Foreign transcriptional enhancers in yeast. I. Interactions of papovavirus transcriptional enhancers and a quiescent pseudopromoter on supercoiled plasmids

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ABSTRACT

We have constructed simple test-plasmids to study transcriptional enhancers in yeast. In this paper the reporter-gene is a plasmid borne deletion-substitution derivative (his-del4) of the *Saccharomyces cerevisiae* HIS3 gene in which the native promoter has been replaced by a dormant, susceptible pseudopromoter. We investigate the function in yeast of foreign control elements, the polyomavirus enhancer and some of its derivatives, inserted in either orientation at the 3' or 5' ends of the reporter gene. The polyoma enhancer (and, although less thoroughly studied, the SV40 enhancer) will strongly activate transcription from latent start sites within the pseudo-promoter sequence. The rules we draw for the polyoma enhancer effect in yeast are, with a few interesting exceptions, remarkably similar to those discovered by experimentation in mammalian cells.

INTRODUCTION

In eukaryotes effective transcription by RNA polymerase II is dependent on DNA sequence domains flanking the mRNA coding region. In the yeast *Saccharomyces cerevisiae* at least three such *cis*-acting elements are known to be required for efficient transcription initiation: the initiation site/s itself (IR), the TATA box, and the upstream activation site (UAS) (1,2,3).

The IR sites, unlike their higher eukaryote counterparts, are preferred sequences (2,3). The TATA box elements restrict initiation to potential IR sequences situated within an initiation window 60-100 bp downstream. The UAS elements, situated 100 bp or more upstream, determine the level of transcription and often, via interaction with cognate TATA boxes, which start sites are used. UAS elements may overlap regulatory sites (URS) or be constitutive (eg polyAT sequences) (4). Some TATA boxes respond to URS elements and some only to constitutive UAS elements (5,6). Yeast UAS elements have been inverted and their distance upstream (but not downstream) varied extensively without appreciable effect on transcription (7,8). The sum of these properties, that distinguish yeast UAS elements from canonical promoter elements, as defined in prokaryotes (9), suggests a loose analogy with so-called transcription enhancer sequences of higher eukaryotes and their viruses.

Enhancer elements were first discovered as components of animal-virus promoter elements (10,11,12,13), in the papovaviruses *polyoma* and SV40, and have been intensively studied because of their surprising properties. By interaction with DNA binding *trans*-acting factors (14,15,16), prototypical enhancers will activate transcription from natural start sites or (when removed from their native sequence environment) from heterologous or hybrid promoters, or from otherwise quiescent pseudopromoters. Furthermore enhancers ge-

nerally function about as effectively in either orientation and at variable distances, even far downstream from transcription initiation sites (10,11,17).

Recently some cellular promoters have been dissected into elements that will stimulate transcription of heterologous transcription units and that are position-independent; the operational distinction between enhancers and canonical promoter elements is thus blurred (18,19). However, viral enhancers are structurally distinctive because of their size (up to 200 bp compared to the 6-10 bp of upstream promoter elements). Such enhancers contain a mosaic of multiple contiguous short sequence elements, some common to the promoters of other viral and cellular genes (20,21,22,23). Functionally the component elements of the enhancer behave additively or antagonistically and contain binding sites for proteins that appear to differ in their cell type specificity.

These features suggest that enhancers are multivalent control elements serving to extend the host range of the viruses by their potential to interact with a multitude of evolutionarily conserved host DNA binding proteins. If such proteins and the underlying mechanism of transcription activation are sufficiently conserved, perhaps viral enhancers will also function in yeast. Work in this organism might uncover elements of a new and interesting level of transcriptional control and expose them to investigation by the rich methodology of yeast molecular genetics.

In this and the accompanying paper we directly address the nature of the analogy between UAS and enhancer elements and ask two basic questions: a) can transcription of a yeast gene be driven by viral enhancers?; b) if so, will the foreign enhancers interact with the other elements of a yeast promoter: the TATA box and IR sites?

By the use of appropriate test-plasmids, we find that the polyoma enhancer is active in yeast and can substitute for deleted UAS sequences. Although less extensively studied also the SV40 enhancer appears to be functional in this organism.

MATERIALS AND METHODS.

Test-plasmids.

pGM8 and pGM8A (Fig. 1A) were constructed from plasmid pAT011 (24), a derivative of pAT153. This 3021 bp plasmid lacks pBR322 sequences between the EcoRI and SalI sites suspected of being responsible for position-effect activation of cryptic promoters (25,26; see below) and provides a cluster of unique adjacent restriction sites (polylinker) where yeast genes and enhancers may be cloned in close proximity without interspersal of plasmid sequences.

A 1.4 kb EcoRI yeast fragment containing the TRP-I gene and an ARS element (ARS-1, 27) cloned at the EcoRI site of PAT011 (pGM1) simultaneously introduces a selectable marker and the ability to replicate autonomously in yeast cells.

At the BamHI site of plasmid pGM1 we inserted either (pGM9) the wild-type HIS3 gene, (as a 1765 bp BamHI fragment; Sc2676; 28,) or (pGM8, pGM8A) a mutant derivative of HIS3 from which, by in-vivo recombination in a lambda vector (29b), nt -447 to nt -10 had been substituted by 247 base pairs from the bacteriophage lambda attachment site. The mutant sequence, also called his3-del4 is contained within a 1575 bp BamHI fragment (Sc2715, 29). The test-plasmid is called pGM8 if the TRP1 fragment is in the orientation shown in fig. 1, and pGM8A if it is in the opposite one. In both these deletion derivatives two TATA-like sequences are generated, by a combination of lambda and yeast sequences, just 14 or 30 bp upstream from one natural RNA initiation site and 25 or 41 bp from the other (28,29a,29b). In pGM8, but not in pGM8A, two additional TATA box at nucleotides 1080-1087 and 1332-1336 of the TRP1 fragment (27), are located 640 and 388 bp upstream from the beginning of the HIS3 structural gene. In some constructs HIS3 transcription is activated from a dormant promoter within the 3' end of the TRP1 fragment (the 1.4 kb transcript; see text). The BamHI fragment includes, downstream from HIS3, about 500 bp of the 5' side (including the promoter) of the ded-1 gene (30). Enhancers and derivatives were cloned as XhoI or BamHI fragments into pGM8 (or pGM8A) at various sites described in the text.

