fragment internal to HIS3.

When transcript size suggested that initiation was taking place upstream of our RNA probes, 5′ ends were mapped by AMV reverse transcriptase (IBI) using a 21 nt synthetic primer (homologous to HIS3 nts +32 to

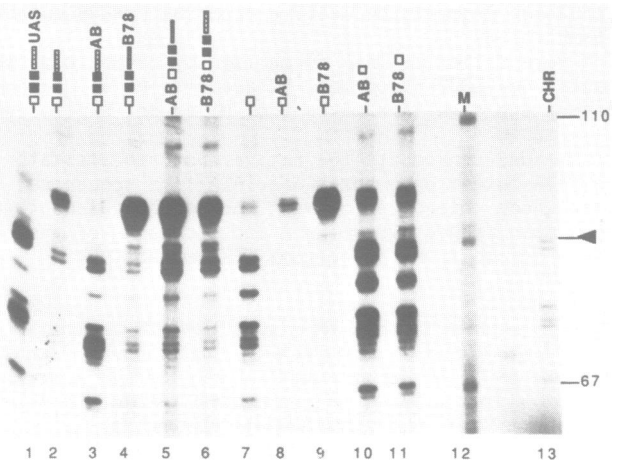


Figure 4. RNAase mapping of 5′ ends of transcripts elicited by the polyoma enhancer. Total RNA was hybridized to a uniformally labelled RNA probe complementary to nts -257 to +82 of his-del4 (Ciaramella et al, accompanying ms). After treatment with ribonuclease (Materials and Methods), protected fragments were run on a 6% sequencing gel.

lane 1, pGM9; lane 2, pGM3181; lane 3, pGM3182-1; lane 4, pGM3182-2; lane 5, pGM3183-1; lane 6, pGM3183-2; lane 7, pGM2809; lane 8, pGM2810-1 ; lane 9, pGM2810-2; lane 10, pGM2811-1; lane 11, pGM2811-2; lane 12, the marker is an end-labelled HpaII digest of pAT011 (Ciaramella et al, accompanying manuscript). lane 13, the HIS3 chromosomal transcript. Fragments predicted from the sequence of pAT011 are in nts: 492, 465, 404, 242, 238, 201, 190, 160, 147, 110, 90, 76, 67, 53, 34, 26, 26. Symbols: see legend to Figure 3.

+52) end-labelled by polynucleotide kinase. RNA (about 30 μg) was denatured at 65°C and annealed to about 1 picomole of primer (10^4 cpm) at 37°C for one hour. Primer extension was carried out at 48°C as described (10). The products of the reaction were separated on a 6% polyacrylamide sequencing gel.

RESULTS

Interaction between the polyoma enhancer and the native yeast TATA boxes.

In rich media the wild type promoter of the yeast HIS3 gene, at its normal chromosomal site, directs transcription to two major RNA start sites at nt +1 and +12. This constitutive base-level HIS3 transcription depends on an upstream polydAT stretch at nt 113-130 and on a TATA box at nts -50 to -54 (Tc); histidine starvation, by GCN4 activation of two TGACTC UAS sequences at nts -263 and -99, induces, via a TATA box at nts -40 to -46 (Tr), transcription specifically from IR at nt +12 (3).

In test-plasmid pGM3181 sequences upstream of nt -80 (comprising the native UAS elements) are deleted and transcription is strongly reduced; Actually, some HIS3 transcripts are detectable in cells containing plasmid borne constructs (Fig. 3; lane 2) and initiate at native start-sites, at nt +1 and more weakly at nt +12 (Fig. 4; lane 2) as well as upstream of nt +1 (Fig. 6B, lane 3). This observation explains the weak histidine prototrophy conferred by pGM3181.

