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Analysis of *Saccharomyces cerevisiae his3* Transcription In Vitro: Biochemical Support for Multiple Mechanisms of Transcription

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The promoter region of the *Saccharomyces cerevisiae his3* gene contains two TATA elements, T_C and T_R, that direct transcription initiation to two sites designated +1 and +13. On the basis of differences between their nucleotide sequences and their responsiveness to upstream promoter elements, it has previously been proposed that T_C and T_R promote transcription by different molecular mechanisms. To begin a study of *his3* transcription in vitro, we used *S. cerevisiae* nuclear extracts together with various DNA templates and transcriptional activator proteins that have been characterized in vivo. We demonstrated accurate transcription initiation in vitro at the sites used in vivo, transcriptional activation by GCN4, and activation by a GAL4 derivative on various *gal-his3* hybrid promoters. In all cases, transcription stimulation was dependent on the presence of an acidic activation region in the activator protein. In addition, analysis of promoters containing a variety of T_R derivatives indicated that the level of transcription in vitro was directly related to the level achieved in vivo. The results demonstrated that the in vitro system accurately reproduced all known aspects of in vivo *his3* transcription that depend on the T_R element. However, in striking contrast to *his3* transcription in vivo, transcription in vitro yielded approximately 20 times more of the +13 transcript than the +1 transcript. This result was not due to inability of the +1 initiation site to be efficiently utilized in vitro, but rather it reflects the lack of T_C function in vitro. The results support the idea that T_C and T_R mediate transcription from the wild-type promoter by distinct mechanisms.

Most promoters of genes transcribed by eucaryotic RNA polymerase II contain both upstream activating elements and TATA elements (reviewed in references 6, 11, and 21). Upstream elements are usually responsible for defining the regulatory properties of a promoter. In many cases, these elements have been shown to be the binding sites for gene-specific transcriptional activator proteins. A number of such activators have been functionally dissected, demonstrating separable domains involved in DNA binding and transcriptional activation (1, 10). Thus, the binding of such activators to upstream elements is viewed as positioning an activation domain in the vicinity of the promoter. The mechanisms by which activation domains stimulate transcription are not known, but they are presumed to involve interaction between the activation domain and some component of the general transcription machinery, affecting either the formation of transcription complexes or the rate of transcription initiation from such complexes.

TATA elements, usually containing the conserved sequence TATAAA, are located near the mRNA initiation site and are specific binding sites for transcription factor TFIID. TFIID is usually described as a general transcription factor that is a required component of the basic RNA polymerase II transcription complex. However, some eucaryotic promoters have been described as "TATA-less" because of their lack of sequences that adhere to the TATAAA consensus, raising the possibility that TFIID is not universally required. Despite extensive mutagenesis of TATA elements (4, 7), the sequence requirements for TFIID binding have yet to be firmly established. Thus, it remains unclear whether these promoters contain TFIID-binding sites and whether binding of TFIID to a promoter is an obligate primary step towards transcription initiation by RNA polymerase II.

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Under normal growth conditions, transcription of the *Saccharomyces cerevisiae his3* gene is initiated at equal frequencies from two major sites, designated +1 and +13 (19). The constitutive levels of both the +1 and +13 transcripts are reduced fivefold by deletion of an upstream poly(dA)-poly(dT) region (19). During conditions of amino acid starvation, *his3* transcription is induced about threefold (23) by binding of the GCN4 activator protein (8, 9). During induction, transcription from the +1 site remains essentially at the basal level, whereas transcription from the +13 site is preferentially elevated fivefold (22). Preferential initiation from the *his3* +13 site can also be induced by the GAL4 activator protein in hybrid promoters when the *gal* enhancer is fused at various positions to the *his3* promoter (18). Deletion analysis and saturation mutagenesis have shown that induction by GCN4 or GAL4 is mediated through the T_R TATA element that contains the classical TATAAA motif and binds TFIID (4, 7, 17).

In the absence of a functional T_R element, constitutive transcription still occurs at normal levels at the +1 site but is abolished by deletion of or appropriate mutations within a second region approximately 25 base pairs (bp) upstream of T_R called T_C (20; S. Mahadevan and K. Struhl, unpublished data). In *his3* promoters containing a functional T_R element, deletion of or appropriate mutations within T_C selectively eliminate the +1 transcript (7; Mahadevan and Struhl, unpublished data). The T_C region does not contain a sequence that fits the classical T_R consensus, and T_C does not respond to GCN4 or GAL4 activation, thus leading to the suggestion that TFIID might not bind to the T_C element. For this and other reasons, it has been proposed that constitutive and inducible *his3* transcription mediated by T_C and T_R occur by distinct molecular mechanisms (20).

