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Interchangeable RNA polymerase I and II enhancers

(*Saccharomyces cerevisiae*/transcription/upstream activation sequence/thymidine-rich element)

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ABSTRACT **The RNA polymerase I (pol I) enhancer of *Saccharomyces cerevisiae* contains at least three elements commonly associated with RNA polymerase II (pol II) enhancers, binding sites for the transcriptional activators general regulatory factor 2 and autonomously replicating sequence-binding factor I, and a thymidine-rich element. When the particular form of the thymidine-rich element found in the pol I enhancer was placed in front of a pol II promoter, transcription was stimulated 43-fold, comparable to the effect of a powerful pol II activator such as Gal4. Conversely, when two copies of a thymidine-rich element from a pol II enhancer were placed upstream of a pol I promoter, transcription was stimulated 38-fold. This functional reciprocity of pol I and II enhancers may reflect similarities in the mechanisms of transcriptional activation. The pol I enhancer also contains an element that appears to be pol I-specific and prevent the activation of pol II.**

Eukaryotic RNA polymerases I and II (pol I and II) have much in common, including a high degree of sequence homology of the two largest subunits (1, 2), three subunits identical in the two enzymes (3, 4), and similarities in promoter structure. Both enzymes are capable of initiation at a minimal promoter, consisting of sequences in the immediate vicinity of the transcription start site; in both cases the frequency of initiation is greatly increased by the presence of additional sequence elements, termed enhancers, which may be located a variable distance from the promoter and in either orientation (5–11). The question arises whether enhancers are functionally interchangeable between pol I and pol II promoters. Others have found (12) that a number of pol II enhancers fail to stimulate initiation by mammalian pol I, suggesting that the two enzymes are activated by different mechanisms. We report here on findings that lead to the opposite conclusion.

RNA polymerase I is responsible for the synthesis of a large precursor of ribosomal RNAs, 35S in yeast and 40S in higher organisms. The 35S precursor is encoded by ≈120 tandem repeats of a gene and 2.5-kilobase (kb) spacer. Two parts of the spacer are involved in initiation of the 35S precursor: ≈210 base pairs (bp) immediately upstream of the initiation site and ≈190 bp located 2.2 kb further upstream (10, 11, 13). The 190-bp segment stimulates initiation 17-fold and functions at a variable distance from the initiation site in either orientation, leading to its designation as an enhancer. Progressive deletions of the 190-bp segment gradually diminish enhancer activity (14), suggesting that the enhancer is made up of multiple DNA elements. Similarities between some of these elements and previously identified pol II enhancer elements prompted us to investigate the question of functional reciprocity. One pol I enhancer element proved highly effective in the activation of pol II transcription, whereas other pol I enhancer elements may play accessory roles at both pol I and pol II promoters.

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MATERIALS AND METHODS

Plasmid DNAs. Plasmids for transcriptional activation of pol II were members of the pCZ family of *Escherichia coli–Saccharomyces cerevisiae* shuttle vectors, containing a polylinker upstream of a yeast *CYC1–E. coli lacZ* fusion gene (15). The synthetic oligonucleotides listed in Table 1 were ligated with the 1-kbp *Eco*RI–*Cla* I and 7.8-kbp *Cla* I–*Bam*HI fragments of pCZ. The control with no oligonucleotide (designated Δ) inserted in the polylinker was as described (15). For construction of a plasmid with a Gal4-binding site and residues 157–180 of the pol I enhancer in pCZ (plasmid designated pCZGALpolI157-180), the synthetic oligonucleotide had the sequence CGGGTGACAGCCCTCCGAAGGCAAAGATGGGTTGAAAGAGAAGG, with termini as described in Table 1.