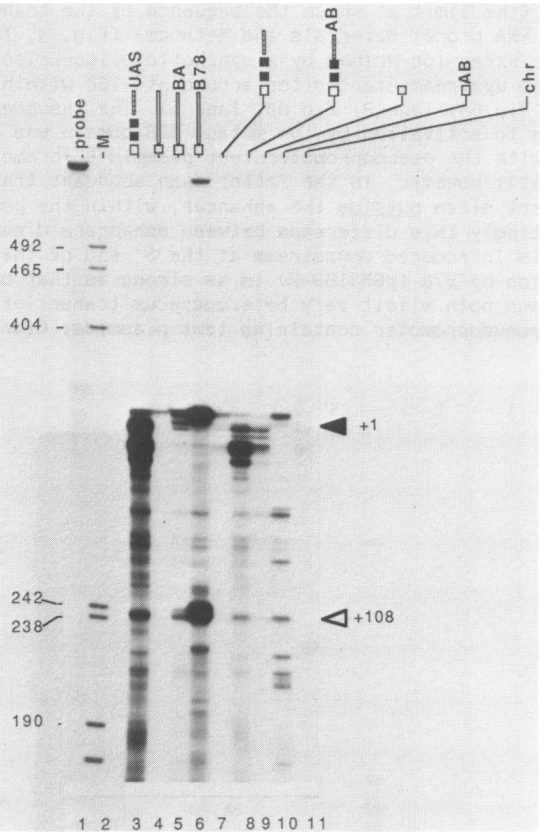
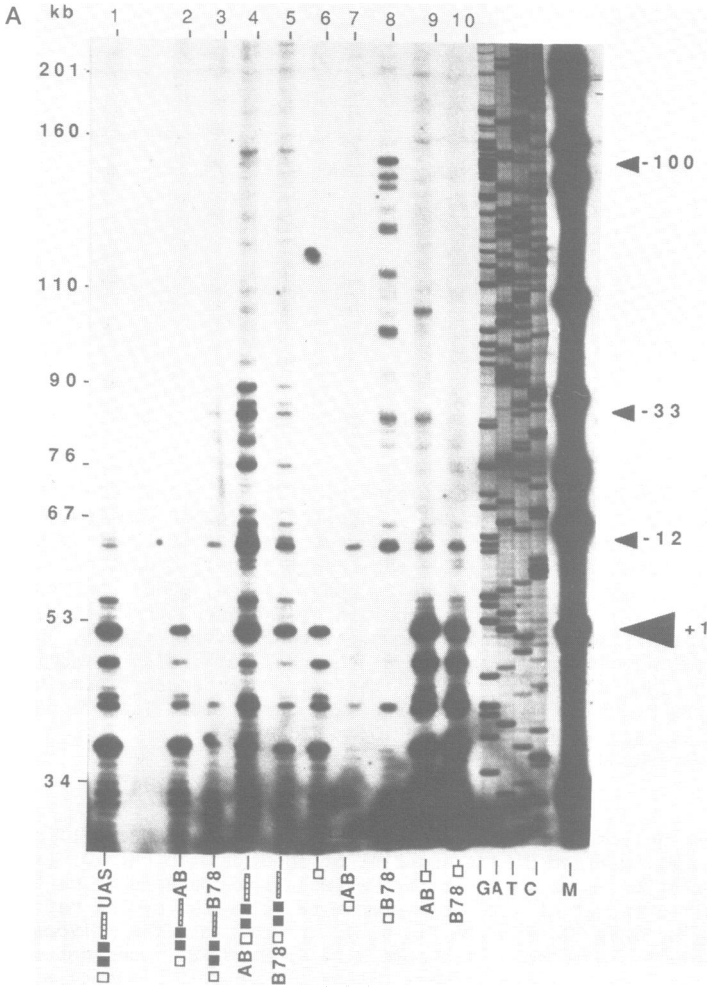


Figure 5. RNAase mapping of the 5′ ends of HIS3 RNA transcribed from transformed cells using a larger uniformally labelled RNA probe complementary to nts -257 to +328 of his-del4 to show far downstream start-sites in pGM2810-1. lane 1, undigested probe; lane 2, end-labelled HpaII digest of pAT011 (Ciaramella et al, accompanying ms); lane 3, pGM9; lane 4, pGM8, the test-plasmid with the 1575 BamHI HIS3 fragment containing the pseudopromoter described in Ciaramella et al (accompanying manuscript) derivative ; lane 5, pGM36 = pGM8 + the polyoma holoenhancer inserted at the -257 BamHI site in early orientation; lane 6, pGM78 = pGM8 + the polyoma enhancer B-domain derivative from the PyFL78 mutant inserted at the -257 BamHI site; lane 7, pGM3181; lane 8, pGM3182-1; lane 9, pGM2809; lane 10, pGM2810-2.
Symbols: see legend to Figure 3.

The polyoma holoenhancer inserted at nt -80 (pGM3182-1) raises the level of a well defined HIS3 transcript of about 0.8 kb (Fig.3; lane 3). 5′ RNAase-protection mapping shows that stimulation concerns primarily transcripts initiating at nt +12 , the same site activated by the native UAS elements (Fig. 4; lane 3) (Fig. 6A, lane 2). In contrast, the polyoma mutant B78 enhancer-domain, inserted at the same site (pGM3182-2), does not increase transcipt level but elicits larger transcripts (Fig. 3, lane 4) starting up-

stream of nt -10 (the limit at which the sequence of the transcript diverges from that of the RNA probe; Materials and Methods) (Fig. 4, lane 4). Precise mapping by primer extension primed by a synthetic oligonucleotide (Materials & Methods) locates upstream start sites around nt -160 within the enhancer fragment itself (Fig.6A; lane 3; Fig.6B, lane 5). The insusceptibility of natural start sites to activation by the mutant B78 domain was also apparent in our experiments with the pseudopromoter test-plasmid (Ciaramella et al, accompanying manuscript); however, in the latter case abundant transcription was elicited from start sites outside the enhancer, within the pseudopromoter.