To begin a biochemical study of *his3* transcription, we assayed the transcription activities of various *his3* templates

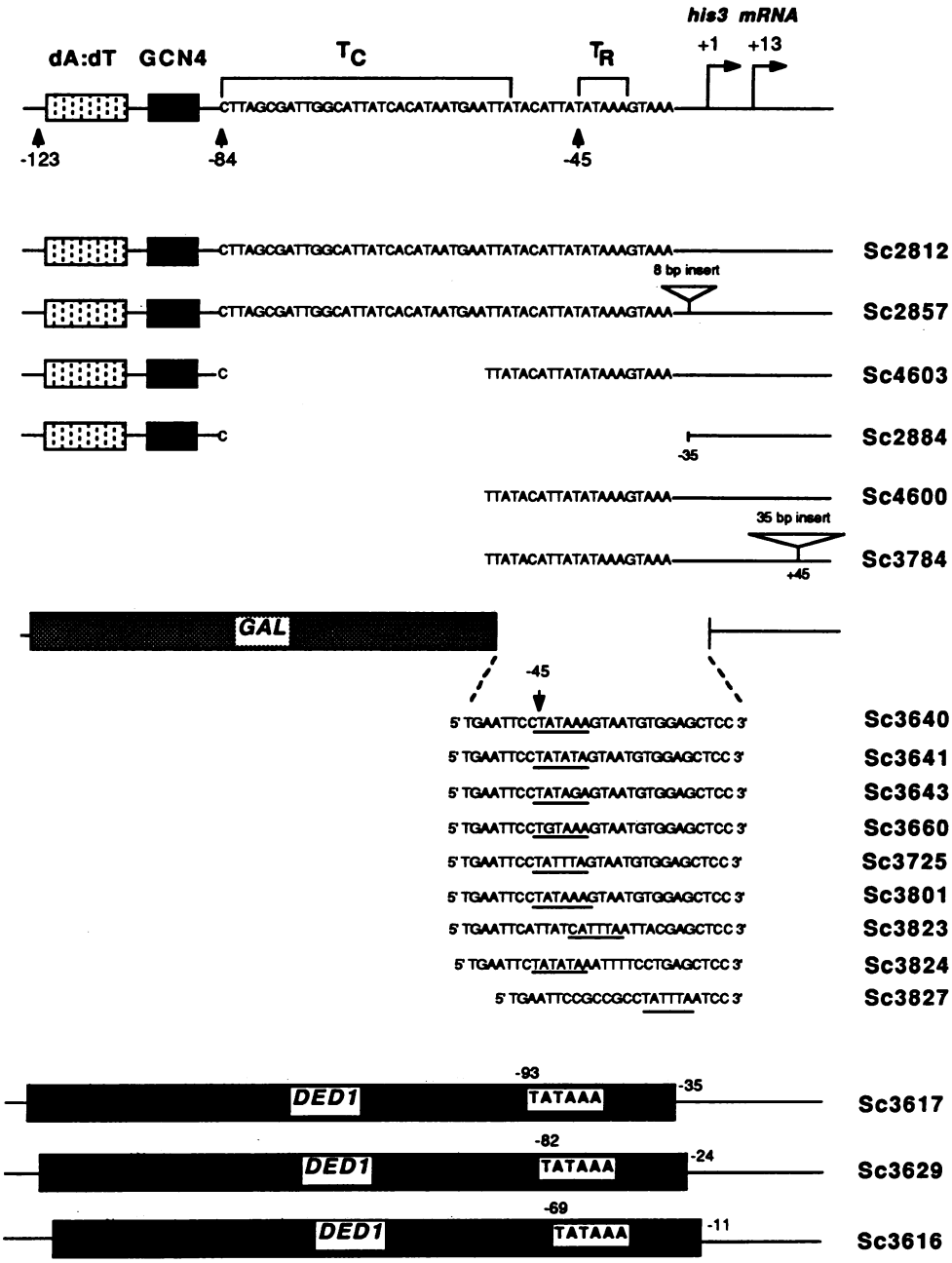

FIG. 1. Yeast *his3* promoter and plasmids used in this study. Diagrammed at the top is the wild-type *his3* promoter region with the poly(dA)-poly(dT) region, the GCN4-binding site, the T_C and T_R TATA elements, and the +1 and +13 mRNA initiation sites indicated. Diagrammed below are the relevant structures of the *his3* promoter regions of the plasmids (listed by their Sc DNA fragment numbers) containing deletions or insertions within the *his3* promoter region, fusions of the *gal* enhancer that binds GAL4 to various T_R sequences, and fusions between a region of the *ded1* promoter to various positions upstream of the *his3* initiation region. Deleted sequences are represented by open spaces, with the exception of the *gal-his3* promoters, in which all of the plasmids have the same basic structure and only the different T_R sequences are listed.

in vitro using unfractionated yeast nuclear extracts. We examined basal transcription of the wild-type *his3* promoter, as well as induced transcription in the presence of added purified GCN4 protein. Variants of the T_R TATA element were examined in the context of a *gal-his3* promoter fusion in which transcriptional activation was induced with a derivative of GAL4 protein. The results demonstrated accurate in vitro reproduction of most aspects of *his3* transcription but indicated differential activities of the T_R and T_C elements in vitro.

