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Activation of yeast RNA polymerase II transcription by a thymidine-rich upstream element *in vitro*

(upstream activation sequences)

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ABSTRACT **A thymidine-rich sequence upstream of the *DED1* gene of *Saccharomyces cerevisiae* activated transcription of the *CYC1* promoter by RNA polymerase II *in vitro*. Activation was inhibited by an excess of an oligonucleotide with the same but not a closely related thymidine-rich sequence, pointing to the involvement of a specific thymidine-rich element-binding factor. The extent of activation was as great as 30-fold and showed a similar distance and orientation dependence and a similar effect of deletions *in vitro* as *in vivo*.**

With the advent of a soluble transcription system for yeast RNA polymerase II (1), it is possible to investigate the initiation and regulation of yeast transcription at the molecular level *in vitro*. Minimal sequence requirements for initiation *in vitro* have been narrowed to a TATA element located 50–160 bp upstream of the initiation sites of the *CYC1* and *PYK1* promoters. Additional sequence elements are needed for regulation *in vivo*, and in yeast these are usually located further upstream of the initiation sites. Positive regulatory elements, termed "upstream activation sequences" (UASs), have been identified as being responsible for both constitutive and inducible transcription (2). Many UASs are binding sites of regulatory proteins, such as the GAL4 (3, 4), HAP2-3 (5), and GCN4 (6) proteins and the heat shock transcription factor (7, 8). We report here on regulation of transcription *in vitro* by a type of UAS consisting of runs of thymidine residues on the coding strand.

A sequence of 15 thymidine residues, interrupted by a C-A dinucleotide, occurs between the divergently transcribed *HIS3* and *PET56* genes (9). Deletions removing this sequence cause a reduction in the constitutive level of transcription of both genes to about one-fifth (10). A sequence consisting of 31 thymidine residues interspersed with 1 guanosine and 7 cytidine residues plays a similar role in transcription of the *DED1* gene (10). Such "T-rich" regions occur upstream of many other genes—for example, regions 37 and 38 base pairs (bp) long, containing 6 and 9 nonthymidine residues, upstream of the small nuclear RNA gene *SNR20* [or *LSR1* (11); ref. 12] and the *YPT2* gene (13), respectively. No deletion analyses or other functional studies of these regions have been reported, and their relationship to transcription is unknown.

It has been suggested that T-rich UASs function by excluding proteins (10, 14, 15). Poly(dA-dT) is refractory to the formation of nucleosomes *in vitro* (16, 17), and extension of this effect to neighboring promoter elements might render them accessible to the transcription apparatus. The data presented here are inconsistent with the hypothesis that protein exclusion is solely responsible for activation by T-rich elements and, instead, point to the involvement of a T-rich DNA-binding transcription factor(s), whose mode of action remains to be elucidated.

MATERIALS AND METHODS

Synthetic Oligonucleotides. Oligonucleotides were produced with an Applied Biosystems DNA synthesizer and were purified and hybridized as described (3). The oligonucleotides contain sequences derived from yeast DNA and sequences on each end that allow ligation to other restriction fragments. Sequences of one strand of most oligonucleotides are shown in Table 1. The complementary strand hybridizes with the one shown so that four-base 5′ protruding ends are created: 5′-AATT-3′ and 5′-TCGA-3′. Sequences of the oligonucleotides designated DED1 (18), PYK1, ENO1, and TEL (19) can be found elsewhere.