Interestingly this difference between enhancers disappears if either enhancer is introduced downstream at the 3′ end of the gene at nt +1340. Potentiation by B78 (pGM3183-2) is as strong as that by the holoenhancer (pGM3183-1) and both elicit very heterogeneous transcripts (as was also observed with the pseudopromoter containing test-plasmids; Ciaramella et al, ac-



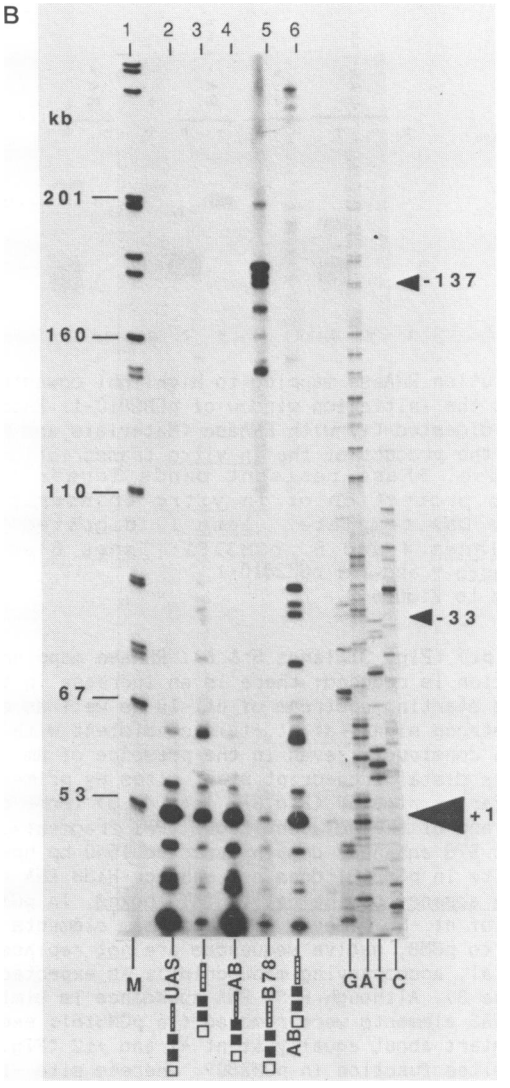


Figure 6. Mapping of 5′ ends of HIS3 transcripts by primer extension (see Materials and Methods).
A) lane 1, pGM9; lane 2, pGM3182-1; lane 3, pGM3182-2 (sample was partly lost; see B for repeat); lane 4, pGM3183-1; lane 5, pGM3183-2; lane 6, pGM2809; lane 7, pGM2810-1; lane 8, pGM2810-2; lane 9, pGM2811-1; lane 10, pGM2811-2; lanes 11, 12, 13, 14, sequence ladder: G, A, T, C; lane 15, the marker is an end-labelled HpaII digest of pAT011 (Ciaramella et al, accompanying manuscript). Symbols: see legend to Figure 3.
B) lane 1, marker; lane 2, pGM9; lane 3, pGM3181; lane 4, pGM3182-1; lane 5, pGM3182-2; lane 6, pGM3183-1; lanes 7,8,9 & 10 sequence ladder. Symbols see legend to Fig.3.

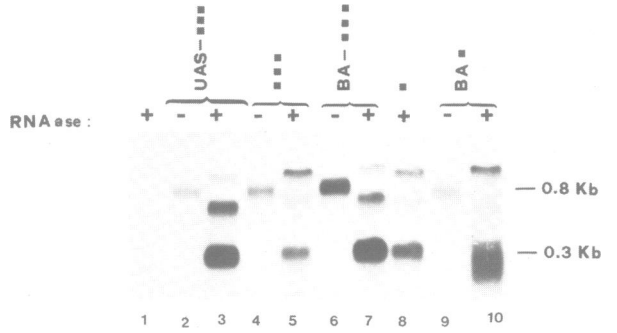


Figure 7. Low resolution RNAase mapping to highlight downstream shift and scatter of sites in the initiation window of pGM2810-1. Each sample is shown undigested (-) and digested (+) with RNAase (Materials and Methods).
In this experiment the product of the in vitro transcription reaction was not treated with DNase. RNase-resisant bands longer than 0.3 kb are artifacts due to protection of in vitro transcripts by annealing to the DNA template. lane 1, digested probe; lanes 2 and 3, pGM9; lanes 4 and 5, pGM3181; lanes 6 and 7, pGM3182-1; lane 8, pGM2809; lanes 9 and 10, pGM2810-1.
Symbols: see legend to Figure 3.