MATERIALS AND METHODS

Plasmids and strains. The *his3* promoter region of each plasmid used in this study is diagrammed in Fig. 1. pUC19-Sc3784 was constructed by subcloning of an *Eco*RI-*Hin*dIII

fragment corresponding to positions −55 to +330 (17) into pUC19, followed by insertion of a 35-bp *Eco*RV-*Bam*HI fragment from the polylinker of pSP73 into the *Mae*I site at position +45. The following plasmids were also used in this study: YIp55-Sc2812 (3); YIp55-Sc2884 (17); YIp55-Sc4600 and YIp55-Sc4603 (Mahadevan and Struhl, unpublished data); YIp55-Sc3640, YIp55-Sc3641, YIp55-Sc3643, and YIp55-Sc3660 (4); YIp55-Sc3725 (7); YCp86-Sc3801, YCp86-Sc3823, YCp86-Sc3824, and YCp86-Sc3827 (16); YRp14-2857 (17); and YIp5-Sc3616, YIp5-Sc3617, and YIp5-Sc3629 (3). KY791 (*matα ura3-52 leu2 trp1 prb1-1122 pep4-3 prc1-407 gal2 his3::TRP1*) was constructed by one-step disruption of the *his3* gene in strain BJ2168.

Nuclear extract. KY791 was grown in 9 liters of YPD medium (1% yeast extract, 2% Bacto-Peptone, 2% glucose) at 30°C to an optical density at 600 nm of 5 to 10, and nuclear extract was prepared by a modification of a previous procedure (12). Cells were harvested by centrifugation at 5,000 × *g* for 10 min, suspended in 600 ml of 50 mM of Tris hydrochloride (pH 7.5)–30 mM dithiothreitol (DTT) and shaken slowly for 15 min at 30°C. The cells were harvested, suspended in 90 ml of YPD–1 M sorbitol, and digested with 150 mg of Zymolase 100T (Miles Laboratories, Inc.) at 30°C until the optical density at 600 nm in 1% sodium dodecyl sulfate was less than 5% of the starting value (45 min). Digestion was stopped by addition of 900 ml of YPD–1 M sorbitol, and the spheroplasts were recovered by centrifugation at 3,000 × *g* for 5 min. The spheroplasts were washed once with 1,200 ml of YPD–1 M sorbitol by gently suspending the pellets initially in a small volume of YPD–1 M sorbitol with a rubber policeman and allowing some small clumps of cells to remain intact. After centrifugation, the spheroplasts were suspended as described above in 1,500 ml of YPD–1 M sorbitol and incubated at 30°C with slow shaking for 30 min. The spheroplasts were recovered and washed once with 1,200 ml of ice-cold YPD–1 M sorbitol and once with 1,200 ml of ice-cold 1 M sorbitol. The spheroplasts were lysed in 600 ml of buffer A (18% [wt/vol] Ficoll 400, 10 mM Tris hydrochloride [pH 7.5], 20 mM KCl, 5 mM $MgCl_2$, 3 mM DTT, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine) containing 1× protease inhibitors (1 mM phenylmethylsulfonyl flouride, 2 μM pepstatin A, 0.6 μM leupeptin, 2 mM benzamidine, 2 μg of chymostatin per ml, diluted fresh from a 100× stock in 95% ethanol) with a motor-driven Teflon-glass homogenizer (four strokes; clearance, 0.15 to 0.23 mm). Cell debris and unlysed spheroplasts were removed by four sequential centrifugations of the supernatant at 3,000 × *g* for 5 min. Nuclei were harvested by centrifugation at 25,000 × *g* for 30 min and suspended in 45 ml of buffer B (0.1 M Tris acetate [pH 7.9], 50 mM potassium acetate, 10 mM $MgSO_4$, 20% glycerol, 3 mM DTT, 2 mM EDTA, 1× protease inhibitors); the nuclear suspension could be frozen in liquid nitrogen at this stage. The nuclei were extracted by dropwise addition of saturated ammonium sulfate (4 M) to a final concentration of 0.5 M with gentle stirring at 4°C. The suspension was kept at 4°C for an additional 20 min and centrifuged in a Beckman type 45 rotor at 33,000 rpm for 2 h at 4°C. The supernatant was adjusted to 75% saturation with ammonium sulfate by slow addition of solid (0.43 g/ml) and neutralized by occasional addition of 1 M KOH (10 μl/g of ammonium sulfate). The ammonium sulfate precipitate was recovered by centrifugation at 10,500 × g for 30 min, and the pellet was suspended in 10 ml of buffer C {20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.6], 10 mM $MgSO_4$, 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 20% glycerol, 5 mM DTT, 1× protease inhibitors}. The suspension was dialyzed against 500 ml of the same buffer by using two 10,000-dalton retention collodion membranes (Schleicher & Schuell, Inc.) until the conductivity of a 1:1,000 dilution in distilled water was less than 15 μS/cm (about 2 h), which is approximately equal to that of buffer C–125 mM ammonium sulfate. Since the transcription reactions require a final protein concentration of 3 to 4 mg/ml and are very sensitive to final ammonium sulfate concentrations greater than 25 mM (unpublished data), it was important to initially suspend the ammonium sulfate precipitate in a small enough volume to ensure a high protein concentration after dialysis (30 to 60 mg/ml). After dialysis, the extract was centrifuged at 14,000 × *g* for 10 min to remove insoluble material, and aliquots of the supernatant (40 mg of protein per ml) were frozen in liquid nitrogen.

Activator proteins. GCN4 and GCN4-C57 proteins were purified to apparent homogeneity as previously described (15). GAL4(1-147) and GAL4(1-147)-B17, purified as previously described (2), were kindly provided by Michael Carey (Harvard University).