Synthetic oligonucleotides containing the minimal enhancer with sequences downstream or upstream of the thymidine-rich (T-rich) element deleted (Fig. 3 downstream del., upstream del.) were also ligated with the 1-kbp *Eco*RI–*Cla* I and 7.8-kbp *Cla* I–*Bam*HI fragments of pCZ. The entire and minimal enhancers [*Eco*RI–*Xba* I and *Eco*RI–*Hin*dIII fragments of pSES5, respectively (ref. 18; gift of S. Roeder, Yale University)] depicted in Fig. 3 were ligated with the 2.2-kbp *Eco*RI–*Bgl* II and 6.6-kbp *Bgl* II–*Xho* I fragments of pCZ and the following *Xho* I–*Xba* I or *Xho* I–*Hin*dIII adapter oligonucleotides:

5′–TCGAGGAAGGGGTTCCCTTCCCCAAGGTCA–5′
and
5′–TCGAGGAAGGGGTTCCCTTCCCCAAGTCGA–5′

(*Xmn* I sites in the adapters are underlined). Orientation of the enhancer fragments was reversed in constructions designated entire$_{rev}$ and minimal$_{rev}$ by ligation of the same fragments of pSES5 with the 6.6-kbp *Eco*RI–*Bgl* II and 2.2-kbp *Bgl* II–*Xba* I or 2.2-kbp *Bgl* II–*Hin*dIII fragments of pCZ. The region adjacent to the minimal enhancer (*Hin*dIII–*Xba* I fragment of pSES5) was ligated with the 6.6-kbp *Xba* I–*Bgl* II and 2.2-kbp *Bgl* II–*Hin*dIII fragments of pCZ.

Plasmids for transcriptional activation of pol I were derivatives of pCZ; the pol I promoter was supplied by a 640-bp *Xba* I (end-filled)–*Bam*HI fragment of pSES5, extending from 212 bp upstream of the initiation site of the 35S rRNA precursor into vector sequences 428 bp downstream. Insertion of the 640-bp pol I promoter fragment between the *Eco*RI (end-filled) and *Bam*HI sites of pCZ(DED48)^2 (17) gave pCpolIZΔ. Insertion of the pol I promoter fragment between the *Xho* I (end-filled) and *Bam*HI sites of pCZ(DED48)^2 gave pCpolIZ-(DED48)^2. For construction of pCpolIZ-ACT, the pol I promoter fragment and large *Eco*RI–*Bam*HI fragment of

Abbreviations: pol I and II, RNA polymerase I and II, respectively; GRF2, general regulatory factor 2; ABFI, autonomously replicating sequence-binding factor I; T-rich, thymidine-rich.

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Table 1. RNA pol I enhancer elements activate RNA pol II transcription

Oligonucleotide	pol I enhancer residues	Relative transcription
Δ		1.0
GRF2 binding		
35SRNA1		3.7
35SRNA2	8–29	2.8
RAP1		1.3
X40		2.5
ABFI binding		
rABFI	40–56	1.8
SPT2		10.7
T-rich		
r(dAdT)	116–156	43
$r(dAdT)_{rev}$	156–116	3.2

The synthetic oligonucleotides listed were inserted in the polylinker of pCZ. Sequences of the 35SRNA1, 35SRNA2, RAP1, and X40 oligonucleotides are given in ref. 16 and that of SPT2 in ref. 17. The rABFI and T-rich oligonucleotides contained the residues listed (Fig. 1), with 5′-GATCC / 3′-G before the first residue and G-5′ / CAATTG-3′ after the last residue. The resulting plasmids were introduced into yeast strain 15C (*α leu2-31, 12, ura3-52, trp1, his4-580, pep4-3*), and β-galactosidase activities in cell extracts (expressed as units per mg of protein) were determined. Relative transcription refers to units of β-galactosidase activity per mg of protein divided by the result obtained for the plasmid with no oligonucleotide (Δ) inserted in the polylinker (0.6 unit per mg of protein). Each value listed is an average of at least two determinations on independent transformants.

pCZ(DED48)[2] were ligated with the following double-stranded oligonucleotide:

AATTCTCTGTCACCCGGCCTCTATTTTCCATTTTCTTCTTTACCCGCCAC
GAGACAGTGGGCCGGAGATAAAAGGTAAAAGAAGAAATGGGCGGTG

[general regulatory factor 2 (GRF2)-binding sites are underlined; ref. 16]. The pol I promoter fragment was inserted between the *Xho* I (end-filled) and *Bam*HI sites of pCZ-3GAL (19) to give pCpolIZ-3GAL with three Gal4-binding sites in front of the promoter.