Plasmids. The pCT family of plasmids contained the *S. cerevisiae CYC1* promoter [nucleotides −248 to +5 in the published numbering scheme (20), lacking the *CYC1* UAS] fused to lacZ protein-encoding sequences as described (1). The *CYC1* UAS is replaced by a polylinker sequence with the following configuration of restriction sites: *Hin*dIII/*Sph* I/*Pst* I/*Sal* I/*Xba* I/*Bam*HI/*Eco*RI/*Sac* I/*Kpn* I/*Sma* I/*Xho* I/*CYC1-lacZ*. The pCZ family of plasmids was obtained from the pCT family by deleting a *Xho* I–*Sph* I fragment of the *CYC1* promoter (nucleotides −248 to −139), blunting the *Sph* I end, attaching a *Xho* I linker, and religating the promoter to the polylinker. Most forms of the pCT and pCZ plasmids have oligonucleotides inserted between the *Eco*RI and *Xho* I sites of the polylinker, with the exception of pCZ-PYK1 (same as pCTdp, ref. 1), which has the oligonucleotide inserted between the *Bam*HI and *Eco*RI sites. pCTΔ and pCZΔ have no inserted oligonucleotide. Plasmid pdAT-2 was a member of the pCZ family with a 116-bp *Acc* I–*Xho* I fragment of *DED1* DNA [nucleotides 765–880 in the published numbering scheme of Struhl (9)] inserted between the *Sal* I and *Xho* I sites of the polylinker. The configuration of regulatory sequences in pdAT-2 places the *DED1* upstream region 10 bp further from the TATA box than in the natural *DED1* gene. In pCS-DED48 and pCS-T39, oligonucleotides were inserted between the *Sal* I and *Eco*RI sites of the polylinker in pCZΔ so that the T-rich elements were in the inverted orientation relative to the *CYC1* promoter. pCZ-DED48/XBL was produced by digestion of pCZ-DED48 DNA with *Xho* I endonuclease, treatment with T4 DNA polymerase and the four deoxynucleoside triphosphates, and ligation of the ends (21).

Transcription *in Vitro*. Nuclear extracts were prepared as described (1) except that (*i*) chymostatin (2 μg/ml) and benzamidine (2 mM) were added to all buffers and (*ii*) after zymolyase treatment, spheroplasts were washed with 400 ml of 1 M sorbitol in YPD medium (1% yeast extract/2% Bacto-peptone/2% dextrose), resuspended in 800 ml of 1 M

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Abbreviations: UAS, upstream activation sequence; GRFI, general regulatory factor I; ABFI, autonomously replicating sequence (ARS)-binding factor I.

Table 1. Activation of transcription by T-rich sequences *in vivo* and *in vitro*

Plasmid	*In vivo* β-Gal units	Relative transcription *in vitro*	% oligo. competition	Oligonucleotide sequence
pCZΔ	0.97	0.8		
pdAT-2	146	8.0		
pCZ-DED48	7.2	9.1	85	AATTCTTTCCTTTTTTCTTTTTGCTTTTTCTTTTTTTTTCTCTTGAAC
pCZ-DED36	3.6	4.4	75	AATTCTTTTTGCTTTTTCTTTTTTTTTCTCTTGAAC
pCZ-DED30	0.72	1.0	40	AATTCTTTCCTTTTTTCTTTTTGCTTTTTC
pCZ-T39	10	1.3	4	AATTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGAAC
pCZ-HIS35	1.8	1.0	0	AATTCGGTAATGATTTTCATTTTTTTTTTCCACC
pCZ-SNR47	15	1.2	36	AATTCTATGTTTCTTTTCTTTTTTCTTTCTTTTTTTTTTTTTTGCCGC
pCZ-YPT47	9.0	2.6	65	AATTCGTTTTTGTTGTTTTTTCTGTTTTTTTCCTTTTTCCTTGTGGC
pCT-DED48	4.4	3.3		
pCZ-DED48/XBL	3.6	3.9		
pCS-DED48	1.0	1.2		

UAS function *in vivo* was determined by measuring β-galactosidase (β-Gal) enzyme levels in extracts from strains containing each plasmid. Units of β-galactosidase activity are in nmol·min^{-1}·(mg of protein)$^{-1}$. Relative *in vitro* transcription efficiency of each template, determined from assays such as those shown in Fig. 5, is given with pCZ-DED30 as the standard. The percentage competition of *in vitro* transcription by oligonucleotides (oligo.) was measured by adding each oligonucleotide (200 ng) to an *in vitro* transcription reaction with pCZ-DED48 as template (200 ng). The sequence of one strand of each oligonucleotide is listed 5′ to 3′ from left to right.

sorbitol in YPD, kept for 30 min at 30°C, washed with 400 ml of 1 M sorbitol in YPD, and washed with 400 ml of 1 M sorbitol before lysis. Transcription was performed with an amount of nuclear extract containing ≈80 μg of protein in a total reaction volume of 24 μl. Other conditions and the mapping of transcripts with ^{32}P-labeled RNA probes were as described (1).

Assays of UAS Function *in Vivo*. Plasmid DNAs were introduced into the yeast strain 5C (a *his3Δ200 ura3-52*) by using a lithium acetate transformation procedure (22) and selecting uracil prototrophy on minimal medium (0.67% yeast nitrogen base/0.5% ammonium sulfate/2% dextrose) supplemented with histidine. For β-galactosidase measurements, cells were grown and disrupted, and extracts were assayed by procedures as described (19).

