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Identification of *cis*- and *trans*-acting elements involved in the expression of cold shock-inducible *TIP1* gene of yeast *Saccharomyces cerevisiae*

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ABSTRACT

Northern blot hybridization analysis of a series of 5′ end, 3′ end and internal deletions has revealed that at least four different regions are involved in the regulation of the expression of *TIP1*, a cold shock-inducible gene of *Saccharomyces cerevisiae*. One of these four regions has negative effect on the expression of the *TIP1* gene, while the others are responsible for the activation and cold shock-induction of the gene. A fragment involved in the cold-shock induction of *TIP1* was used as a probe in gel retardation assays to identify the cold shock-factor. The cold shock-factor could be detected in cells grown at 30°C as well as 10°C, but both the amount of the factor and its affinity to DNA were found to increase 2–3-fold after cold shock. In addition, another factor was found to bind just upstream of the cold shock element, in a region where a transcriptional activator was predicted to function by Northern blot hybridization analysis. The amount of this activating factor and its affinity for DNA was not affected by temperature. Implications of our data on possible mechanisms of transcriptional regulation of the *TIP1* gene by cold shock are discussed.

INTRODUCTION

During the past decade a great amount of information has been compiled concerning proteins that are induced after heat shock and the factors involved in the expression of these proteins in prokaryotes, lower eukaryotes and higher organisms. The function of several heat shock proteins and the mechanism of the regulation of their expression is well known (1). More recently, characterization of proteins induced after down shifting growth temperature and the regulation of the cold shock response was studied, especially in *Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae* (see ref.2 for review). In *E.coli*, thirteen specific proteins have been found to be induced upon shifting the temperature from 37°C to 10°C (3). The gene for the major cold shock protein, *cspA*, has been cloned and characterized (4) and the *cis*- and *trans*-acting elements that regulate the expression of this gene have been analyzed (5). CspA has also been reported to be a DNA-binding protein involved in the expression of H-NS (6) and the A subunit of DNA gyrase (7), two proteins of the cold shock regulon of *E.coli*. In *B.subtilis* a homolog of CspA, CspB, has been reported. This protein is also induced severalfold by cold shock and a strain with a deletion of this gene shows reduced viability at freezing temperatures (8). The three dimensional structure of CspB has been recently determined, and it was found that this protein preferentially binds single-stranded DNA (9, 10).

In *S.cerevisiae* several genes have been found by differential hybridization to increase their levels of expression after shifting the temperature from 30°C to 10°C (11) and two of them have been further characterized. One of these genes, *NSR1*, shows homology with human nucleolin and is involved in pre-rRNA processing and ribosome biosynthesis. Its level of expression increases 3–4-fold after cold shock (12, 13). *TIP1* is another gene whose expression is induced 6–8-fold after cold shock at 10°C. This gene is also induced by heat shock (11). The function of *TIP1* is unknown since a disruption mutation shows no detectable phenotype. This gene encodes a protein rich in serine and alanine and belongs to a multigene family of 3 or 4 members (11). It was shown that the TIP1 protein is heavily glycosylated and localized in membrane and two other genes of this family, *TIR1* and *TIR2* havé also been cloned and characterized (Kowalski, Kondo and Inouye, manuscript in preparation). These two genes, whose functions are presently unknown, are also induced by cold shock. One of them, *TIR1* (previously designated *SRP1*), has also been found to be induced by glucose (14, 15).

In this report we have focused our attention on the expression

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of *TIP1*, the gene that shows the highest induction by temperature shift, and identified several *cis*- and *trans*-acting elements involved in the cold shock induction of this gene. A model for the mechanisms of the cold shock induction of *TIP1* is proposed.

MATERIALS AND METHODS

Strains and growth conditions

S.cerevisiae S288C (*a mal gal2*) was used to prepare extracts for gel retardation assays and KN052, a *TIP1* disruption mutant derived from strain TD4 (*a his4 ura3 trp1 leu2 ade8 gal2 can^r*) (11), was used for transformation with a series of plasmids containing 5′ end, 3′ end and internal deletions, and also for analysis of *TIP1* expression by Northern blot hybridization. Transformation was carried out by the method of Ito *et al.* (16). Yeast culture media was prepared as described by Rose *et al.* (17) and cells were incubated at 30°C unless otherwise indicated. *E.coli* CL83 (*ara* Δ(lac-proAB) rpsL ϕ80 lacZΔM15 recA56) was used as a host for transformation. Colonies were selected on LB plates containing 50 μg/μl ampicillin. *E.coli* cultures were incubated at 37°C.

Construction of 5′ end, 3′ end and internal deletions

For the construction of a series of deletions, the 2.5-kb-*Hin*dIII−*Xho*I fragment which contains the entire *TIP1* gene and 1.15-kb 5′-untranslated region and 0.62-kb 3′-untranslated region (see Fig.1) was cloned into the *Hin*dIII and *Sal*I sites of the centromere plasmid pRS315 (18). To construct a set of nested deletions extending downstream from the *Hin*dIII site, the resultant plasmid (5′0; see Fig. 1) was linearized with *Hin*dIII and *Pst*I, digested with exonuclease III, treated with mung bean nuclease and then with Klenow as described by Sambrook *et al.* (19). The resulting mixture was ligated and used for transformation of *E.coli*. The size of deletion of the plasmids thus obtained was checked by digestion with *Xba*I and later the exact position of deletion was determined by DNA sequencing using the dideoxynucleotide method (20). Twelve deletions were selected and designated 5′1 to 5′12 from shorter to longer.