Assays of Transcriptional Activation. Plasmid DNAs were introduced into yeast by the lithium acetate-transformation procedure (20). Transformants were picked, grown, harvested, and disrupted, and β-galactosidase assays were done as described (16). Values of relative transcription or -fold activation represent β-galactosidase units per mg of protein for the fragment or oligonucleotide in question divided by that for no insert (Δ, 0.6 unit per mg).

RESULTS

The pol I Enhancer Contains Three Sequence Elements Commonly Associated with pol II Promoters. Residues 14–23 of the pol I enhancer (Fig. 1) match the consensus sequence YNNYYACCCG (Y = C or T; N = A, G, C, or T) for recognition by the pol II activator protein GRF2 (16) and bind the protein *in vitro* (16). A protein termed REB1 that binds this region of the pol I enhancer has been identified (21) and appears identical with GRF2 (16). Binding sites for GRF2 occur upstream of many pol II promoters and function synergistically with other upstream activation sequence elements, possibly by excluding nucleosomes from these elements (16, 22, 23). A gene for GRF2 has been isolated from yeast and is essential for viability; the deduced amino acid sequence of the protein exhibits some homology to that of human c-*MYB* (39).

Residues 39–58 of the pol I enhancer match the consensus sequence RDCNYNNNNNACGAD (R = A or G, D = A, G, or T) for recognition by the pol II activator protein autonomously replicating sequence-binding factor I (ABFI) (17), except for the substitution of thymidine for cytidine at the third position. Substitution of adenosine for cytidine at this position was previously shown to reduce, but not abolish, ABFI binding (17), and an oligonucleotide with the sequence of residues 39–58 (Table 1, rABFI) competes with other ABFI sites for ABFI-binding *in vitro* (16). A protein termed REB2 that binds this region of the pol I enhancer has been identified (21) and is probably identical with ABFI. Like GRF2, ABFI binds to many pol II promoters and exerts a modest effect on transcription on its own but functions synergistically with other activators (17).

The third region of the pol I enhancer that resembles previously described pol II activation sequences is the thymidine-rich (T-rich) stretch of residues 116–155. Although the sequence of this region varies somewhat among the many copies of the pol I enhancer in the yeast genome, the first part (Fig. 1, residues 116–131), containing three stretches of 2–5 thymidines, and the last part (Fig. 1, residues 143–153), containing nine contiguous thymidine residues, are conserved among all sequences so far determined (10, 24, 25). Similar T-rich sequences occur upstream of many yeast pol II promoters and contain runs of 3–11 thymidine residues, with as few as 15 thymidines sufficing for activation of transcription (26–30).

From the previous deletion analysis of the pol I enhancer (14), it is apparent that all three regions, the GRF2 and ABFI sites and the T-rich element, contribute to activating pol I transcription. Deletion of residues 7–21, invading the GRF2 site (Fig. 1), reduced transcription 2.7-fold. Extending the deletion of residue 32, removing the rest of the GRF2 site, had