RESULTS

Stimulation of Transcription by the *DED1* T-Rich Sequence *in Vitro*. *In vitro* transcription was performed with yeast nuclear extracts and with plasmids containing the *CYC1* promoter fused to *lacZ* coding sequences as described (1) except in two regards. Spheroplasts resulting from Zymolase digestion were washed extensively before the isolation of nuclei to reduce proteolysis by residual Zymolase. Additional protease inhibitors (chymostatin and benzamidine) were included in all buffers. With these modifications, the amount of nuclear extract needed in transcription reactions was reduced about 67%, and the efficiency of the reaction was increased from ≈10^{-4} to 10^{-3} transcript per template in 40 min at 25°C.

Appreciable transcription, initiating at two major *CYC1 in vitro* start sites, was obtained from a template (pCTΔ) with a TATA element but no positive regulatory elements (Fig. 1 *Top* and *Bottom*). Transcription was stimulated by insertion of a 116-bp fragment of *DED1*-flanking DNA immediately upstream of the TATA element (template pdAT-2). The stimulatory effect was greater at lower template concentrations, ranging from about 20-fold at 2.5 μg/ml to 5-fold at 20 μg/ml (Fig. 1 *Middle*). Both the basal transcription and the upstream sequence-enhanced transcription were sensitive to low levels of α-amanitin, indicative of polymerase II transcription.

The *DED1* fragment in template pdAT-2 contained the T-rich sequence mentioned above as well as binding sites for autonomously replicating sequence (ARS)-binding factor I (ABFI) (18). To distinguish the contributions from these elements, the *DED1* fragment was replaced, in template pCZ-DED48, by a 48-bp synthetic oligonucleotide containing only the T-rich sequence (Fig. 1 *Bottom*). A marked stimulation of transcription was again observed (Fig. 2 *Left*), ranging from 5- to 30-fold depending on the template concentration (Fig. 2 *Right*).

The DED48 oligonucleotide was located 24 bp upstream of the TATA element in pCZ-DED48. Moving the oligonucleotide 103 bp further from the TATA element (in pCT-DED48) diminished the level of transcription 50–75% (Fig. 2). This moderate distance dependence of the DED48 effect *in vitro* agrees with the behavior of the element *in vivo* (see below).

Inhibition of Transcription from a Template Containing the *DED1* T-Rich Sequence by an Oligonucleotide with the Same Sequence. The involvement of specific protein-binding in the stimulation of transcription by the *DED1* T-rich sequence was investigated by adding synthetic oligonucleotides as competitors to the transcription reaction. Addition of the DED48 oligonucleotide diminished the level of transcription from pCZ-DED48 (Fig. 3; see below for quantitation). Other oligonucleotides failed to suppress transcription—for example, oligonucleotides with the sequences of the T-rich region upstream of the *HIS3* gene (HIS35), the ABFI-binding site upstream of the *DED1* gene (DED1), and the general regulatory factor I (GRFI)-binding site upstream of the *ENO1* gene (ENO1) (18, 19). None of the oligonucleotides, including DED48, diminished the lower level of transcription from a template containing a GRFI-binding site (pCZ-PYK1). Thus, the DED48 oligonucleotide does not compete for one of the basic transcription factors required for accurate initiation in the absence of UASs.

The inhibitory effect of the DED48 oligonucleotide was quantitated in reactions containing 0.2 μg of pCZ-DED48 and 0.2 μg of total oligonucleotide, with various ratios of the DED48 and ENO1 oligonucleotides (Fig. 4). The total oligonucleotide was held constant because the level of transcription increased slightly with increasing DNA concentration. Inhibition by the DED48 oligonucleotide was half-maximal at a molar ratio of DED48 oligonucleotide to template of about 40:1. This result is indicative of a transcription factor in excess over the template or of a factor that binds more tightly to the template than to the oligonucleotide. A greater affinity for the template could be due to interaction with other factors on the template or simply to preferential binding to a longer length of DNA.