Enhancer fragments. The structural and biological properties of the enhancers we study are described elsewhere in this paper (Introduction, Results).

The polyoma holoenhancer (Fig. 1B). This 244 bp fragment had been provided with XhoI linkers (10). We have added BamHI linkers for some of the constructions by cloning into the the Sall site of the symmetric polylinker of pPKL1 (31) and excising with BamHI. The 134 bp B-domain 'hemienhancer' (PvuII fragment) and a B-domain derivative from polyoma mutant Py-FL78 (32), are provided with BamHI linkers. Orientation of the polyoma holoenhancer was determined by cutting recombinant plasmids with PvuII (that cuts once, within the polyoma enhancer between the A- and B-domains) and either XhoI or HindIII. The restriction digest was separated on 0.8% agarose, blotted and hybridised to a B-domain specific probe.

The A-domain was inserted at the 3' end of his-del4 by substituting the 580 bp XhoI PvuII fragment of pGM7A (3' insertion of the polyoma holoenhancer in Late-orientation) with the 446 bp XhoI to HincII (in Sall) fragment from pGM8. The A-domain was inserted at the 5' end of his-del4 by substituting the 1271 bp PvuII XhoI fragment of pGM35 (5' insertion of the polyoma holoenhancer in Early-orientation) with the 1140 bp SmaI to XhoI fragment of pGM8. Recombinants were checked by sequencing or by restriction mapping and hybridization. In some cases (pGM7A and B) plasmid were transferred back into E. coli and checked for possible rearrangements by restriction mapping and hybridisation.

The SV40 holoenhancer (Fig. 1C). This 196 bp fragment was provided with XhoI linkers and an internal EcoRI linker adjacent to one extremity (bp 294 on the SV40 map) (10,21). Orientation of the enhancer was determined by cutting with EcoRI and XhoI. We have constructed a 'hemienhancer' by digestion with SphI and re-ligation.

Insertion of non-enhancer sequences.

As a control that sequence, rather than spacing, is important in HIS3 activation by insertion of enhancers at flanking sites, we introduced a 209 bp Sau3A Adenovirus-2 restriction fragment (nt 9467-9666) overlapped by three possible open reading frames (33). This fragment, cloned into the 3' (not shown) and 5' BamHI sites of pGM8 (Fig. 3A, lane h), did not stimulate appreciable his-del4 RNA synthesis. Insertion at the 5' site however did restore weak histidine prototrophy (aminotriazole sensitivity is retained, Table 1). We have also inserted various 200-300 bp lambda fragments into the adjacent 5' Sma site and find that they all restore histidine prototrophy. Apparently, at the 5' end of his-del4, sequence-independent (however see below) spacer effects somehow restore weak HIS3 RNA synthesis.

Bacterial and yeast strains.

Plasmids were transformed and maintained in E. coli in the rec A strain HB101 (F⁻, hsd S20, (r_m⁺, m_m⁺), recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Sm^r), xyl15, mtl-1, supE-44, λ⁻) selecting for Ampicillin resistance. The strain was checked periodically for the RecA phenotype.

Plasmids were usually transformed and maintained in yeast in strain ScKY117 (a, ara3-52, tryp1-del1, his3-del200, ade2-101_{oc}, lys2-801_{am}, tryp1-del1 deletes all sequences corresponding to the 1.4 kb EcoRI TRP-1 ARS-1 fragment (27,30), his3-del200 completely deletes chromosomal HIS3 sequences (corresponding to nt -178 to nt +855 of the 1765 bp wild-type BamHI fragment (34,8,4).

The phenotype of yeast cells harboring plasmids was determined by standard procedures (35) on SD minimal plates lacking appropriate nutrients. In some cases the ability of recombinant plasmids to synthesize abundant imidazoleglycerolphosphate dehydratase (IGPD), the product of the HIS3 gene, was tested by growth by on SD plates containing 10mM aminotriazole, a potent competitive inhibitor of IGPD.

Yeast cells were transformed by the LiCl protocol (36).

Nucleic acid biochemistry Yeast RNA, chromosomal and plasmid DNA were prepared and assayed by capillary blotting as described (34,35,37). For quantitation Northern blots were reprobed with uniformly labelled LEU2 or DED1 specific probes and/or by methylene blue staining of ribosomal RNA transferred to the filters. Because, in some constructs (Fig. 9), enhancers exert effects on copy number and on over-all plasmid transcription, transcripts from plasmid-borne genes were not used in normalization.

Preparation of radioactive probes.

In most cases probes were double-stranded and prepared from restriction fragments uniformly ³²P labelled by nick-translation. To detect the various transcripts the following probes were used: for TRP1, the complete 1.4 kb EcoRI fragment; for HIS3, an internal HindIII fragment (nts +328 to 515); for DED1 the segment from the XhoI site at nt +880 to the flanking vector Sall site (Fig. 1A).

Single stranded uniformly labelled RNA probes were prepared in-vitro with T7 RNA polymerase by run-off transcription of appropriate HIS3 fragments cloned into vector pGEN4 (Promega) (Fig. 2) as described (38). See figure legends for details.

Figure 1. Structure of the recombinant test plasmid, pGM8 and of the papova virus enhancers. A) The test-plasmids (pGM8 and pGM8A) were constructed by inserting a 1.4 kb EcoRI fragment containing TRP-I and ARS I, and a 1.6 kb BamHI fragment containing his-delta4 into the contiguous EcoRI and BamHI sites of plasmid PAT011 (24), a derivative of pAT153. The test-plasmid is called pGM8 if the TRP-I fragment is in the orientation shown in fig. 1, and pGM8A if it is in the opposite one.

In his-delta4 247 bp of extraneous DNA replaces native flanking sequences up to nt -10 thus removing UAS elements and TATA boxes but not native IR sequences. Two TATA sequences are generated, by a combination of bacteriophage λ (cfr Materials and Methods) and yeast sequences, just 14 or 30 bp upstream from one natural RNA initiation site and 25 or 41 bp from the other (29a,29b,30):

GGATCCGCCTACCTTTACGAGTTGCGCAGTTTGTCTGCAAGACTCTATGAGAAGCAGATAAGCGATAAGTTT
GCTCAACATCTTCTCGGCATAAGTCGGACACCATGGCATCACAGTATCGTGATGACAGAGGCAGGGAGTGGG
ACAAAATTGAAATCAAATAATGATTTTATTTGACTGATAGTGACCTGTTCTGTCACAAATTGATAAGCAA
TGCTTTTTTATAATGCCAACTTAGTATATAAAAAATGAGCAGGCCAAAGATGACAGAG.