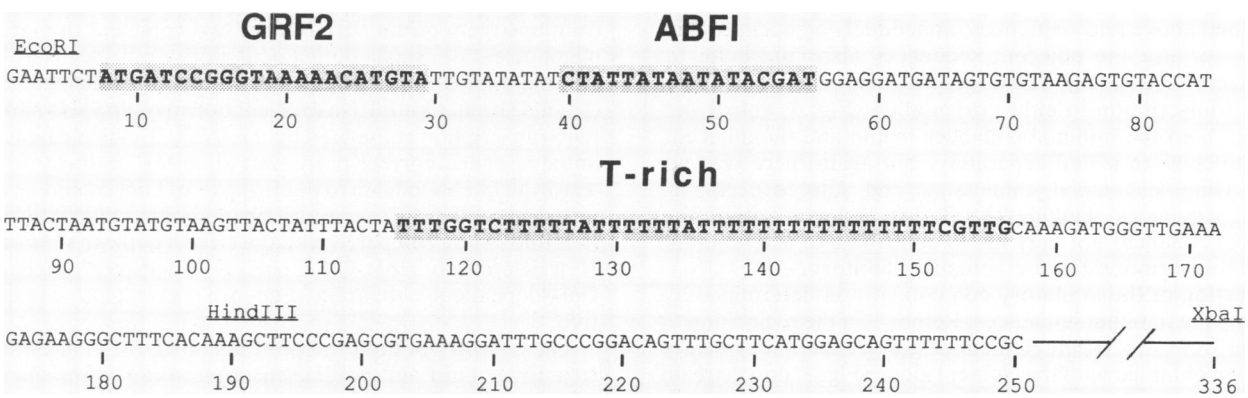

FIG. 1. Nucleotide sequence of an RNA polymerase I enhancer from *S. cerevisiae*, as determined by Stewart and Roeder (18). GRF2-binding, ABFI-binding, and T-rich sequences analyzed as synthetic oligonucleotides in the present study are shaded and in boldface type.

no greater effect, but deletion of residues 7–46, encroaching on the ABFI site, reduced transcription a further 2.4-fold. An even larger deletion, removing the entire T-rich element, caused an additional 2.3-fold drop in transcription.

The T-Rich Region of the pol I Enhancer Activates pol II Transcription. In view of the similarity of the GRF2, ABFI, and T-rich regions of the pol I enhancer to pol II activation sequences, we tested the capacity of the regions to stimulate pol II transcription *in vivo*. Synthetic oligonucleotides with sequences of all three regions were inserted in a polylinker 16 bp upstream of the major TATA element of the yeast *CYC1* promoter fused to the *E. coli lacZ* gene in a yeast centromeric plasmid. Levels of β-galactosidase activity were determined in extracts of yeast harboring the various constructions (Table 1). The T-rich sequence from the pol I enhancer activated pol II transcription to a remarkable extent [r(dAdT) oligonucleotide, 43-fold stimulation above background with no oligonucleotide], more than a T-rich sequence from a pol II promoter (the *DED1* T-rich element, in the form of the DED48 oligonucleotide, 7.4-fold stimulation; ref. 15). Activation by the pol I T-rich sequence was strongly orientation dependent [reduced from 43- to 3.2-fold stimulation on reversing orientation in the $r(dAdT)_{rev}$ oligonucleotide], as observed previously for the *DED1* T-rich element (15). The effect of the pol I T-rich sequence was comparable to those of the most potent pol II activation sequences known, such as a single Gal4-binding site (≈100-fold; ref. 31).

Two controls were done to confirm that transcription from the *CYC1* promoter constructs was from pol II and not from pol I recruited to the promoter by the pol I T-rich sequence. (*i*) A form of the *CYC1* promoter was used from which the major TATA element had been deleted (pCTbal, ref. 32). This deletion abolishes pol II transcription from the promoter *in vitro* and *in vivo* (32) and reduced transcription stimulated by the pol I T-rich element 5.8-fold. The low level of transcription remaining may be attributable to two TATA-like sequences (TATTTT) within the pol I T-rich element. (*ii*) The second control was to compare the transcription start sites obtained with a known pol II activation sequence to those found in the presence of the pol I T-rich element. For this purpose, RNA was isolated from cells bearing plasmids with either a Gal4-binding site or the pol I T-rich element in front of the *CYC1* promoter. The 5′ ends of *CYC1* transcripts, revealed by RNase protection mapping with ^{32}P-labeled RNA probe, were the same in the two cases (Fig. 2), with a larger amount of RNA probe protected for the Gal4-binding plasmid, congruent with the somewhat greater enhancer activity of the Gal element than the T-rich sequence. The pattern of *CYC1* transcription start sites was the same as reported (32).

Despite the capacity of the pol I T-rich sequence to activate pol II transcription, the entire pol I enhancer failed to do so. The enhancer was initially tested in the form of a larger fragment, including the 190-bp minimal enhancer region described above and sequences immediately adjacent to this region because the adjacent sequences also contribute to enhancer function (18). The larger fragment failed to activate pol II transcription in either orientation (Fig. 3, entire and $entire_{rev}$), so the minimal enhancer region and the adjacent sequences were tested separately, but again there was no effect (Fig. 3, minimal, $minimal_{rev}$, and adjacent). Apparently, sequences flanking the T-rich element within the minimal enhancer not only fail to contribute to activation of pol II transcription but are actually inhibitory.