Effects of Various T-Rich Sequences on Transcription *in Vivo*. Oligonucleotides containing various T-rich sequences

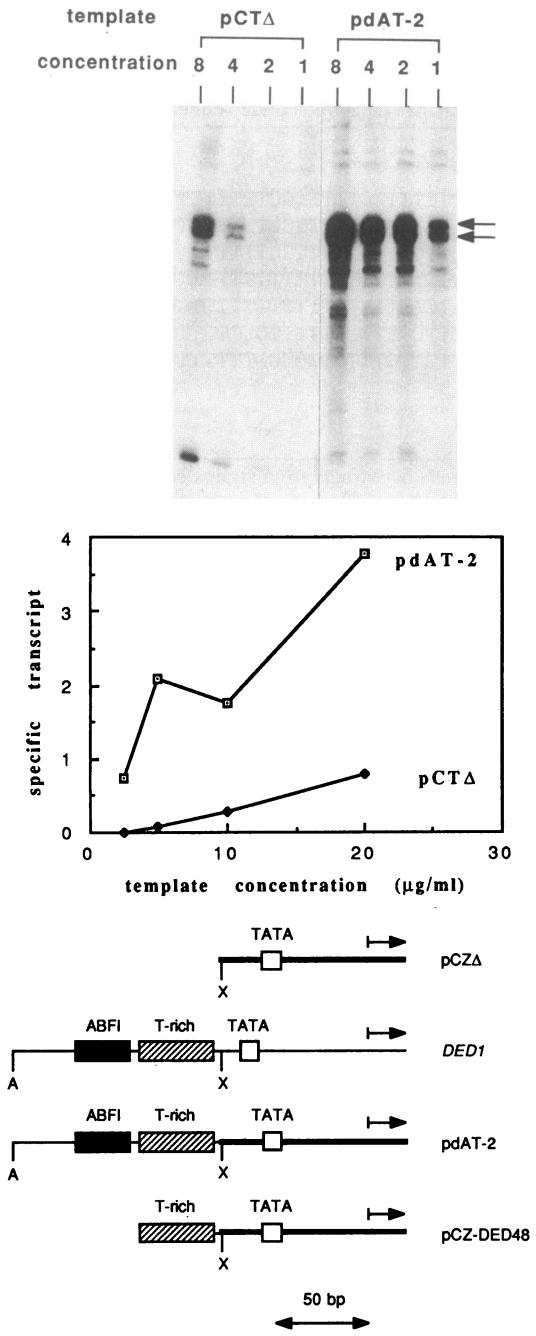

FIG. 1. Effect of a region upstream of the *DED1* gene on transcription from the *CYC1* promoter *in vitro*. (*Top*) Templates with the *DED1* upstream region (lanes pdAT-2) or lacking any upstream regulatory elements (lanes pCTΔ) were used at concentrations of 2.5 μg/ml times the numbers given. Products were hybridized to complementary ^{32}P-labeled RNA probes, digested with RNase, and electrophoresed in a 7% polyacrylamide/urea gel, which was dried and autoradiographed. Bands due to transcription from the major *CYC1* start sites (235–255 bases) are indicated by arrows. (*Middle*) Data from *Top* was quantitated by densitometry. (*Bottom*) Structures of the promoter regions of the *DED1* gene and the pCZΔ, pdAT-2, and pCZ-DED48 templates. *CYC1* promoter sequences are shown with the thick black line; and *DED1* promoter sequences, by the thin black line. Arrows indicate the start sites for transcription. TATA sequences are indicated with a white box; T-rich sequences, by hatched boxes; and the ABFI-binding region, with a black box. Restriction sites are indicated below the promoter maps: A, *Acc* I; X, *Xho* I.

were tested for activation of *CYC1-lacZ* expression *in vivo* by transformation into yeast and measurement of β-galactosidase activity in cell extracts (Table 1). The DED48 oligonucleotide increased expression 4.5- and 7.5-fold when located 127 and 24 bp upstream of the TATA element, respectively (compare pCT-DED48 and pCZ-DED48 with pCZΔ). This degree of activation is comparable to that of the *DED1* and *HIS3* T-rich sequences in their natural contexts, but it is an order of magnitude less than that reported for UASs such as GAL4- and GRFI-binding sites (19). T-rich sequences may generally function alongside other regulatory elements. For example, the region upstream of the *HIS3* gene contains both a T-rich sequence and GCN4-binding sites, which also activate expression (23). And as mentioned above, the region upstream of the *DED1* gene also harbors at least one additional type of element, binding sites for ABFI. When the entire *DED1* upstream region (in the form of the 116-bp fragment of *DED1*-flanking DNA described above) was placed in front of the *CYC1-lacZ* fusion gene, it activated expression 150-fold (Table 1). The role of ABFI in this effect and its relationship to that of the putative T-rich sequence-binding factor remain to be determined.