In pGM8, but not in pGM8A, two additional TATA boxes at nucleotides 1080-1087 and 1332-1336 of the TRP-I fragment (27), are located 642 and 390 bp upstream from the beginning of the HIS3 structural gene. In some constructs HIS3 transcription is activated from a dormant promoter within the 3' end of

the TRP1 fragment (the 1.4 kb transcript; for details see text).

■ natural and recombinant TATA boxes

□ UAS

terminator= T

The BamHI fragment includes, downstream from HIS3, about 500 bp of the 5' side (including the promoter) of the *ded-1* gene.

Enhancers and derivatives (see below) were cloned, as XhoI or BamHI fragments, in Early and Late orientations into pGM8 (or pGM8A) at various sites described in the text and indicated schematically in the figure. Early and Late orientations mean respectively that either the early or the late coding strand of the *polyoma* insert is in the same orientation as the HIS3 coding strand.

Restriction sites: EcoRI (E); SmaI (Sm); Sau3A/MboI (Sau); BamHI (Bm); HindIII (H); BglII (B); PstI (P); XhoI (X); SalI (S).

B) The *polyoma* enhancer shown in Early orientation. The 244 bp BclI--PvuII fragment had been provided with XhoI linkers (10). We have added BamHI linkers for some of the constructions. A 134 bp B-domain "hemi-enhancer" (PvuII fragment) and a mutant B-domain from PyFL78 (B-78), are provided with BamHI linkers. B-78 contains a transposition of an altered version (by 4 base substitutions) of the 30 bp box A motif (32) into the B-domain: CTGGCCGTGAGTCACTTAGGCAGGAAGTCACTAGCTGACCGCCGACATCCTCTTTTAATTAGTTGCTAGGCAAC-TGGCTCCAGAGGGCAGTGTGTTTTCGAAGAGGAAGCAAACGCCTCTCCACCCAGGCCTAGAAATGTTTCCACCCAATCATTACTATGACAAACAGCTG. The 30 bp altered box A sequence is underlined.

C) A segment of DNA containing the SV40 holoenhancer in Early orientation. The 196 bp fragment was provided with XhoI linkers at both extremities and with a EcoRI linker at one extremity (10). We have constructed a "hemi-enhancer" by digestion with SphI and re-ligation.

(●) Transcription start-sites.

(Δ) deletion that removes one of the 72 bp repeats.

Restriction sites in parenthesis were destroyed by linker addition

"E1a" and "SV40" indicate homologies, respectively to the Adenovirus E1a and SV40 enhancers.

RESULTS

Effects of the enhancers on transcription

Polyoma and SV40 enhancers were tested in yeast after insertion into an appropriate test-plasmid. We briefly summarise salient features of the test-plasmid and of the enhancer fragments (for further details see Materials and Methods)

The test-plasmid. The test-plasmid we used in most constructs, pGM8 (M&M, Fig. 1A) carries two adjacent yeast sequences: a) a 1575 bp BamHI fragment containing a transcriptionally inert deletion-substitution derivative of the yeast HIS3 gene (*his3-del4*), and b) a 1460 bp EcoRI fragment containing the TRP1-ARS1 sequences we use for plasmid selection and maintenance.

In *his3-del4* the native HIS3 promoter sequences, excepting the transcription start-site signals, are replaced by 257 bp of AT-rich, non-yeast DNA. This replacement fragment contains quiescent pseudopromoters (26,40) that are known to be susceptible to activation by the proximity of yeast enhancer-like elements present in transposable element Ty1 (41,42).

Position-effect interactions on transcription between *his3-del4* and the TRP1-ARS1 EcoRI restriction fragment have been extensively explored (26).

To avoid introducing further sequence complexities in the ARS1 test plasmid we chose not to stabilize it by introducing an additional CEN con-

Figure 2. Origin of RNA probes for RNA mapping. The fragments shown were cloned in pGEM4™ and used as templates for the *in vitro* synthesis of uniformly labeled RNA probes.

taining fragment. Thus in any given experiment only about 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 unnatural has the advantage of reproducing conditions for transient expression assays in mammalian cells following $\text{Ca}_3(\text{PO}_4)_2$ transformation.

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

Two important advantages of the way pGM8 is constructed are: restriction sites, appropriate for insertion of enhancer fragments, flank both the 5' and 3' ends of the *his-del4* BamHI fragment; strong transcription termination sites are situated about 940 bp upstream and about 100 bp downstream from the HIS3 structural sequence (30,43; Fig.1A) thus preventing readthrough of HIS sequences from active promoters and pseudopromoters in the vector.

The enhancers. The *polyoma* enhancer is a functionally composite structure, contained within a segment of 244 base pairs (nucleotides 5265-5021) (Fig. 1B) shown to be essential for viral transcription and replication (11,22,44,45). At least two domains have been identified within this region: the A domain (109 base-pairs) located between the BclI (nucleotide 5021) and PvuII (nucleotide 5130) restriction sites, and the B domain (134 base-pairs) located between the two PvuII sites (nucleotides 5131-5265) (20). The isolated A domain has considerable activity in stimulating RNA synthesis (by a linked *polyoma* or heterologous gene) in mouse fibroblasts while the isolated B domain has almost none (20). In other cell types activity of the isolated A domain is much weaker (such that *polyoma* virus with a truncated enhancer will not grow).

By deletion analysis and testing for the capacity to promote viral replication Veldman et al (22) dissect the A domain into two distinct elements: the A element (nts 5108-5130), essential for replication and the partially dispensable D element (nts 5021-5098). The B domain may contain yet other two separable elements, C and B that play an auxiliary role in replication activation.

TABLE I
Characteristics of test-plasmids.