companying manuscript) (Fig. 3; lanes 5 & 6). RNAase mapping confirms that specificity of initiation is reduced: there is an increase in the level of heterogeneous transcripts starting upstream of nt -10 as well as at nt +1 (Fig. 4; lanes 5 & 6). The strong signal at nt +1 is consistent with rapid growth of cells carrying such constructs, even in the presence of aminotriazole. We have mapped the more distal transcript start sites by primer extension to positions upstream as far as nt -100 (Fig.6A, lane 4, 5) (some of these sites are within the 3′ end of the adjacent EcoRI TRPI fragment).

The mutant B78 enhancer domain inserted 1540 bp upstream of HIS3 at the distal EcoRI site in pGM3181 does not enhance HIS3 RNA (Figure 3; lane 7). Enhancer effects in absence of the native TATA boxes. In pGM2809 deletion of sequences upstream of nt -35 removes both upstream elements and the TATA boxes (in contrast to pGM8, native sequences are not replaced by a pseudopromoter, Ciaramella et al, accompanying manuscript); as expected transcription is reduced (Fig.3, lane 8). Although HIS3 RNA abundance is similar to that observed when only the UAS elements were removed (in pGM3181; see above), residual HIS3 transcripts, start about equally at nt +1 and +12 (Fig. 4; lane 7). We do not know why such sites function in pGM2809, whereas site -10 is prevalent in pGM3181. Perhaps AT rich tracts in the flanking TRPI-ARS1 fragment are responsible. Such transcripts suffice for the relatively weak histidine prototrophy observed on plates lacking histidine and containing aminotriazole.

Unexpectedly, when the polyoma holoenhancer (or the B78 domain enhancer, see below) is inserted at nt -35 (pGM2810-1), histidine prototrophy is almost abolished, even under less stringent conditions of histidine deprivation (i.e. on plates lacking histidine but with no addition of aminotriazole). As visualized on Northern blots, the holoenhancer inserted in pGM2809 appears to shift the size distribution without raising transcript levels (Fig. 3; lane 9). Indeed as evidenced by low resolution RNAase mapping (Fig. 7) the holoenhancer potentiates very heterogeneous and clearly shorter transcripts.

High resolution RNAase mapping using the longer 584 nt riboprobe

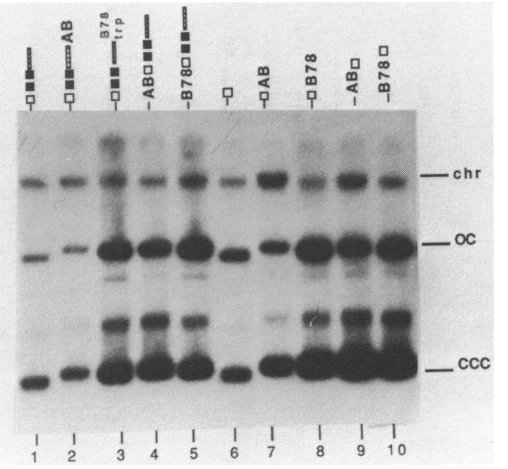


Figure 8. Effects of enhancer inserts on plasmid copy-number. "Southern" blots of yeast DNA minipreps hybridized to the ^{32}P-labelled XhoI- SalI fragment (Figure 1A, Ciaramella et al, accompanying ms) comprising the begining of the DED1 gene. lane 1, pGM3181; lane 2, pGM 3181-2; lane 3, pGM3184-2; lane 4, pGM3183-1; lane 5, pGM3183-2; lane 6, pGM2809; lane 7, pGM2810-1; lane 8, pGM2810-2; lane 9, pGM2811-1; lane 10, pGM2811-2.
CHR, chromosomal DNA; OC, open circular plasmid; CCC, covalently closed circles. P = uncleaved pGM8 DNA.
Symbols: see legend to Figure 3.

(cfr. Fig. 2, Ciaramella et al, accompanying ms.) confirms this observation and demonstrates that the enhancers are effectively shifting start sites from nt +1 and +12 to various sites upstream of nt -10 (Fig. 6A, lanes 7 & 8) and downstream of nt +44 (Fig. 5, lane 10) (precise downstream coordinates were determined in a separate experiment; not shown).