In vitro transcription reactions. Transcription reactions (25 μl) contained 10 mM HEPES (pH 7.6), 100 mM potassium glutamate, 10 mM magnesium acetate, 2% polyvinyl alcohol, 5 mM EGTA, 2.5 mM DTT, 4 mM phosphoenolpyruvate, 10% glycerol, each of the four ribonucleoside triphosphates at 0.4 mM, 0.5 U of Inhibit-Ace RNase inhibitor (5 Prime-3 Prime, Inc.), 100 μg of nuclear extract protein, and a template as indicated in the figure legends. Reactions were incubated at room temperature for 1 h, terminated by addition of 0.3 ml of a stop mixture (0.1 M sodium acetate [pH 5.2], 10 mM EDTA, 1% sodium dodecyl sulfate, 75 μg of tRNA per ml), extracted twice with an equal volume of phenol-chloroform (1:1), and precipitated by addition of ammonium acetate (to 2.5 M) and ethanol (2.5 volumes).

Primer extension analysis. The precipitated reaction products were suspended in 15 μl of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA and 5 μl of a mixture containing 0.7 M KCl, 0.5 U of Inhibit-Ace, 100 fmol of 5′-end ^{32}P-labeled *his3* oligonucleotide primer was added. The primer contained the sequence 5′-GGTTTCATTTGTAATACGC-3′, corresponding to the bottom strand of the *his3* sequence from +50 to +68. The mixtures were incubated at 37°C for 1 h. A 30-μl volume of a solution containing 83 mM Tris hydrochloride (pH 8.0), 16.8 mM DTT, 16.8 mM $MgCl_2$, each of the four deoxyribonucleoside triphosphates at 833 μM, 7 U of avian myeloblastosis virus reverse transcriptase (Promega Biotec) was added, and the reaction was incubated at 42°C for 15 min. The primer extension products were ethanol precipitated (50 μl of 5 M ammonium acetate, 250 μl of ethanol), suspended in formamide-loading dyes, and analyzed on 10% acrylamide–7 M urea gels. All of the experiments described here were performed at least twice, and in most cases, three times.

RESULTS

In vitro transcription of *his3*. Nuclear extract was prepared from strain KY791, and in vitro transcription was carried out with a DNA template containing the wild-type *his3* promoter (YIp55-Sc2812). To compare the in vitro transcription products with those made in vivo, total RNA was isolated from strain KY114 (18) grown in rich medium; under these growth conditions, GCN4 activation does not occur. Products of the in vitro transcription reactions and the in vivo RNA were then analyzed by primer extension with a ^{32}P-labeled *his3*

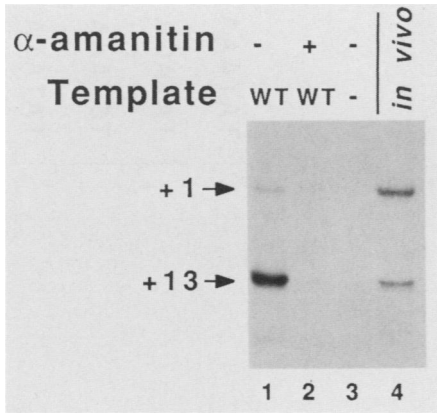

FIG. 2. In vitro transcription of *his3*. Transcription reactions were performed by using a template containing a wild-type (WT) *his3* promoter (YIp55-Sc2812; 80 fmol) under standard conditions (lane 1) with addition of 40 μg of α-amanatin per ml (lane 2) or with no template (lane 3). Shown in lane 4 are the primer extension products from 30 μg of total yeast RNA obtained from strain KY114, which contains the wild-type *his3* gene. The mobilities of the primer extension products corresponding to the accurately initiated +1 and +13 transcripts are indicated.

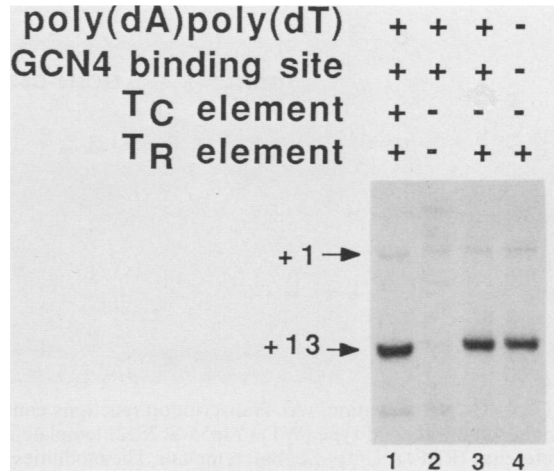

FIG. 3. Analysis of templates containing deletions in the *his3* promoter region. Transcription reactions contained 80 fmol of plasmid YIp55-Sc2812 (wild-type; lane 1), YIp55-Sc2884 (deletes −35 through −83; lane 2), YIp55-Sc4603 (deletes −56 through −83; lane 3), or YIp55-Sc4600 (deletes −56 through −123; lane 4). The mobilities of the primer extension products corresponding to the +1 and +13 transcripts are indicated.

primer corresponding to the bottom strand from positions +50 to +68. Transcription in vitro initiated at the same sites as those used in vivo (Fig. 2, lane 1 versus lane 4). The +13 transcript had previously been designated +12 on the basis of S1 nuclease mapping; analysis of the primer extension products shown in Fig. 2 alongside dideoxynucleotide sequencing markers indicated that both the in vivo and in vitro start sites are at +13 (data not shown). Transcription was dependent upon the presence of the template (lane 3) and was >95% inhibited by α-amanitin (40 μg/ml; lane 2), demonstrating that the transcripts were synthesized by RNA polymerase II. Interestingly, however, transcription in vitro yielded approximately 20 times more of the +13 transcript than the +1 transcript, in sharp contrast to the equal levels of these transcripts produced in vivo. The ratio of the +13 and +1 transcripts observed in vitro was reminiscent of in vivo analyses of *his3* or *gal-his3* promoters that lack the T_C element, in which the T_R element directed transcription preferentially, but not exclusively, to the +13 site (4, 7, 14, 18).