Deletion of the longest uninterrupted stretch of thymidine residues, nine residues, from the 3′ end of the DED48 sequence abolished the activating effect *in vivo* (pCZ-DED30), whereas the deletion of nine residues from the 5′ end reduced expression by only half (pCZ-DED36). Extending the uninterrupted stretch of thymidine residues by converting all guanosine and cytidine residues to thymidine residues increased the stimulatory effect (pCZ-T39). Other naturally occurring T-rich sequences upstream of the *SNR20* (pCZ-SNR47) and *YPT2* (pCZ-YPT47) genes activated expression to a comparable extent *in vivo*. The *HIS3* T-rich element (pCZ-HIS35) was less effective.

In contrast to the behavior of many UASs, the function of T-rich elements was strongly orientation dependent. Inverting the DED48 sequence in relation to the promoter reduced the level of *CYC1-lacZ* expression to background (pCS-DED48). Inverting the all-thymidine oligonucleotide (T39) had a similar effect (not shown). The function of some upstream elements is affected by rotation about the helix axis brought about by insertion or deletion of odd multiples of half-helical turns (24, 25). Insertion of 4 bp between the DED48 oligonucleotide and TATA element reduced expression by 50% (pCZ-DED48/XBL). Since insertion of 103 bp reduced expression by only 40% (pCT-DED48), the effect of the 4-bp insertion was likely due to rotational misalignment rather than increased distance between the T-rich element and the promoter.

Effects of Various T-Rich Sequences on Transcription *in Vitro*. Stimulation of *CYC1-lacZ* transcription by the DED48 element and derivatives *in vitro* paralleled the activation of expression *in vivo* (Fig. 5, Table 1): as already mentioned, there was a moderate distance dependence of activation *in vitro* (compare pCZ-DED48 and pCT-DED48); insertion of 4 bp between the DED48 oligonucleotide and TATA element reduced transcription by 50% *in vitro* (pCZ-DED48/XBL); the 3′ but not the 5′ end of the oligonucleotide was required for activation *in vitro* (pCZ-DED36 and pCZ-DED30); and oligonucleotide inversion abolished activation *in vitro* (pCS-DED48). However, other T-rich sequences were much less effective *in vitro* than *in vivo*. The *YPT2*-associated sequence gave a small degree of activation (pCZ-YPT47), while the *HIS3*- and *SNR20*-associated sequences and the all-thymidine oligonucleotide gave no activation at all (pCZ-HIS35, pCZ-SNR47, pCZ-T39). The effects of the various T-rich oligonucleotides as competitors of DED48-dependent transcription paralleled their stimulatory activities *in vitro*.

DISCUSSION

The activation of transcription by the *DED1*-associated T-rich sequence was somewhat greater *in vitro* than *in vivo*.