Results with pGM2809 carrying an insert of the enhancer mutant B78 domain at nt -35 (pGM2810-2) differ from those with the holoenhancer and are reminiscent of the effect of the B78 fragment inserted into pGM3181 (see above). Histidine protrophy is even weaker than that observed with the holenhancer at nt -35. Furthermore, average length of HIS3 RNA increases (by more than 100 nts) instead of decreasing (Fig. 3; lane 10) (again, there is no potentiation of transcript level). RNAase mapping locates transcription initiation sites upstream of nt -10 (Fig. 4; lane 9). Accurate mapping by primer extension positions multiple start sites between nt -10 and -102 (Fig. 6A; lane 8); the latter is within the mutant enhancer itself.

When inserted downstream at 1340 bp from the natural initiation sites, both the holoenhancer (pGM2811-1) and the B78 domain (pGM2811-2) regain their ability to strongly potentiate transcript level. By RNAase protection mapping, the major start sites activated by the downstream enhancers are at nt+1 and +12. By primer extension weaker upstream start sites are located between nts -16 and -110. It is consistent with this pattern of relatively abundant transcription that pGM2809, with enhancer inserts at nt +1340, confers vigorous prototrophy to his3 deficient cells even on plates containing aminotriazole.

Plasmid copy number. We have determined enhancer function on multicopy plasmids. The polyoma enhancer increases plasmid copy number three- or four-fold

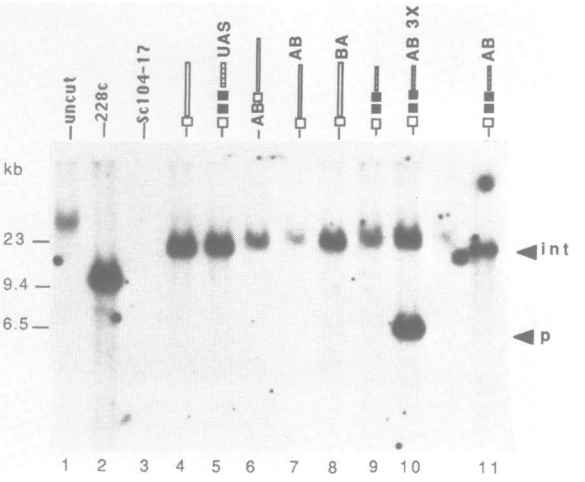


Figure 9. Copy number of integrants in S. cerevisiae strain Sc104-17.
Southern blot of genomic XhoI digests hybridized to a ^{32}P-labelled riboprobe synthesized in vitro with T7 RNA polymerase from a restriction fragment internal (nt +121 to +328) to the HIS3 gene, cloned in pGEM4™. In all strains integration was at the TRP1 chromosomal locus (Materials and Methods).
lane 2, S. cerevisiae 288c; lane 3, Sc104-17; lane 4, pGM8 single copy; lane 5, pGM9 single copy; lane 6, pGM7A single copy; lane 7, pGM35 single copy; lane 8, pGM36 single copy; lane 9, pGM3181 single copy; lane 10, pGM3182-1 three copies; lane 10, pGM3182-1 single copy.
Symbols: see legend to Figure 3. int: XhoI fragment from integrant at TRP1; p: plasmid size XhoI fragment from multiple tandem integrants.

in some pseudopromoter containing constructs (Ciaramella et al, accompanying manuscript); we have failed to detect a correlation between plasmid copy-number and RNA enhancement (eg both upstream and downstream enhancer insertions stimulate transcript levels, but only downstream inserts raise copy number (Ciaramella et al, accompanying manuscript).

Although increase in copy number cannot account for differential initiation it might contribute to increases in transcript level in some constructs. For this reason we looked for effects on copy number in the new constructs. As shown in fig. 8 the results are similar to those described in the preceding paper. In general copy number is significantly raised by 3′ but not by 5′ inserts of the polyoma holoenhancer. The mutant enhancer domain B78 raises copy number in all 3′ and 5′ inserts in pGM3181 and pGM2809 independently of the extent to which it enhances transcription.

Effect of chromosomal integration on enhancer function

One reason for using autonomously replicating plasmids in our experiments is that in mammalian cells viral enhancer potency is markedly enhanced by supercoiling (11). The degree and distribution of supercoils in the yeast genome is unknown; in the chromosomes of higher eukaryotes negative supercoiling appears to be localized and the bulk of DNA is not under torsional strain (beyond that attributable to nucleosome formation) (eg 12).