PLASMID	Enhancer/control Fragment			Phenotypes: enhancement						
	domain	site	orient.	-His	ATR	HIS RNA	TRP1 RNA	DED1 RNA	DNA	
pGM9	/	/	/	+	+	++	+	+	1	
pGM4	/	/	/	+	+	++	+	+	1	
pGM8	/	/	/	-	-	-	+	+	1	
pGM8A	/	/	/	+	nt	-	+	+	1	
pGM7A	Py-H	3'Sall	Late	+	-	+	++	++	3	
pGM7B	Py-H	3'Sall	Early	+	-	++	++	++	3	
pGM35	Py-H	5'BamHI	Late	+	+	++	+	+	1	
pGM36	Py-H	5'BamHI	Early	+	+	+	+	+	1	
pGM63	Py-H	3'BglII	Early	-	nt	-	+	+	0.9	
pGM13	Py-H	3'XhoI	Late	-	nt	-	+	+	1.9	
pGM59	- A	3'Sall	Late	+	-	(+)	(-)	+	1.3	
pGM40	- B	3'Sall	nt	-	-	-	(+)	+	1.6	
pGM60	- A	5'BamHI	Early	+	-	+	nt	(+)	1	
pGM39	- B	5'BamHI	nt	-	-	-	+	+	1	
pGM78	-B78	5'BamHI	nt	+	+-	++	+	+	1	
pGM61	-B78	3'BamHI	nt	+	-	++	++	++	3	
pGMAd2	Ade	3'BamHI	/	-	nt	-	+	+	1	
pGMAd1	Ade	5'BamHI	/	+	nt	-	+	+	1	
pGM33	SV40	3'Sall	Late	+	nt	++	++	++	0.9	
pGM33A	SV40	3'Sall	Late	+	nt	++	++	++	1	
pGM33B	SV40	3'Sall	Early	+	nt	+	nt	nt	nt	
pGM64A	Py-H	3'BglII Sall	Early Late	-	nt	-	nt	nt	nt	

Table I: Characteristics of recombinant plasmids used:

pGM9 and pGM4 contain a BamHI fragment with the wild-type HIS3 gene. In pGM9 the orientation of the BamHI fragment is the same as in pGM8, in pGM4 the BamHI fragment is inverted.

Other plasmids are derivatives of pGM8 and were obtained by ligating electroeluted DNA fragments to appropriate restriction sites.

Enhancer/control fragments: Py-H, polyoma holoenhancer; BoxA, BoxB, B-78 are derivatives of the polyoma enhancer described in the text. SV40H, simian virus 40 holoenhancer; SV40h, is a partial enhancer with a single 72 bp element. Ade is the 209 bp Adenovirus control fragment, described in the text.

Growth in absence of histidine was determined on SD plates with appropriate supplements at 30 °C. Growth in the presence of 10 mM Aminotriazole was determined after 4 days at 30 °C. Scoring is relative to growth supported by pGM8

Orientation: the Early orientation refers to the HIS3 gene and is the same as that shown in fig.1. Late is the opposite orientation.

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

Northern blots of total RNA hybridised to the ³²P-labelled HindIII restriction fragment internal to HIS3 (Fig. 1A). A) Effect of 5' inserts: lane a, pGM9, contains the wild-type promoter; lane b, pGM8, the test-plasmid with the 1575 BamHI fragment containing the promoterless HIS3 derivative his-del4 (Fig. 1A); lane c, pGM35 = pGM8 + the polyoma holoenhancer inserted at the -257 BamHI site in Late orientation; lane d, pGM36 = pGM8 + the polyoma holoenhancer inserted at the -257 BamHI site in early orientation; lane e, pGM60 = pGM8 + the polyoma enhancer A-domain inserted at the -257 BamHI site; lane f, pGM39 = pGM8 + the polyoma enhancer B-domain inserted at the -257 BamHI site; lane g, pGM78 = pGM8 + the polyoma enhancer B-domain derivative from the PyFL78 mutant inserted at the -257 BamHI site; lane h, pGMAd1 = pGM8 + a

209 bp *Sau*III restriction fragment from Adenovirus-2 (nt 9467-9666) inserted at the -257 *Bam*HI site;

B) Effect of 3' *polyoma* enhancer inserts: *lane a*, pGM9, contains the wild-type promoter; *lane b*, pGM8, the test-plasmid with the 1575 *Bam*HI fragment containing the promoterless HIS3 derivative *his-del4* (Fig. 1A); *lane c*, pGM7A = pGM8 + the *polyoma* holoenhancer inserted at the +1324 *Sal*I site in *late* orientation; *lane d*, pGM7B = pGM8 + the *polyoma* holoenhancer inserted at the +1324 *Sal*I site in *early* orientation; *lane e*, pGM59 = pGM8 + the *polyoma* enhancer A-domain inserted at the +1324 *Sal*I site; *lane f*, pGM40 = pGM8 + the *polyoma* enhancer B-domain inserted at the +1324 *Sal*I site; *lane g*, pGM61 = pGM8 + the *polyoma* enhancer B-domain derivative from the PyFL78 mutant inserted at the +1318 *Bam*HI site;

C) Same filter as in A rehybridized to a LEU2 probe (Materials and Methods).

D) Same filter as in B rehybridized to a LEU2 probe (Materials and Methods).

Symbols: A and B mean respectively the A and B domains of the *polyoma* enhancer. AB (read from top to bottom) indicates the Early orientation, BA the late. B78 is the B-domain from the *polyoma* FL78 mutant.

Viral mutants selected for growth on cells that are nonpermissive for wild-type *polyoma* carry rearrangements in the enhancer region. One mutant (PyFL78), selected for growth in Friend erythroleukemic cells, carries a 30 bp duplication of the element A region spanning nt 5096 to 5128, inserted between nt. 5139 and 5140 in element C (B domain); four base substitutions are also present (legend to Fig. 1) (32). The mutant PyFL78 B domain (elements C and B plus the A insert) is an efficient enhancer in mouse fibroblasts (44) even if separated from the A domain.

The intact Simian virus 40 holoenhancer is contained within a segment of 194 base pairs (nucleotides 100-294) and contains two 72 base-pair repeat "hemienhancers" (Fig. 1C). A single copy of the 72 base pair repeat, obtained by cutting the enhancer with *Sph*I and religation, is sufficient for enhancement of transcription in HeLa cells (12).

We cloned intact, partial and mutant *polyoma* and SV40 enhancers, as *Xho*I fragments or as *Bam*HI fragments (after linker addition), in both *Early* and *Late* orientations (Fig. 1B) into pGM8 at various sites upstream and downstream of (as well as within) the promoterless HIS3 derivative, *his-del4* (Fig. 1A).