Location of the inhibitory component was determined by deleting the flanking sequences. Removal of the region downstream from the T-rich element restored function to nearly the level obtained with the element alone (Fig. 3, downstream del.). On the other hand, addition of only the downstream residues 157–180 to the T-rich element abolished function (Fig. 3, upstream del.). We conclude that residues 157–180 prevent activation of pol II transcription. This conclusion is supported by the effect of inserting residues 157–180 between a Gal4-binding site and the *CYC1* promoter (in pCZGAL polI157-180, see *Materials and Methods*). Activation by Gal4 protein was reduced from >100- to only 7-fold.

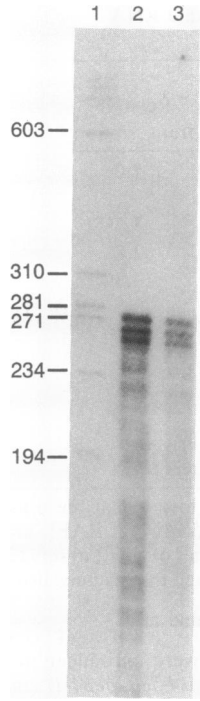

FIG. 2. Patterns of transcription start sites with pol I and pol II enhancer elements upstream of the *CYC1* promoter. Synthetic oligonucleotides containing a Gal4-binding site (same as G4-1 oligonucleotide of ref. 22, here designated G01P) (lane 2) or the pol I T-rich element [same as r(dAdT) oligonucleotide of Table 1, here designated HOT1] (lane 3) were inserted in the polylinker upstream of the *CYC1* promoter in pCZ. The resulting plasmids were introduced into yeast strain 15C (see Table 1), and RNA was isolated and subjected to RNase protection mapping with RNA probe from pSPCTB as described (32). Markers run in lane 1 were from an *Hae* III digest of φX174 restriction fragment DNA; sizes in nucleotides are indicated at left.

The GRF2- and ABFI-binding sequences associated with pol II promoters exert only modest effects on transcription (2- to 10-fold; refs. 16 and 17), so it was not surprising to find that the GRF2- and ABFI-binding sequences from the pol I enhancer barely stimulated pol II transcription at all. The GRF2-binding sequence (35SRNA2 oligonucleotide) stimulated slightly (2.8-fold), as did a second GRF2-binding sequence from immediately upstream of the pol I transcription start site (35SRNA1 oligonucleotide, oppositely oriented to 35SRNA2, 3.7-fold stimulation). The ABFI-binding sequence from the pol I enhancer (rABFI oligonucleotide) also activated pol II transcription slightly (1.8-fold).

The *DED1* T-Rich Element Activates pol I Transcription. Identification of pol I enhancer elements that stimulate pol II transcription led us to investigate the possibility that some pol II enhancers might activate pol I transcription. Various pol II enhancers were placed in front of the 35S rRNA initiation region (212 bp upstream of the start site of pol I transcription), and levels of transcription in yeast harboring the constructions were determined by RNase protection mapping of cellular RNA (Table 2). A pair of T-rich elements from upstream of the *DED1* gene (two copies of the DED48 oligonucleotide) was a potent activator of pol I transcription (38-fold stimulation), more so than the pol I enhancer itself (17-fold stimulation). RNase protection mapping also showed that transcription stimulated by the *DED1* T-rich element was initiated at the correct pol I start site (34), so transcription was in all likelihood due to pol I. A pair of GRF2-binding sites upstream of the *ACT* gene stimulated pol I transcription less (3.8-fold) when compared with the effects of these sites on pol II transcription (16). Other pol II enhancers, such as a Gal4-dependent activation sequence, were also less effective.