To determine which *his3* promoter elements were involved in generating the in vitro transcripts, we tested the transcription activities of templates containing various deletions of the *his3* promoter region. Shown in Fig. 3 are results obtained with a wild-type template (lane 1) or templates deleted for both the T_R and T_C elements (−35 through −83; lane 2); deleted only for the T_C element (−56 through −83; lane 3); or deleted for the T_C element, the GCN4 binding site, and the poly(dA)-poly(dT) tract (−56 through −123; lane 4). Transcription of the template deleted for T_C and T_R yielded a severely reduced level of the +13 transcript (lane 2). A small amount of the +1 transcript was seen with this template, comparable to the level observed with the wild-type template, as well as two additional weak starts in the initiation region. This residual transcription seen with this template lacking both the T_C and T_R elements might be due to a cryptic TATA element being brought closer to the initiation region as a result of the deletion. Transcription of the template deleted for only the T_C element yielded transcription products identical to those obtained with the wild-type template (lane 3 versus lane 1), indicating that the T_C element apparently directed no transcription in vitro. Moreover, transcription of a template deleted for the T_R element but containing the T_C element yielded products identical to those obtained with the template deleted for both T_R and T_C, which is also consistent with T_C not directing any transcription in vitro (data not shown). Transcription of the template deleted from −56 through −123 demonstrated that deletion of the GCN4-binding site or the poly(dA)-poly(dT) region had no significant effect on the level of the in vitro transcripts (lane 4). Thus, these results demonstrated that the wild-type *his3* promoter was transcribed accurately in the nuclear extract but suggested that the T_C element did not function in vitro.

Activation by GCN4. To determine the significance of the above-described results, we sought to better establish the relevance of the in vitro system by examining other aspects of *his3* transcription. Initially, we tested whether purified GCN4 protein would stimulate transcription in vitro as it does in vivo. For these and subsequent analyses, we constructed a variant *his3* template to serve as an internal reference. The reference template, pUC19-Sc3784, was deleted for the upstream poly(dA)-poly(dT) tract, the GCN4-binding site, and the T_C region, retaining only the T_R element upstream of *his3*. In addition, this plasmid contained an insertion of approximately 35 bp at position +45 so as to yield distinct primer extension products 35 nucleotides larger than those generated from standard *his3* templates. The wild-type and reference templates were transcribed either separately or together (Fig. 4A). Equal levels of +13 transcription from these templates were observed, as expected from the previous finding that the dA-dT region, the GCN4-binding site, and the T_C region did not contribute to initiation at the +13 site. Moreover, transcription of the reference template still resulted in the small amount of the +1 transcript seen with the wild-type template, also consistent with no contribution to +1 transcription by the T_C element in vitro. It should be noted that a number of smaller transcripts were observed with the reference template (either downstream starts or degradation products of full-length transcripts, and these presumably occur with the wild-type template but were not seen because the small products were

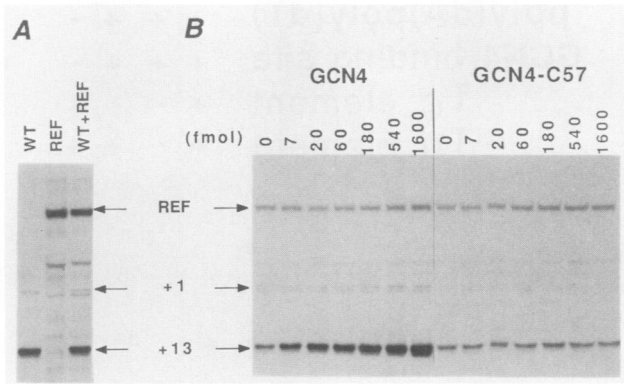

FIG. 4. GCN4 activation. (A) Transcription reactions contained 80 fmol each of the wild-type (WT) (YIp55-Sc2812) template and/or the reference (REF) (pUC19-Sc3784) template. The mobilities of the primer extension products corresponding to the +1 and +13 transcripts from the wild-type template and the +13 transcript from the reference template are indicated. (B) Transcription reactions contained 80 fmol each of the wild-type and reference templates under standard conditions or with addition of purified GCN4 or GCN4-C57 protein as indicated.