The plasmid constructs were transformed into yeast strain ScKY117, selecting for tryptophan prototrophy. In preliminary screenings the recombinant plasmids were tested for their ability to confer Histidine prototrophy upon ScY117 by growth on SD media lacking Histidine and on the same media containing 10 mM aminotriazole, a competitive inhibitor of the HIS3 gene product (IGPD); in the presence of aminotriazole yeast will only grow if very large amounts of IGPD are synthesized (Materials and methods).

Plasmid structures and phenotypic properties are summarized in Table I. Total RNA was prepared from yeast cells transformed by the recombinant plasmids. Since we ignored what potential native or foreign transcription initiation sites on the double stranded segment of DNA, situated between the two known termination sites in the test-plasmid, might be activated by the enhancers, "Northern" blotting and hybridisation to a double stranded probe prepared from a *Hind*III fragment taken from the center of the HIS3 gene (Fig. 1A) was chosen as the most reliable and unbiased preliminary measure of HIS3 RNA (Materials and Methods).

Figure 4 enhancement of HIS3 transcripts by the SV40 enhancer.

Northern blots of total RNA hybridised to the ^{32}P -labelled HindIII restriction fragment internal to HIS3 (Fig. 1A). The holoenhancer is the element shown in Fig. 1C. The hemienhancer, containing a single 72 bp repeat, was obtained by cutting with SphI and religation.

lane a, pGM9; lane b, pGM8; lane c, pGM33A = pGM8 + the 196 bp SV40 holoenhancer inserted at the +1324 SalI site in late orientation; lane d, pGM33B = pGM8 + the 196 bp SV40 holoenhancer inserted at the +1324 SalI site in early orientation; lane e, pGM33 = pGM8A + the 196 bp SV40 holoenhancer inserted at the +1324 SalI site in late orientation; lane f, pGM34 = pGM8A + a single 72 bp repeat of the SV40 enhancer inserted at the +1324 SalI site in late orientation; lane g, pGM8A, a variant of test-plasmid pGM8 with the TRP1 fragment in opposite orientation to that shown in Fig. 1A.

On plasmids containing the intact promoter sequence, the HIS3 gene is traversed by a transcript of approximately 0.8 kb corresponding to the size determined for chromosomal HIS3 RNA (Fig. 3, lane a). The size and abundance of this transcript is not affected by inverting the wild-type HIS3 BamHI fragment (data not shown). All sequences required for HIS3 RNA initiation and termination are contained within the BamHI fragment. This transcript is dependent on the integrity of the HIS3 promoter and is greatly reduced when the native 5' flanking sequences of HIS3, including the promoter, are substituted by non-yeast DNA (his-del4) as in pGM8 (Fig. 3, lane b) and pGM8A (Fig. 4, lane g). Faint new higher molecular weight transcripts of 1.4 and 1 kb, homologous to the HindIII (HIS3) probe appear in pGM8 (in pGM8A only a 1 kb species is sometimes visible); these RNAs must start at weak cryptic initiation sites in the new 5' flanking sequences.

Intact enhancers. The polyoma holoenhancer stimulates transcription when inserted in Late or Early orientation (Fig. 3A, lanes c & d) at the BamHI site at the 5' end of his-del4, 257 bp upstream from the beginning of the gene. The length of the major transcript driven by both orientations of the polyoma enhancer is 0.8 kb suggesting that one of the same initiation sites used by the native UAS element are being activated. This suggestion is strengthened by the strong histidine prototrophy (aminotriazole resistance) conferred by these plasmids (Table I). The apparent greater efficiency of the Late orientation is at least in part due to a loading artifact (Fig. 3C; lanes c & d).

Introduction of the polyoma holoenhancer at the 3' SalI site (nt +1324), in both orientations (Fig. 3B, lanes c & d), leads to an increase in the amount of HIS3 RNA. The Early orientation is considerably more effective

Figure 5. Strandedness of enhanced transcripts.

Northern blots of total RNA hybridised to ^{32}P -labelled sense (A) and antisense (B) single-stranded RNA probes transcribed in vitro with T7 or SP6 RNA polymerase from a restriction fragment internal (nt +121 to +328) to the HIS3 gene (Fig. 2), cloned into pGEM4TM (Promega, Biotec) (Materials and Methods).

lane a, pGM9; lane b, pGM8; lane c, pGM36; lane d, pGM60; lane e, pGM78; lane f, pGM7A; lane g, pGM59; lane h, pGM61.

Symbols: UAS represents the native upstream activator; closed boxes are the native TATA-boxes; open boxes are TATA-like sequences; bars represent "spacer" sequences (native: cross-hatched; recombinant: open) interposed between the control elements under consideration. A and B mean respectively the A and B domains of the *polyoma* enhancer. AB indicates the Early orientation, BA the late. B78 is the B-domain from the *polyoma* FL78 mutant.

than the *Late*. The transcripts are heterogeneous in size; 1 and 1.4 kb RNAs are predominant, while the 0.8 kb species is rarer.

The relatively weak prototrophy (aminotriazole sensitivity) conferred by these plasmids is consistent with this pattern of transcription (Table I).

Dissected and mutant *polyoma* enhancers.

We have dissected the enhancer sequences to determine whether the sequence specificities observed in mammalian cells are retained in yeast. In our experiments, when the *polyoma* holoenhancer is dissected into the A-domain and the B-domain and inserted either at the 5' or 3' BamHI sites of *his-del4*, detectable HIS3 specific transcripts are directed by the A-domain (the effect of the A-domain is weaker at the 3' site) but not by the B-domain (Fig. 3A and B: lanes e & f). In contrast, inserts of the mutant B-domain from Py-FL78, at the 5' end (pGM78) (Fig. 3A, lane g) or at the 3' end (pGM61) (Fig. 3B, lane g) of *his-del4*, stimulate transcription almost as effectively as the holoenhancer. Apparently the modules of the *polyoma* enhancer behave in the reactivation of the HIS3 yeast gene much as they do in mouse fibroblasts.

Interestingly, the size distribution of the enhanced transcripts

Figure 6 RNAase mapping of 5' ends of transcripts elicited by the polyoma enhancer.