It was of interest to test some of the enhancer containing plasmids also after integration into the chromosome.
We directed integration of pseudopromoter and TATA box containing test plasmids to the TRP1 locus by standard procedures (Material and Methods). Nor-

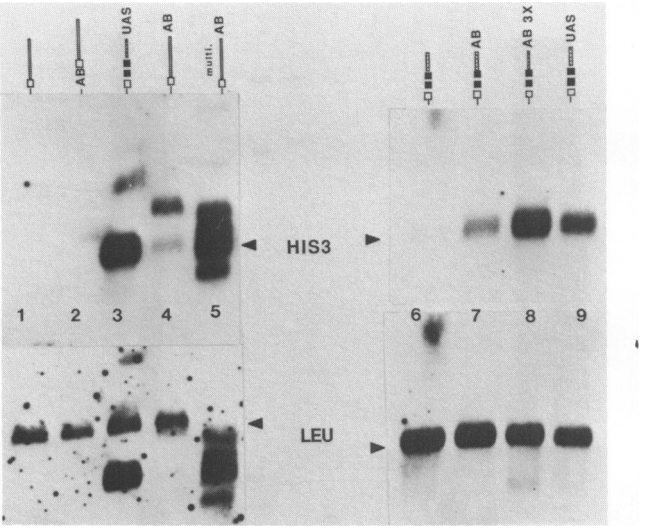


Figure 10 Northern blots of total RNA synthesized by the integrated transformants hybridised to ^{32}P-labelled antisense single-stranded RNA probe transcribed in vitro with T7 RNA polymerase from a restriction fragment internal (nt +121 to +328) to the HIS3 gene, cloned into pGEM4™ (Promega, Biotec) (Ciaramella et al, accompanying ms).
Left panels: Pseudopromoter (Ciaramella et al, accompanying ms) containing constructs. lane 1, pGM8 single copy; lane 2, pGM7A (late orientation) single copy; lane 3, pGM9 single copy; lane 4, pGM35 single copy (late orientation); lane 5, pGM36 (early orientation) multi-copy; Right panels: TATA box containing constructs: lane 6, pGM3181; lane 7, pGM3182-1 single copy; lane 8, pGM3181-2 three copies; lane 9, pGM9.
Bottom panels are the same filters rehybridized to a ^{32}P-labelled antisense LEU2 riboprobe (Ciaramella et al, accompanying ms).
Symbols: see legend to Figure 3.

thern blot analysis of total RNA shows that, after integration, the polyoma holoenhancer cloned upstream of pGM8 (containing the pseudopromoter) (Fig. 9, lane 4, 5) or of pGM3181 (containing the native TATA boxes) (Fig. 9, lane 7, 8) potentiates transcription to somewhat less than the basal level attained by the wild type gene, at its normal location or after translocation to the TRP1 locus (Fig. 9B, lane 2). RNAase protection mapping shows that the two integrated test genes use the same start sites favored in the autonomously replicating state (Fig 11, lane 4, 5). A three copy integrant of pGM3182-1 promotes transcription to a level expected from the copy-number (Fig. 9; Fig. 10, lane 7, 8).

In contrast, the polyoma enhancer inserted downstream at nt +1340 in pGM8 loses its ability to potentiate transcription once integrated at TRP1 in single (Fig. 10, lane 2) or in multiple copies (data not shown).

DISCUSSION

Experiments described in our previous work (5; Ciaramella et al, accompanying manuscript) showed that the polyoma holoenhancer (A-domain + B-