The capacity of both the pol I and *DED1* T-rich elements to activate pol I and pol II transcription raised the question whether the effects of these elements are mediated by a common protein factor. Insertion of the *DED1* T-rich element upstream of a pol II promoter activates transcription 10- to

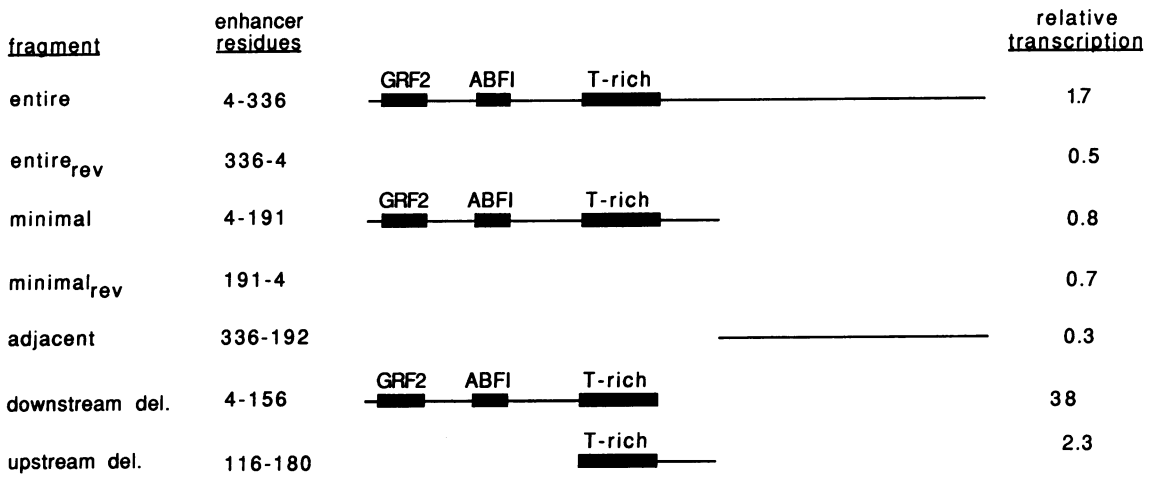

FIG. 3. Identification of a region of the pol I enhancer that prevents activation of pol II transcription. The fragments indicated (residue numbers as in Fig. 1) were incorporated in the polylinker of pCZ, and their effects on transcription were analyzed as in Table 1. Fragments are designated entire and minimal for entire and minimal enhancers, entire$_{rev}$ and minimal$_{rev}$ for the same fragments in the opposite orientation, adjacent for the region immediately downstream of the minimal enhancer, and downstream del. and upstream del. for synthetic oligonucleotides containing the minimal enhancer with sequences downstream or upstream of the T-rich element deleted.

30-fold *in vitro*, and activation is inhibited by addition of free *DED1* T-rich element (DED48 oligonucleotide) to the reaction (Fig. 4; ref. 15), indicating involvement of a *DED1* T-rich-binding factor. By contrast, the pol I T-rich element caused no activation of transcription *in vitro*, and the low level of transcription observed was unaffected by addition of the free element [r(dAdT) oligonucleotide, Fig. 4]. Thus, action of the pol I fragment is probably mediated by a distinct factor, as appears so for other T-rich elements that have proved ineffective *in vitro* (15). Consistent with this interpretation, the pol I element was a poor competitor of transcription from a *DED1* T-rich template, and the inhibition that did occur (25% of that with the *DED1* T-rich element; data not shown) probably reflects low affinity of the *DED1* T-rich-binding factor for the pol I element.

DISCUSSION

Dissection of the pol I enhancer was essential for revealing the capacity of one sequence, the T-rich element, to activate pol II transcription because another sequence, between residues 157–180, was inhibitory. These residues probably contain a factor-binding site, as deletion of residue 166 and insertion of a linker greatly diminishes activity of the enhancer (18). It has also been noted that residues 166–173 nearly match the simian virus 40 enhancer core consensus (11). Factor-binding between the T-rich element and promoter may block activation of pol II, much as was shown for protein-binding sites interposed between a Gal4-dependent activation sequence and promoter (37).

The minimal pol I enhancer, containing GRF2, ABFI, and T-rich elements, but lacking residues 157–180, gave no increase in activation of pol II transcription above the level obtained with the T-rich element alone (Fig. 2). Both GRF2 and ABFI sites exert synergistic effects when located adjacent to T-rich elements in other constructs (16, 17), but in the case of a GRF2 site, the effect has been shown to be strongly distance-dependent, declining by 85% when spaced from a T-rich element only half that in the pol I enhancer. The distance-dependence of the ABFI effect as well as possible synergism between GRF2 and ABFI elements have not been investigated.