A) Total RNA was hybridized to a uniformly labelled RNA probe complementary to nts -257 to +82 of his-del4 (BamHI-MboI fragment in Fig. 2). After treatment with ribonuclease (Materials and Methods), protected fragments were run on a 6% sequencing gel.

lane a, pGM9; lane b, pGM8; lane c, pGM35; lane d, pGM36; lane e, pGM60; lane f, pGM78; lane g, pGM7A; lane h, pGM61. The marker is an end-labelled HpaII digest of pAT011. Fragments predicted from the sequence are in nt: 492, 465, 404, 242, 238, 201, 190, 160, 147, 110, 90, 76, 67, 53, 34, 26, 26.

B) Total RNA was hybridized to a uniformly labelled RNA probe complementary to nts -257 to +328 of his-del4 (BamHI to HindIII fragment in Fig. 2).

lane a, pGM9; lane b, pGM8; lane c, pGM35; lane d, pGM78; lane e & f, increasing amounts of HIS3 chromosomal transcripts from the wild-type strain, Sc288c; lane g, undigested probe; lane h, pAT011 HpaII marker (see above).

Symbols: see Legend to Fig. 5.

depends not only on the position but also, for the 5' inserts, on which particular enhancer derivative is introduced. The prevalent transcript directed by the mutant B-domain hemienhancer from PyFL78 differs in size from that directed by the wild-type holoenhancer at the same 5' position. In fact, in spite of abundant transcript, this recombinant displays relatively weak prototrophy (partial aminotriazole sensitivity, Table I). Also, transcripts driven by the A-domain at the 5' position differ strikingly in size and heterogeneity from those driven by the holoenhancer at the same position. On the contrary, the size distribution of transcripts elicited by the various 3' inserts does not vary between constructs.

The SV40 holoenhancer and "hemienhancer". The synthesis of heterogeneous HIS3 RNA species is also activated by the SV40 holoenhancer inserted at the 3' Sal-I site (5' inserts were not tested) (Fig. 4, lane c and d). The Late orientation is more effective than the Early. The major transcripts driven by the SV40 holoenhancer are reduced to a single species of about 1 kb when the TRP-ARS1 fragment is inverted in the GM8A configuration, suggesting that 1.4 kb HIS3 RNA is initiated (or terminated) within this fragment (Fig. 4, lane e). A SV40 "hemienhancer", obtained by SphI digestion and religation of the holoenhancer (Fig. 1C) contains a single copy of the 72 bp repeat but is as effective as the intact element in stimulating the 1 kb transcript in pGM8A (Fig. 4, lane f).

Strandedness of enhancer driven transcripts. Which DNA strand functions as template in the different enhancer containing constructs was determined using a high specific activity single stranded RNA probes transcribed in-vitro either with T7 RNA polymerase (antisense strand) or with SP6 RNA polymerase (sense strand) from a Sau3A (nt +121)/ Hind3 (nt +328) HIS3 fragment cloned into the polylinker of plasmid pGEM4 (Promega Biotec) (Fig. 2). HIS3 sense transcripts are in general identical in size and relative abundance (Fig. 5B, lanes a-e) to those monitored with the double stranded probe, with the exception of the c. 1.4 kb RNA transcribed from pGM8 and enhanced by 3' enhancer inserts (Fig. 3B, lane b, f, g and h). 1.4 kb sense-strand RNA is a minor transcript (Fig. 5B; lanes f and h); most transcripts of this size are anti-sense (Fig. 5A lane f and h).

Mapping RNA start-sites. Transcripts driven by the polyoma enhancer are of heterogeneous size and often differ from the size of the wild type transcript (0.8 kb). Furthermore the size-heterogeneity appears to depend on the position and sequence of the enhancer itself. This complexity in our results is not unexpected since in his-del4, all promoter sequences except start sites are replaced by extraneous AT rich DNA containing a number of potential TATA boxes (Legend to Fig. 1A).

We have used RNAase mapping to determine the positions of the start-sites (Materials & Methods) and find that indeed the size heterogeneity of transcripts reflects the heterogeneity of the RNA start-sites.

The holoenhancer sequence inserted at nt -257 in the 5' flanking region of his-del4 potentiates transcription from start sites at nts -154, -9 and +1 (Fig. 6A; lanes c & d). The natural HIS3 start sites are little used: only the +1 site coincides with a wild-type start site and most transcripts start 9 bp upstream at the joint between yeast and sequences or even further upstream inside the substitution fragment. The mutant B78 domain at the same position, besides activating strong start sites at nt -154 and -9 (Fig. 6A, lane f), also potentiates transcription from nt +108 as evidenced by the longer probe (Fig. 6B; lane d).

RNA start sites stimulated by wild type and mutant enhancers cloned downstream at nt +1324 are even more heterogeneous: both the wild-type holoenhancer and the mutant B78 domain potentiate transcription from start sites at nt -9, -48, -92 and -154 in the 257 bp pseudopromoter sequence in his-del4 (Fig. 6A; lanes h & i).

Comparison of the RNAase protection map to the Northern blots (Fig. 3A & B) suggests that the bias for initiation at nt -9 is likely to be a hybridization artifact. Nucleotide +9 is in a T-rich stretch within the break-point between relatively AT rich upstream sequences and the more GC rich HIS3 structural sequence. The apparent initiation site might reflect a discontinuity caused by different optimal annealing temperatures of upstream and downstream portions of the transcripts.

Figure 7. Unproductive sites.

Northern blots of total RNA hybridised to the 32 P-labelled HindIII restriction fragment internal to HIS3 (Fig. 1A).

lane a, pGM9; lane b, pGM8; lane c, pGM13 = pGM8 + the polyoma holoenhancer inserted at the +880 XhoI site in late orientation; lane d, pGM63 = pGM8 + the polyoma holoenhancer inserted at the +419 BglII site in early orientation; lane e, the Sandwich: pGM64A = pGM8 + two copies of the polyoma holoenhancer one inserted at the +419 BglII site in early orientation, the other at the + 1324 SalI site in late orientation; lane f, pGM7A.
For further information see to the Legend of Fig. 3.

Unproductive sites: enhancer inactivity, messenger instability or transcriptional occlusion? Some of the enhancer insertions in pGM8 fail to activate transcription probably for different causes.