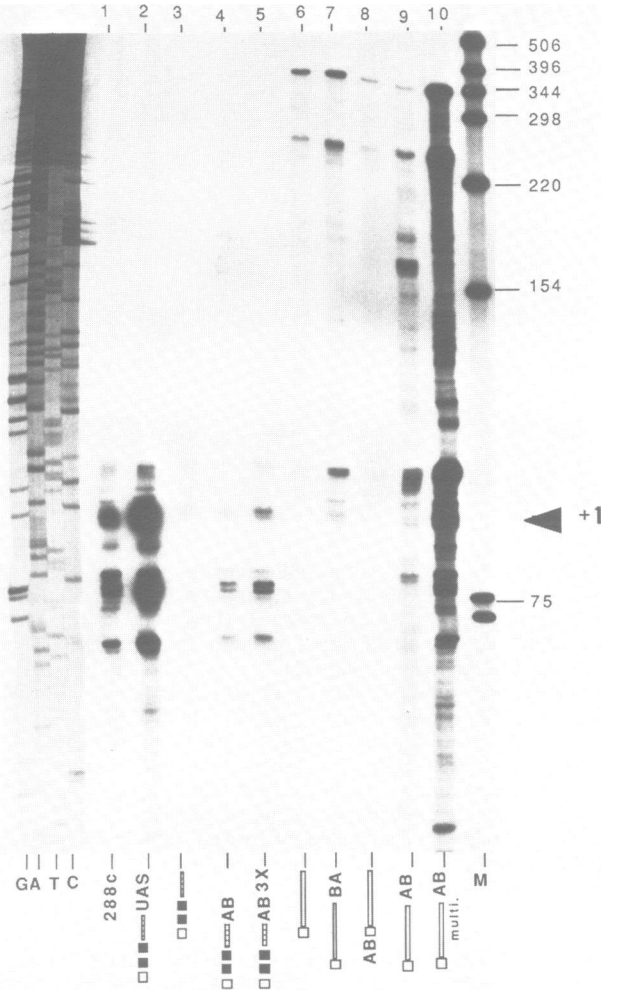


Figure 11. RNAase mapping of 5′ ends of transcripts elicited by the polyoma enhancer after chromosomal integration. Total RNA was hybridized to a uniformally labelled RNA probe complementary to nts -257 to +92 of his-del4 (Ciaramella et al, accompanying ms). After treatment with ribonuclease (Materials and Methods), protected fragments were run on a 6% sequencing gel. G, A, T, C: sequence ladder; lane 1, Sc 288c; lane 2, pGM9, single copy; lane 3, pGM3181, single copy; lane 4, pGM3182-1 single copy; lane 5, pGM3182-1, three copies; lane 6, pGM8 single copy; lane 7, pGM36 single copy; lane 8, pGM7A, single copy; lane 9, pGM35, single copy; lane 10, pGM 35, three copies. The marker is an end labelled HinfI digest of pBR322.

domain), the A-domain partial enhancer and a mutant B-domain partial enhancer (B78) raise transcript levels in yeast when inserted into a test-plasmid containing as reporter the S. cerevisiae HIS3 gene flanked by a quiescent,

susceptible pseudopromoter sequence. The main initiation sites of the potentiated transcripts did not coincide with natural initiation sites (at nt +1, +12 +22), but were scattered within an initiation window 10 or more base pairs further upstream within the pseudopromoter sequence. Interestingly, the size and position of the initiation window shifted with the position (upstream or downstream) and nature (holoenhancer, A-domain or mutant B-78 domain) of the enhancer insert.

In this paper we have replaced the pseudopromoter with truncated versions of the HIS3 promoter to examine the interactions of the polyoma enhancer with native yeast TATA boxes and IR sites.

Interactions of the polyoma holoenhancer with HIS3 promoter elements.

A major result of our present work is that the polyoma enhancer potentiates accurate initiation when inserted 5′ to the reporter gene flanked by a truncated derivative of the natural promoter that retains the native yeast TATA-box elements. The preferred initiation of the potentiated transcripts is at nt +12. The ability to potentiate transcription from this site is lost upon removal of the TATA boxes. This result fits nicely within the framework of current ideas about yeast promoters and depicts the enhancer as just another UAS-like element. In fact, the observation of a physiological interaction between foreign enhancer and "canonical" promoter elements controlling RNA initiation at nt +12 suggests that the enhancer contains a binding site for a yeast protein that potentiates RNA synthesis by interacting with the rest of the transcription complex. In the wild type HIS3 promoter, differential activation of the +12 site depends on a protein-protein interaction of the GCN4 protein bound at the URS and some component of the transcription apparatus (perhaps a TATA-binding protein at Tr) (4).

That transcriptional enhancement and selectivity in initiation are retained after single or multiple copy integration into a yeast chromosome of test plasmids with 5′ enhancer inserts is also consistent with this view. That integrants (in single or multiple copy) containing 3′ inserts of the enhancer do not transcribe the reporter gene is consistent with the finding of others on the properties of UAS elements at chromosomal sites (13,14).
Note however that we have only tested integrants with a 3′ enhancer insert associated with a pseudopromoter; it will be interesting to see what happens in constructs with native TATA elements.

On the whole our observations suggest strongly that the underlying molecular mechanism of polyoma enhancer function in yeast is similar to that in mammalian cells.

Ideas about the mechanism of enhancer function in yeast.

We will consider three types of mechanisms whereby enhancers might potentiate transcription; two of these have been proposed by Struhl (4) to explain UAS function.

1) enhancers increase chromatin accessibility of a potential initiation window by clearing DNA of nucleosomes. In transfected mouse cells 20-25% of polyoma minichromosomes exhibit nucleosome free enhancer-promoter regions (15); whether this effect is connected with transcription (or replication) potentiation is unknown. In yeast, naturally occuring poly(dA-dT) sequences promote constitutive transcription from the three contiguous HIS3 initiation sites, +1, +12 and +22) perhaps by nucleosome exclusion (16). If this mechanism is responsible for all enhancer function in our experiments, it is difficult to understand why, when we replace the natural HIS3 UAS elements with the holoenhancer at nt -80, just one (IR +12), among the three contiguous sites is favoured by enhancer-dependent potentiation.

Perhaps nucleosome clearing is the mechanism whereby the B78-domain (5′ or 3′) and the holoenhancer (only when inserted far downstream) potentiate transcription from a very wide (100 bp or more) initiation window in

pGM3181, pGM2809 and pGM8. Note that the latter constructs also raise plasmid copy-number suggesting a more general effect on plasmid structure.