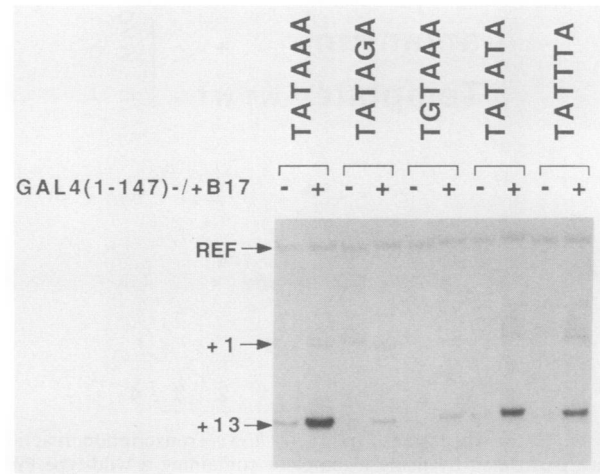

FIG. 5. Analysis of T_R derivatives. Transcription reactions contained 17 fmol of the reference (REF) (pUC19-Sc3784) template and 80 fmol of the indicated *gal-his3* template with addition of either GAL4(1-147) or GAL4(1-147)-B17 protein. The amount of GAL4 derivative added was that which completely protected the GAL4-binding sites in the *gal* enhancer from DNase I digestion. The *gal-his3* DNA molecules were YIp55-Sc3640 (TATAAA), YIp55-Sc3643 (TATAGA), YIp55-Sc3660 (TGTAAA), YIp55-Sc3641 (TATATA), and YIp55-Sc3725 (TATTTA). The mobilities of the primer extension products corresponding to the +1 and +13 transcripts from the *gal-his3* template and the +13 transcript from the reference template are indicated.

electrophoresed off the bottom of the gel); hence, in reactions containing both the reference template and a test template, these smaller products should not be mistaken for transcripts coming from the test template.

Equimolar amounts of the wild-type and reference templates were transcribed together in a standard reaction or with addition of increasing amounts of purified GCN4 protein or GCN4-C57 protein, which retains the DNA-binding domain but lacks the acidic activation domain. The molar equivalents of the two proteins added were determined also to possess equivalent DNA-binding activities as determined by mobility shift assays (data not shown). Transcriptional activation of the wild-type template was observed only with GCN4 protein containing the activating region (Fig. 4B). Densitometric scanning of the autoradiogram revealed that the GCN4 activation was about fourfold in the reaction with 1,600 fmol of GCN4, which correlates well with the approximately fivefold stimulation observed in vivo. Reactions containing higher amounts of GCN4 resulted in activation of the reference template lacking a GCN4 binding site, presumably because of nonspecific binding of GCN4 at high concentrations (data not shown).

Analysis of T_R TATA derivatives. To further establish the relevance of the in vitro system, we assayed the transcription of templates containing various T_R elements. The sequence requirements for in vivo T_R function have previously been examined in the context of a *gal-his3* hybrid promoter in which prospective T_R elements were placed downstream of an enhancer that binds the GAL4 activator protein (4, 7). In this study, we tested the in vitro transcription of *gal-his3* templates containing a wild-type T_R (TATAAA), two mutants which show intermediate transcription levels in vivo (TATATA and TATTTA), and two mutants which have no activity in vivo (TATAGA and TGTAAA). Transcriptional activation mediated by T_R was assayed by adding to the reactions either GAL4(1-147) protein, representing the DNA-binding domain of GAL4, or GAL4(1-147)-B17 protein, which contains the DNA-binding domain of GAL4 fused to an acidic activating region encoded by an *Escherichia coli* genomic fragment (2, 13). Compared with the reactions containing GAL4(1-147) (which showed no stimulatory activity relative to reactions with no added GAL4 derivative; data not shown), GAL4(1-147)-B17 stimulated transcription of the *gal-his3* templates about 10-fold, while having no effect on transcription of the reference template, pUC19-Sc3784, which lacks a GAL4-binding site (Fig. 5). Stimulation was observed for both the +1 and +13 transcripts, consistent with the low level of the +1 transcript coming from T_R-directed events. Basal and induced transcript levels were highest with the template containing the wild-type T_R TATAAA, followed by intermediate levels with the templates containing TATATA and TATTTA, and much reduced levels with the templates containing TAT AGA and TGTAAA.

As an additional test of how well the in vitro system reproduced TFIID-directed events, we examined the transcription of three *gal-his3* templates whose T_R elements were functionally selected in vivo from random-sequence oligonucleotides. These functional T_R elements have also been shown to serve as substrates for yeast TFIID in a reconstituted system with purified mammalian transcription factors in vitro (16). By assaying transcription of these templates, we tested whether templates containing nonconsensus T_R sequences are transcribed in the yeast nuclear extract when they are in slightly different positions relative to the start of transcription and are surrounded by different flanking sequences. Compared with the *gal-his3* template containing the wild-type T_R sequence (CTATAAAGTAAT GTG; template Sc3801), these templates contained the sequences ATTATCATTTAATTAC (template Sc3823), TA TATAAATTTTCCT (template Sc3824), and CGCCGCCTA TTTAATCC (template Sc3827) (Fig. 1). Reactions were performed with addition of GAL4(1-147) or GAL4(1-147)-B17 as described above. Relative to the wild-type control, all three templates showed efficient basal and induced transcription (Fig. 6); template Sc3823, in fact, showed higher transcription levels than the wild-type, consistent with previous in vivo and in vitro results (16). Therefore, we conclude on