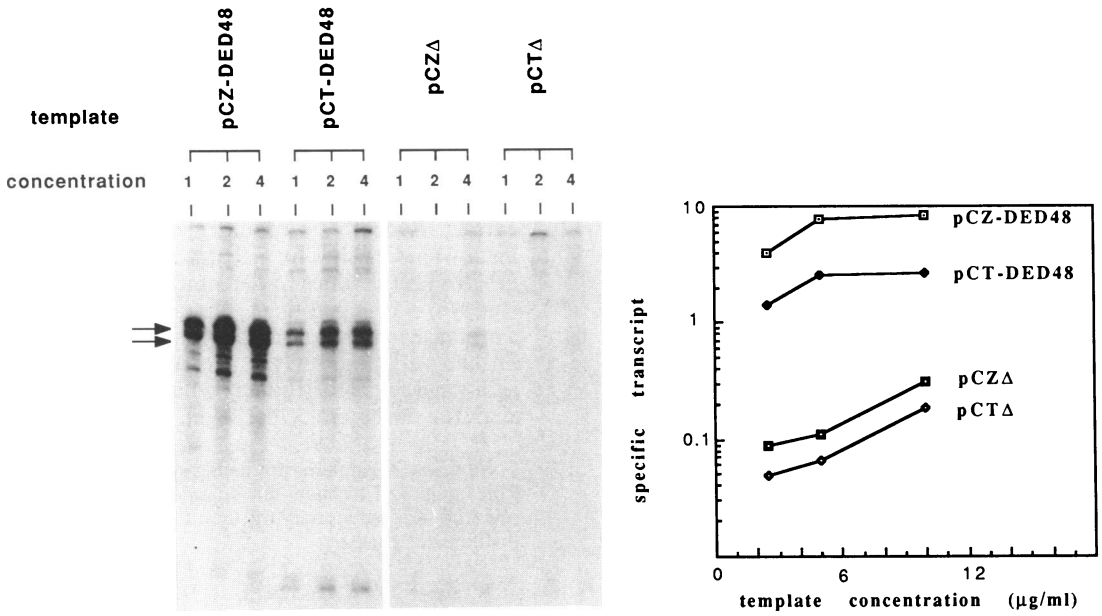

FIG. 2. Effect of the T-rich sequence upstream of the *DED1* gene on transcription from the *CYC1* promoter *in vitro*. (*Left*) Templates with a 48-bp synthetic oligonucleotide (DED48) containing the *DED1* T-rich sequence, placed 24 bp (pCZ-DED48) or 127 bp (pCT-DED48) upstream of the *CYC1* promoter, were used at concentrations of 2.5 μg/ml times the numbers given. Bands due to transcription from the major *CYC1* start sites are indicated by arrows. (*Right*) Data from *Left* was quantitated by densitometry.

On the other hand, the dependence on distance from the TATA element, the effects of deletions in the T-rich sequence, and the effect of inverting the sequence were similar *in vitro* and *in vivo*. Therefore, it seems likely that the mechanism of activation *in vitro* was similar to that *in vivo*.

A mechanism of activation by T-rich sequences based on the exclusion of nucleosomes rather than the binding of a transcription factor has been put forward (10, 14, 15). Three observations argue against this proposal in its simplest form.

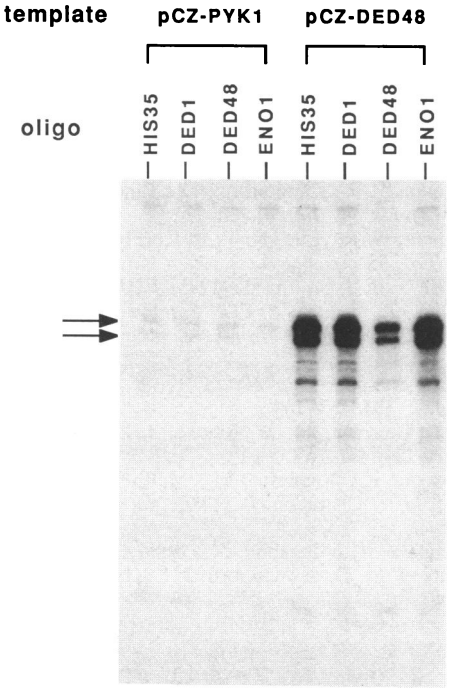

FIG. 3. Inhibition by a synthetic oligonucleotide with the *DED1* T-rich sequence of transcription stimulated by that sequence. A template with an oligonucleotide containing the *DED1* T-rich sequence (pCZ-DED48) or with an oligonucleotide containing a GRFI-binding site upstream of the *PYK1* gene (pCZ-PYK1) was used at 4 μg/ml, and 100 ng of the oligonucleotides indicated were added to the reaction mixture.