2) enhancers increase transcript levels by binding an activator. By analogy with the proposed function of known UAS-binding proteins perhaps activation occurs by contact with other elements of the transcription complex. In yeast it has been shown that specificity of UAS binding proteins resides in the DNA binding moiety (17); the effector that contacts the rest of the transcription complex is basically a surface of acidic aminoacids that may vary in composition without loss in efficacy (18). This basic structure of DNA binding activators appeares to hold also in higher eukaryotes: very recently it has been shown, by introducing appropriate binding sites proximal to reporter genes, that the yeast control element GAL4 will activate transcription in mammalian cells (19,20) and conversely that a mammalian oncogene protein (fos) will activate transcription in yeast (21). Implications of these findings have been discussed by Guarente (23).

A specific version of this model of transcriptional stimulation posits that the acidic surface of the activator protein contacts a TATA-box bound protein; which initiation site will be potentiated depends on the position and type of the TATA box and cognate binding factor (4).

There are difficulties in interpreting our results by this version of the model. In some cases we have observed that the enhancer will raise transcript level even when the TATA boxes are deleted (3′ inserts in pGM2811-1 and pGM2811-2). Possibly the natural sequences are replaced by surrogate TATA boxes fortuitously present in the flanking sequences; in higher eukaryotes substitute sequence elements not obviously related to the consensus TATA can replace natural TATA boxes (24). It is also puzzling that in some constructs preferred RNA initiation sites appear to be dictated by the nature and position of the enhancer.

3) Enhancers actively participate in the alignment of the transcription machinery on the DNA template. In general the presence of TATA boxes at their normal locations restricts initiation to the natural IR sites. This however is not always so: the mutant B78 domain inserted at nt -80 overcomes the directives of the TATA box and induces start sites upstream of the natural IR sites, perhaps because B78 itself contains a preferred yeast initiation window. Yet when the B78 domain is inserted upstream of a pseudopromoter in the experiments described in the accompanying paper, it potentiates transcription from start sites located outside the enhancer itself and different from those elicited by the holoenhancer at the same position.
Further support for a more direct role in start-site selection comes from the observation that the holoenhancer inserted at nt -35 in pGM2809 (Fig.5) very effectively diplaces transcription initiation from the natural IR sites.
Perhaps sets of yeast proteins that we imagine bind to the enhancer favour a given initiation window (as presumably do the TATA binding proteins) by influencing the assembly of the transcriptional complex and thus the alignment of the polII initiation domain on the DNA template. The geometry of the complex may differ between holoenhancer and B-78 mutant domain. Very recently it has been observed that polyoma variants bearing rearranged enhancers promote transcription from heterogeneous unnatural start-sites when transfected into certain mouse cell types (25). Differences in initiation disappear (whatever the flanking sequence) when the enhancers are inserted far downstream at nt +1318; perhaps distance relieves constraints imposed upon the assembly of the complex by the rigidity of the shorter DNA spacer between enhancer and other promoter elements in the 5′ constructs.

Whatever the mechanism, our results suggest that DNA binding domains of some higher eukaryote regulatory proteins are conserved in yeast. Such conservation has been described between the yeast GCN4 protein and the mammalian proteins Jun (26) and AP1 (27). Indeed, very recently factors from S.

cerevisiae (28) and Schizosaccharomyces pombe (29) have been shown to bind specifically in vitro to an AP1 binding site (TTGCTGACTAAT) in the SV40 enhancer; these proteins appear to be the yeast homologs (yAP1) of the mammalian AP1 factor. The S. cerevisiae yAP1 factor shows a twenty-fold higher affinity in vitro to a synthetic copy of its cognate site than to a GCN4 (ATGACTCAT) binding site (28), while the S. cerevisiae GCN4 protein binds equally to both.

There is also, in fact, an AP1 binding site in the A-box of the polyoma enhancer; it is lost by point mutation in the A-box transposition into the B-domain of the PyB78 mutant (eg Ciaramella et al, accompanying manuscript). It is unlikely that GCN4 participates significantly in enhancer function, at least in wild-type yeast cells, since the function of the polyoma holoenhancer in single of multicopy integrants, is not stimulated by the addition of 10 mM aminotriazole to the growth medium (V. Rocco , unpublished results). Aminotriazole is a competitive inhibitor of the HIS3 gene product and induces GCN4 synthesis. Perhaps the lack of response of the enhancer to aminotriazole is due to a relatively low level of GCN4. By induction the level of this protein increases only by a factor of six (30) and any effect would be masked by a higher basal level of yAP1 that is not inducible by aminotriazole (28).

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