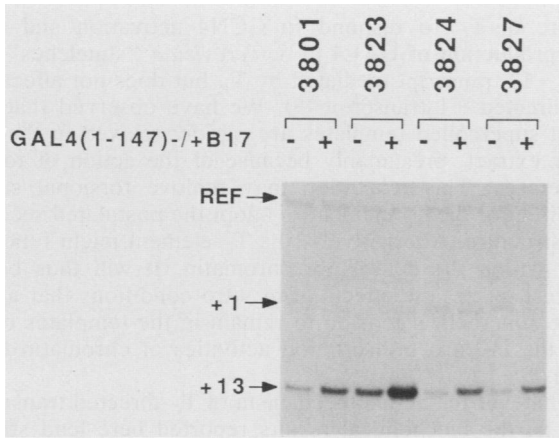

FIG. 6. Analysis of nonconsensus T_R derivatives with altered position and flanking sequences. Transcription reactions were performed as described in the legend to Fig. 5. The mobilities of the primer extension products corresponding to the +1 and +13 transcripts from the wild-type template and the +13 transcript from the reference (REF) template are indicated.

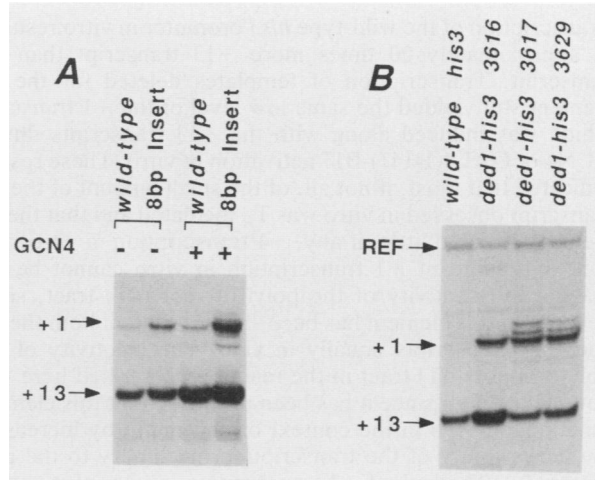

FIG. 7. Efficient utilization of the *his3* +1 initiation site in combination with a T_R element. (A) Transcription reactions contained 80 fmol of the wild-type template (YIp55-Sc2812) or YRp14-Sc2857, which contains an 8-bp *Eco*RI linker insertion at position −35. Reactions were performed under standard conditions or with 1,600 fmol of GCN4 protein. The mobilities of the primer extension products corresponding to the +1 and +13 transcripts are indicated. (B) Transcription reactions contained 35 fmol of the reference (REF) template (pUC19-Sc3784) and 80 fmol of the wild-type template (YIp55-Sc2812) or 80 fmol of the indicated *ded1-his3* template. The T_R element in the wild-type *his3* promoter is located 45 bp upstream of the +1 initiation site; the hybrid promoters position the *ded1* TATA element 69 bp (Sc3616), 82 bp (Sc3629), or 93 bp (Sc3617) upstream of the +1 site. The mobilities of the primer extension products corresponding to the +1 and +13 transcripts from the wild-type or *ded1-his3* template and the +13 transcript from the reference template are indicated.

the basis of the combined results of the T_R analysis that the in vitro system accurately reproduces TFIID-dependent transcription.

Efficient utilization of the +1 initiation site in combination with a T_R element. The disparity in the levels of the +1 and +13 transcripts seen with the wild-type *his3* template in vitro might be due to lack of function of the T_C element. However, this result might also be due to the fact that the +1 site is an inherently poor initiation site in vitro. To address this possibility, we examined the transcription of templates in which the distance between a T_R element and the *his3* initiation region was increased relative to the distance between these two elements in the wild-type *his3* promoter. It has previously been shown that such a situation in vivo allows T_R-directed transcription to initiate at the +1 site. This occurs because the strong preference for the +13 site in T_R-directed transcription of the wild-type promoter is not due to an inherent property of the +13 initiator but rather is due to the fact that the +1 site is too close to the T_R element (3, 20).

Initially, we tested the basal and GCN4-activated transcription of a template containing an insertion of an 8-bp *Eco*RI linker at position −35, resulting in an increase of the distance between the T_R element and the +1 site by 8 bp. The increased distance between the T_R element and the +1 site resulted in an increase in +1 transcription in vitro as it did in vivo (Fig. 7A; 20). We also examined the transcription of three templates containing a yeast *ded1-his3* hybrid promoter, in which a region containing the upstream and TATA (TATAAA) elements of the *ded1* promoter is fused to the *his3* initiation region (3). Compared with the wild-type *his3* gene, in which the T_R element is located 45 bp from the +1 initiation site, the hybrid promoters position the *ded1* TATA element 69 bp (template Sc3616), 82 bp (template Sc3629), or 93 bp (template Sc3617) upstream of the +1 site. Transcription of these templates in vitro showed efficient utilization of the +1 initiation site (Fig. 7B). The overall level of transcription of these templates was about three- to fivefold greater than that of a wild-type *his3* template (lane 1), consistent with previous in vivo results (3). Moreover, as the TATA element is moved to position −82 or −93, additional transcripts with start sites just upstream of +1 are produced in vitro as they are in vivo (3). These results indicate that the +1 initiation site can be used in the in vitro system, and hence, the failure of efficient +1 transcription from the wild-type *his3* promoter is most easily explained by inactivity of the T_C element in vitro.