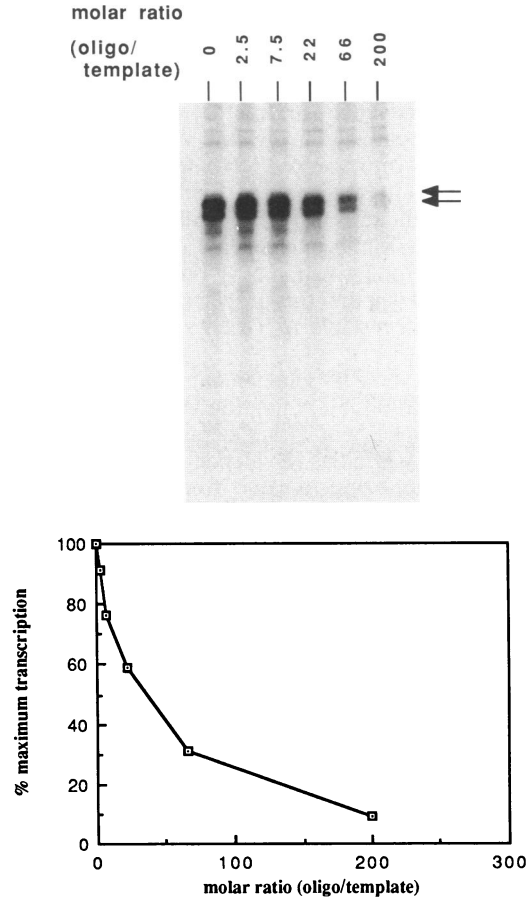

FIG. 4. Concentration dependence of inhibition by the *DED1* T-rich oligonucleotide of transcription stimulated by the oligonucleotide. (*Upper*) Transcription reaction mixtures contained 200 ng of pCZ-DED48, with amounts of the DED48 oligonucleotide giving the molar ratios to the template indicated. The total amounts of oligonucleotide added to the reactions were held constant at 200 ng by the addition of appropriate amounts of the ENO1 oligonucleotide. Bands due to transcripts from the major *CYC1* start sites are indicated by arrows. (*Lower*) Data from *Upper* was quantitated by densitometry.

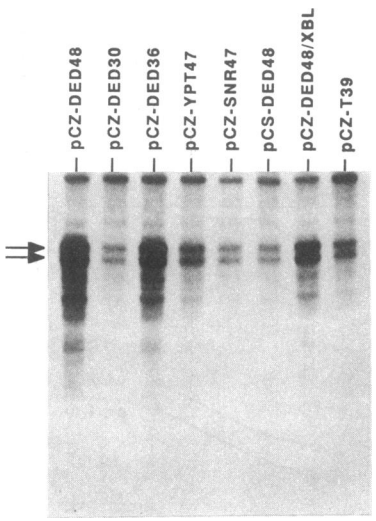

FIG. 5. Effect of various T-rich sequences at several locations upstream of the *CYC1* promoter on transcription *in vitro*. Transcription was performed with the indicated templates at 3 μg/ml. Bands due to transcripts from the major *CYC1* start sites are indicated by arrows.

First, the capacity of an excess of T-rich oligonucleotide to inhibit the activation of transcription by the same T-rich sequence in a template is indicative of factor-binding to the T-rich sequence. Second, the loss of activation upon inverting either the DED48 or the T39 T-rich element cannot be understood in terms of the nucleosome exclusion idea, since a poly(dA-dT) sequence is refractory to nucleosome formation in both directions. Finally, the very observation of an activating effect with a naked DNA template *in vitro* under conditions that do not support nucleosome formation is inconsistent with activation by inhibition of nucleosome formation alone. While nucleosome exclusion is not sufficient to account for activation by T-rich elements, it remains to be determined whether this property plays a role in making these elements accessible to T-rich sequence-binding transcription factors.

The failure of some T-rich elements to activate transcription *in vitro* or to inhibit activation by the *DED1* sequence points to a multiplicity of T-rich element recognition factors with different specificities. Evidence for diverse T-rich element-binding factors also came from gel electrophoretic mobility shift experiments revealing different DNA–protein complexes formed by different T-rich sequences in crude extracts (A.R.B., unpublished data). Thus, there does not appear to be one type of T-rich element whose activating effect is mediated by a single factor, but rather there may be a family of elements and multiple factors.

T-rich elements often occur in conjunction with other activation sequences, such as binding sites for GCN4 protein (9), GRFI (26), and ABFI (18). The effects of the T-rich and ABFI-binding elements upstream of the *DED1* gene are synergistic *in vivo* (A.R.B., unpublished data), indicative of interactions between factors bound to the elements or to different mechanisms of transcriptional activation by the different elements.

As mentioned above, some of the sequences that activated expression *in vivo* failed to show an effect *in vitro*. There are several possible explanations for this observation. For example, different upstream elements might operate by different mechanisms, and only a subset of these effects may be revealed under the present assay conditions. Alternatively, some of the factors that mediate these processes may be of low abundance or may be damaged during preparation of the extract. Recently, we found that a different upstream element—binding sites for GRFI—could stimulate transcription *in vitro* (19). Failure to detect a GRFI effect in our original study (1) was in part due to the susceptibility of this factor to a protease in the enzyme used to lyse yeast cell walls. Thus, further variations of the methods used here may reveal additional regulatory effects whose mechanisms can then be analyzed at the molecular level.

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