One inactive insert is at the XhoI site, 200 bp downstream from the 3' end of the HIS3 structural gene (Fig. 1A), between the UAS and TATA box elements of the DED1 gene (30) (Fig.7, lane c). Sequences in the DED1 promoter surrounding the XhoI site probably inhibit enhancer activity much as HIS3 upstream promoter sequences inhibit activity of the yeast enhancer-like GAL1-GAL10 UAS (8).

The second inactive insert is at the BglII site #1 (Fig. 1A) 419 bp downstream from the native HIS3 RNA initiation window (Fig. 7, lane d). This observation is surprising since the polyoma enhancer is quite active much further downstream at nt +1324 from 1400 bp downstream. It is interesting that also yeast enhancer-like UAS elements, fail to stimulate transcription when inserted relatively close downstream from mRNA start sites (7,8). In particular the GAL1-GAL10 UAS is ineffective 300 bp downstream from HIS3 mRNA start sites. Local sequences, RNA destabilization or topology may be adduced to explain downstream inactivity.

We have tested some of these possibilities. To see whether the polyoma enhancer at the internal BglII site might be actually preventing transcription we used a "sandwich" construct in which transcription of the HIS3 gene containing the internal enhancer is driven by an enhancer at the "active" external 3' SalI site. The internal enhancer strongly inhibits transcription (Fig. 7, lane e). Thus, either mRNA destabilisation or transcriptional occlusion by the enhancer could be the principal reasons for our inability to

Figure 8. Transcription of sequences complementary to the polyoma enhancer.

Northern blots of total RNA hybridised to the 32 P-labelled XhoI-polyoma enhancer fragment (Fig. 1B).

lane a, pGM9; lane b, pGM7A; lane c, pGM59; lane d, pGM61; lane e, pGM35; lane f, pGM36; lane g, pGM60; lane h, pGM78.

Symbols: refer to the legend to Fig. 3.

detect HIS3 RNA driven by the +419 bp insert. A comparable result was obtained by Struhl (8) using a GAL1-GAL10 UAS "sandwich".

We have also tested whether RNA is destabilised by the presence of transcribed polyoma enhancer sequences. Using an enhancer-specific probe we find that the polyoma holoenhancer (Fig. 8, lane b), as well as the B-domain from the PyFL78 mutant (Fig. 8, lane d) are efficiently transcribed when inserted at the "active" 3' SalI site 400 bp downstream from the promoter of the truncated DED1 gene in pGM8 and conclude that RNA molecules containing polyoma enhancer sequences are not inherently unstable.

Effects of the enhancer on plasmid copy number and on the transcription of other plasmid sequences.

Our experiments are carried out on multicopy plasmids. Enhancers might increase the level of RNA transcripts by increasing plasmid copy-number rather than by activating transcription. The polyoma holoenhancer, in both orientations, and the mutant B-domain from PyFL78 do in fact increase plasmid copy number three- or four-fold, when inserted at the 3' SalI site (Fig. 9A, lanes b, d, h) but not at the 5' BamHI site (Fig. 9B, lanes d, e, h).

The SV40 enhancer has no effect on copy number (Fig. 8, lane e).

The increase in HIS3 RNA attributable to the enhancer is not correlated to copy-number. Even in the 3' inserts, the relationship between HIS3 transcript levels and copy number is not consistent; Early and Late late orientations of the enhancer raise copy-number to the same extent (Fig. 9, lane b, d), yet the late orientation is much more efficient than the Early in raising HIS3 RNA levels (Fig. 3, lane c, d).

We have also measured transcript level of two other yeast genes present on the test plasmid: TRP1 and the 3' truncated DED1 gene (Fig. 1A). Transcription patterns are complex since these genes as cloned, in contrast to the HIS3 test-gene, are not flanked on both sides by efficient transcription

Figure 9. Effects on plasmid copy-number. "Southern" blots of yeast mini-preps hybridised to the ^{32}P -labelled XhoI-SalI fragment (Figure 1A) comprising the beginning of the DED1 gene.

A) Upper panel includes enhancer inserts at nt +1324 3': lane a, pGM9; lane b, pGM7A; lane c, pGM8; lane d, pGM7B; lane e, pGM33A; lane f, pGM13; lane g, pGM40; lane h, pGM61; lane i, pGM59; lane j, uncleaved pGM8.

B) Lower panel includes enhancer inserts at nt -257: lane a, pGM8; lane b, pGM7A; lane c, pGM7B; lane d, pGM35; lane e, pGM36; lane f, no plasmid; lane g, pGM60; lane h, pGM78; lane i, uncleaved pGM8.

CHR, chromosomal DNA; OC, open circular plasmid; CCC, covalently closed circles. P = uncleaved pGM8 DNA.

barriers. Transcription from the DED1 promoter proceeds into and around the plasmid sequences; transcripts from plasmid promoters and pseudopromoters encroach upon TRP1 (43).

In brief, the 3' holoenhancer inserts raise heterogeneous DED1 (Fig. 10B, lane c, d, i) and TRP1 (Fig. 10C, lane a, c, f) transcript levels relative to the enhancerless test plasmid (Fig. 10B, lane h). This correlation is however imperfect: the *Late* is much more efficient than the *Early* in raising RNA levels (Fig. 10, lane c, d) although the DNA standards are the same (Fig. 9, lane b, d). We do not know why some of the inserts appear to reduce transcript levels (Fig. 10A, lane g; Fig. 10B, lane h). Furthermore the increase in copy number in the 3' inserts is too small to contribute significantly to restoration of HIS3 transcription.

Copy number increase could be due to dosage compensation if the *polyoma* enhancer somehow interferes with expression of TRP1, the marker we select for plasmid maintenance. Other possible causes are: an improvement in plasmid segregation directed by ARS1 (46) or, more interestingly, an escape from

Figure 10 Transcription of TRP1 and DED1.

Northern blots of total RNA hybridised to the ^{32}P -labelled XhoI-SalI fragment (Figure 1A) comprising the beginning of the DED1 gene or to the entire ^{32}P -labelled 1.4kb TRP1 EcoRI restriction fragment.

A. Effect of 5' enhancer insertions on DED1 RNA. lane a, pGM33A; lane b, pGM35; lane c, pGM36; lane d, chromosomal DED1 transcript; lane e, pGM78; lane f, pGM39; lane g, pGM60.