DISCUSSION

Accurate reproduction of T_R-mediated *his3* transcription in vitro. We have shown that the yeast in vitro transcription system, using unfractionated nuclear extracts, accurately reproduces *his3* transcription events that are mediated by the T_R TATA element, a known binding site for transcription factor TFIID (16; C. R. Wobbe and K. Struhl, submitted for publication). These include (i) accurate transcription initiation with a strong preference for the +13 site over the +1 site, (ii) transcriptional activation by GCN4 that depends on the acidic activation domain, and (iii) transcriptional activation by GAL4(1-147)-B17 of *gal-his3* hybrid promoters containing various derivatives of the T_R element. The T_R elements tested in vitro included point mutations with partial or severe transcriptional defects, as well as nonconsensus elements with changes in the flanking sequences or the relative position of the element with respect to the transcriptional start site. The relative basal and induced transcriptional activities of the T_R elements tested in vitro correlated well with their known transcriptional activities in vivo. Thus, we conclude that the in vitro conditions described here do not impose any unusual constraints on T_R function but rather allow faithful reproduction of TFIID-dependent transcription.

Differential activities of the T_C and T_R elements in vitro.

Transcription of the wild-type *his3* promoter in vitro resulted in approximately 20 times more +13 transcript than +1 transcript. Transcription of templates deleted for the T_C element still yielded the same low level of the +1 transcript which was induced along with the +13 transcripts during GCN4 or GAL4(1-147)-B17 activation in vitro. These results indicated that most, if not all, of the small amount of the +1 transcript observed in vitro was T_R mediated and that the T_C element directed little, if any, +1 transcription in vitro. The lack of significant +1 transcription in vitro cannot be explained by inactivity of the poly(dA)-poly(dT) tract, since deletion of this element has been shown to affect both the +1 and +13 transcripts equally in vivo. The inactivity of the poly(dA)-poly(dT) tract in the reactions described here was not unexpected, since it has been proposed that this element functions in vivo in the context of chromatin by increasing the accessibility of the transcription machinery to the promoter (19). The lack of +1 transcription in vitro also cannot be explained by an inherent inability of RNA polymerase II to initiate efficiently at this site in vitro, since efficient +1 transcription was observed with templates containing a T_R element positioned further upstream from the initiation region. Thus, the results indicate that the lack of +1 transcription in vitro is due to inactivity of the T_C element under conditions in which all known TFIID-dependent transcription events mediated by the T_R element are accurately reproduced.

Implications for mechanisms of *his3* transcription. The differential activity of the T_C and T_R elements in vitro lends biochemical support to the idea that these elements use different mechanisms in promoting transcription. Since the T_R element is a known binding site for TFIID, the results described here support the view that TFIID does not bind the T_C element. However, it remains possible that TFIID binds to the T_C element, but in an unusual and fundamentally different manner than it does to a traditional TATA sequence, such that (i) the bound TFIID is unresponsive to transcriptional activation by GCN4 or GAL4 and (ii) TFIID fails to bind to the T_C element and promote transcription under the in vitro conditions used.

Another possible explanation for the lack of in vitro T_C function is that an unidentified transcription factor that binds to the T_C element is preferentially lost in the preparation of the nuclear extract or is inactive under the in vitro assay conditions used. We think that this possibility is made less likely by results of recent mutagenesis experiments of the T_C element. These have demonstrated that although the T_C element cannot be replaced by random sequences and thus is a sequence-dependent element, the sequence requirements for in vivo T_C function are quite complex, with many T_C derivatives containing multiple point mutations still having function (Mahadevan and Struhl, unpublished data). The results suggest that the T_C element may not be a binding site for a transcription factor (TFIID or otherwise) but rather may play a structural role in promoting transcription by adopting some secondary structure in vivo.

If the T_C element functions via an altered secondary structure in vivo, how might this structure promote transcription? One possibility is that localized torsional stress near the *his3* promoter in vivo causes the T_C element to unwind partially or adopt some other secondary structure. Such a structure might then facilitate assembly of a transcription initiation complex, perhaps circumventing the requirement that TFIID bind to the template. If TFIID is the target for the acidic activation domains of GCN4 and GAL4, such a mechanism for T_C function could explain both the failure of T_C to respond to GCN4 activation and why overproduction of GAL4 in *S. cerevisiae* "squelches" the *his3* +13 transcript mediated by T_R but does not affect the T_C-directed +1 transcript (5). We have observed that the input supercoiled templates are rapidly relaxed in the nuclear extract, presumably because of the action of topoisomerases. This relaxation may remove torsional strain required for the T_C element to adopt the postulated secondary structure. Alternatively, the T_C element might function only within the context of chromatin. It will thus be of interest to test the effects of in vitro conditions that allow some superhelical tension to remain in the templates or to test the in vitro transcription activities of chromatin templates.

Whatever the actual mechanism of T_C-directed transcription is, the biochemical results reported here lend strong support to the idea that it is distinct from that directed by the traditional TATA element T_R. Establishing the conditions that allow for T_C-directed +1 transcription in vitro will prove invaluable in gaining a better understanding of how these two elements differ in their mechanisms of promoting transcription.

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