Table 2. Effects of RNA pol II enhancers on RNA pol I transcription

Sequence	Relative transcription
Δ	1.0
(DED48)^2	38
ACT	4.0
3GAL	3.8

The sequences listed (Δ, no insertion; DED48, 48-bp *DED1* T-rich element; ACT, two GRF2-binding sites; 3GAL, three GAL4-binding sites) were placed in front of a pol I promoter in derivatives of pCZ designated pCpolIZΔ, pCpolIZ-(DED48)^2, pCpolIZ-ACT, and pCpolIZ-3GAL. Plasmids were introduced into yeast strain 5C (a *his3Δ200*, *ura3-52*); transformants were isolated and grown as described (15) to an A_{600} value of 0.5 and RNA was isolated (33); transcripts initiated at the 35S precursor start site in the plasmids were quantitated by hybridizing with an RNA probe, RNase digestion, and gel electrophoresis as described (34), and then counted with an AMBIS (San Diego) radioanalytic imaging system.

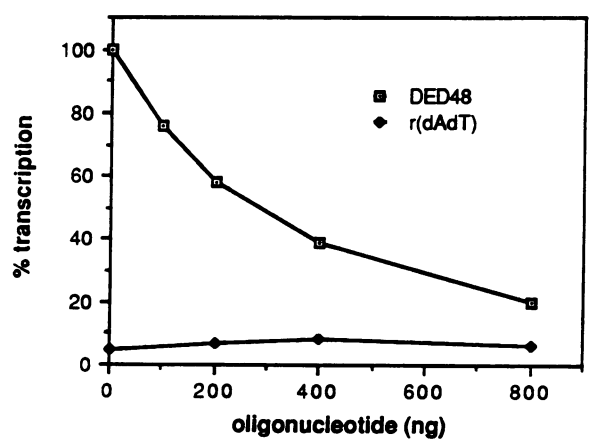

FIG. 4. Effects of T-rich elements from upstream of the *DED1* gene (DED48 oligonucleotide) and from the pol I enhancer [r(dAdT) oligonucleotide, Fig. 1] on pol II transcription *in vitro*. Templates for transcription were p(DED48)^{2}CG-, with two copies of the DED48 oligonucleotide (48-bp *DED1* T-rich element) in front of the yeast *CYC1* promoter (35), and pr(dAdT)CG-, with the r(dAdT) oligonucleotide. pr(dAdT)CG- was constructed by insertion of r(dAdT) between the *Xho* I and *Hin*dIII sites of pGAL4CG- (35). Transcription reactions with p(DED48)^{2}CG- (□) contained 200 ng of template and the amounts of DED48 oligonucleotide indicated, whereas reactions with pr(dAdT)CG- (◆) contained 380 ng of template and r(dAdT) oligonucleotide [levels of transcription from pr(dAdT)CG- were divided by 1.8 to correct for the greater quantity of DNA used]. Procedures were as described (36).

The evidence presented here for reciprocal actions of pol I and pol II enhancers can be interpreted in two ways. (*i*) pol I and pol II enhancers may function by similar mechanisms. Stimulatory proteins that bind to these enhancers, such as GRF2, ABFI, and T-rich binding factors, may interact with common components of the pol I and pol II transcription machineries. The selection of one or the other type of polymerase at a particular promoter would be determined by additional sequences. For example, the pol II-inhibitory component of the pol I enhancer described above, or the TATA element specific to pol II promoters, might fulfill this role.

(*ii*) An alternative interpretation would be that different proteins bind to the same enhancer sequence, depending on whether it is located in front of a pol I or pol II promoter. The detailed mechanisms of transcriptional activation might then be different at the two types of promoter. Although there is precedent for multiple transcription factors recognizing the same DNA sequence in higher cells (38), a single factor appears to bind a particular sequence in most cases. The question of whether one or multiple factors are involved in the present examples could be addressed with the use of mutant binding sequences, such as those described for ABFI (17) and GRF1 (31).

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