B. Effect of 3' enhancer insertions on DED1 RNA. lane a, chromosomal DED1 transcript; lane b, pGM8; lane c, pGM7A; lane d, pGM7B; lane e, pGM13; lane f, pGM63; lane g, pGM40; lane h, pGM59; lane i, pGM61. For details refer to the legend to Fig. 3.

cell-cycle control on plasmid replication. A different experimental design will be needed to explore these possibilities.

DISCUSSION

We have demonstrated that a 244 bp polyoma virus DNA fragment, (nucleotides 5021 to 5262 on the polyoma map; 47), comprising a prototypical mammalian enhancer element (13), strongly increases the level of RNA synthesised from a plasmid-borne promoterless yeast HIS3 gene (his-del4). The enhancer is effective, in both orientations at sites upstream and downstream from the test gene, at a distance of up to 1300 bp. Operationally, the polyoma fragment fulfills in yeast all the prerequisites of an enhancer element. The SV40 enhancer, which we have studied in less detail, also potentiates HIS3 RNA.

In principle there are three possible ways in which enhancers might increase HIS3 RNA transcribed from his-del4:

a) by stimulating transcription, thus compensating for the lack of the native UAS sequence.

b) by increasing the stability of RNA molecules transcribed from weak pseudopromoters.

c) by increasing the copy number of weakly transcribed HIS3 genes.

Although we have no direct measure of the rate of HIS3 transcription, our data argue against mechanisms b and c playing an important role in increasing the level of HIS3 RNA. First, in most cases HIS3 and enhancer se-

quences are not co-transcribed, and in the one case where they are, HIS3 RNA is not augmented. Second, although, in some constructions, the polyoma enhancer raises the plasmid copy number, the increase (3 to 4-fold) is insufficient to account for the observed increase in HIS3 RNA. Third, in all constructions carrying 5' inserts of the enhancer, RNA is stimulated without an increase in template. Fourth, different RNA start sites are favoured in different constructs.

Probably transcription activation is the principal mechanism by which the viral elements raise the level of HIS3 RNA in our experiments. True transcriptional activation by the SV40 enhancer has been demonstrated in in-vivo HeLa cell nuclear transcription assays: the enhancer increases the number of RNA polymerase II molecules transcribing a linked γ -globin gene (48). Our study of the dissected components of the polyoma and SV40 enhancers, and of a mutant derivative of the polyoma enhancer, show that the sequence specificity requirements observed in mammals are conserved in yeast, suggesting that the same processes are involved. This conclusion is further supported by very recent reports that a mammalian AP1 binding sequence from the SV40 enhancer binds a specific protein in *S. cerevisiae* (49) and *Schizosaccharomyces pombe* (50) and that this sequence functions in both yeast cells as an efficient UAS element when introduced into a hybrid promoter. Interestingly, in the B78 B-domain the transposed A-box AP1 site is destroyed by base substitution (see legend to Fig.1B). The fact that the mutant B-78 domain is active in yeast suggests that there is at least one other enhancer binding protein, besides AP1 in this organism.

We will comment now on some aspects of enhancer function that, at least superficially, appear to be peculiar to yeast.

a). Alternate orientations of the polyoma and SV40 enhancer differ reproducibly in the efficacy with which they raise HIS3 RNA levels: this difference is not generally observed in mammalian systems. The reason may be that it is easier to quantify transcription in transformed yeast cells than in transient expression assays of transfected cells. More interestingly the topological properties of plasmids in yeast might favour one orientation over the other. In prokaryotes orientation dependence in the interaction of distant sites on the same molecule, in site-specific recombination systems, is strongly dependent on DNA supercoiling (51). If enhancers drive transcription by physically contacting (via interaction of bound protein factors) sequences close to RNA start sites, orientation preference might depend on the degree of supercoiling of the DNA; plasmids in yeast are supercoiled in vivo while supercoiled plasmids used in testing enhancer function by transient expression assays in mammalian cells are rapidly linearised following transfection (52). In this context, it is of interest that replication and to a lesser extent transcription of polyomavirus derivatives, bearing enhancers that had been rearranged in vitro, exhibit strong tissue-specific dependence on the polarity of some of the constituent sequence elements (53). Mechanistic analogies between site specific recombination systems and aspects of promoter/operator function have been discussed in general terms by Ptashne (54).

b). Although the SV40 and polyoma enhancers function in many heterologous systems they do show some species or cell-type preference in mammals indicating that while some trans-acting factors are common to rodents and primates, others must be specific. The similar efficiency of the two enhancers in yeast is simply accounted for if yeast shares some factors with higher eukaryotes but lacks negative control elements that might determine species- and tissue- specificity in mammals.

c). The polyoma enhancer fails to function in yeast when cloned inside the HIS3 gene about 400 bp downstream from RNA start sites, but does function quite well further downstream (about +1400). Although typically en-

hancers are supposed to function from downstream positions, it has been reported that, in recombinant plasmids transfected into HeLa cells, the SV40 72 bp repeat fails to stimulate effectively RNA synthesis when cloned about 100 bp downstream from a potential promoter (17).

This behaviour is reminiscent of that of the yeast GAL and CYC1 UAS elements. The GAL UAS element does not activate transcription if inserted 100 or 300 bp downstream from the HIS3 initiation sites (8). The CYC1 UAS element is inactive when inserted 200 bp downstream of its target promoter (7).

Aside from the sequence environment, local structural constraints might prevent efficient start-site/enhancer interaction if the downstream distance of the enhancer is too short (400 bp or less).

"Sandwich" type experiments (8,17, this paper) show that enhancers/UAS elements actually prevent transcription driven by a second enhancer element, suggesting that the internal insert destabilises mRNA or somehow occludes the transcription unit (by protein binding?). Obstruction is not observed when the enhancer is cloned just downstream of the DED-1 promoter (a strong polydAT promoter (4)).

d). Surprisingly different enhancers (for instance: the polyoma ho-loenhancer, the A-domain and B-domain from the PyFL78 mutant) introduced at the same site stimulate different sized transcripts. This is novel for enhancers but not for UAS elements. The native HIS3 polydAT UAS, the HIS3 URS and the GAL UAS, located at equivalent sites upstream of the HIS3 gene activate transcription from different initiation sites (5,6,8) probably by interacting with different TATA box elements. In the AT rich pseudopromoter flanking our reporter gene there are a number of TATA like sequences (Legend Fig. 1A) We further consider initiation-site selection by the enhancers in the accompanying paper.

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