The series of 3′ end deletions were constructed starting at the *Eco*NI site which is located 180-bp upstream of the start codon. Plasmid 5′0 was digested with *Eco*NI and *Kpn*I and the 2.6-kb fragment containing the upstream region of *TIP1* plus part of *LEU2* gene was purified and treated with exonuclease III, mung bean nuclease and Klenow as above to originate the series of nested deletions. To avoid insertion of this fragment in two orientations (see the map of vector of pRS315; 18), the mixture of fragments was digested with *Cla*I, which cuts inside the *LEU2* gene. The mixture of fragments was then separated from the small fragment released after the digestion by electrophoresis on a 5% polyacrylamide gel. Plasmid 5′0 was also digested with *Cla*I and *Pvu*I and the 2.75-kb fragment containing the remainder of the *LEU2* gene and part of the *amp* gene was purified. Plasmid 5′0 was also digested with *Eco*NI and the ends were made blunt with T4 DNA polymerase. This fragment was then digested with *Pvu*I and the 3.1-kb fragment containing the rest of the *amp* gene and the entire *TIP1* gene plus a portion of upstream region was purified. The two purified fragments the mixture of deleted fragments were then ligated. After transformation of *E.coli* and analysis of the plasmids, the size of deletion was checked by digestion with *Xba*I. Several plasmids containing deletions of the desired sizes were selected and the exact position of the deletion was determined by DNA sequencing using a synthetic oligonucleotide as a primer. Eleven deletions were selected and designated 3′1 to 3′11 from shorter to longer (see Fig. 1). In the case of deletions 3′1 to 3′10 the 2.3-kb-*Hin*dIII fragment was also inserted at the *Hin*dIII site of each plasmid to obtain plasmids with a longer upstream region (see Fig. 1). These new plasmids were designated 3′1H to 3′10H, where 3′1 and 3′1H contain the same size deletion, 3′2 the same size deletion as 3′2H and so on.

Two different internal deletions have also been constructed using plasmid 5′0 as the starting material. This plasmid was digested with *Hin*dIII and *Nco*I and the two fragments obtained were separated and purified in a 0.8% agarose gel. The large fragment (7 Kb) was saved and the small one (1.43 Kb) was used for the construction of the internal deletions. For the construction of plasmid 5′0ES, the 1.43 kb *Hin*dIII−*Nco*I fragment was digested with *Eco*RV and *Sca*I; the 75-bp *Hin*dIII−*Eco*RV and the 1070-bp *Sca*I−*Nco*I fragments were then purified and ligated to the 7-kb *Hin*dIII−*Nco*I fragment. For the construction of the plasmid 5′0S, the 1.43-kb *Hin*dIII−*Nco*I fragment was digested with *Sau*3AI and the 510-bp *Hin*dIII−*Sau*3AI and the 750-bp *Sau*3AI−*Nco*I fragments were each purified and ligated to the 7-kb *Hin*dIII−*Nco*I fragment. After construction of plasmid 5′0ES and 5′0S, they were digested with *Hin*dIII and the 2.3-kb *Hin*dIII fragment of the upstream region of *TIP1* gene (see Fig. 1) was inserted in both the right and the wrong orientation. The plasmids thus obtained have been designated as 1ES and 1S when the *Hin*dIII fragment was in the right orientation and as 1ESW and 1SW when the *Hin*dIII fragment was inserted in the opposite orientation.

Preparation of RNA and Northern blot hybridization analysis

After transformation of *S.cerevisiae* KN052 with the series of 5′ end, 3′ end and internal deletions, colonies were selected on SC plates lacking leucine. Colonies were then transferred to a fresh plate of SC medium lacking leucine and incubated at 30°C for 2 days. New colonies were then grown overnight in YPD medium. The cultures were diluted into 40 ml of fresh YPD medium to 7 Klett units and grown at 30°C while shaking to a cell density of 30−40 Klett units (A_{600} is approximately 1). A sample of 20 ml was then withdrawn and used to prepare RNA. The other 20 ml were transferred immediately to a water bath at 10°C and incubated at this temperature for 3 h while shaking. The remaining culture was then withdrawn to prepare RNA. All the samples collected at either 30°C or 10°C were centrifuged at 2000 rpm at 4°C for 5 min. The cells were resuspended in 100μl of LETS buffer (0.1M LiCl, 10 mM EDTA, 10 mM Tris−HCl [pH 7.4], 0.2% SDS) and transferred to an eppendorf tube containing glass beads and 120 μl of phenol equilibrated in LETS buffer. The cells were broken by vortex for 3 min in 30-s bursts with 30 s intervals on ice. Then 200 μl of LETS buffer were added to each tube and the aqueous phase was separated from phenol and glass beads by centrifugation. The aqueous phase was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and RNA was precipitated for 3 h at −20°C after addition of 40 μl of 5M LiCl and 1ml of ethanol. The pellet was then washed in 1 ml of 70% ethanol and resuspended in 50 μl of water. The concentration of RNA was determined by absorbance at 260 nm. 10 μg of RNA was then loaded on a 1.3% agarose/formaldehyde gel. After electrophoresis the gel was washed twice for 20 min each in 10×SSC (1×SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and the RNA was transferred to a Hybond N+ membrane (Amersham) overnight

in 10×SSC. After blotting, the RNA was fixed to the membrane with 0.05 N NaOH. Hybridization was carried out in 6×SSPE (1×SSPE: 0.15 M NaCl, 1 mM EDTA, 10 mM sodium phosphate, pH 7.4), 5×Denhardt's solution (1×Denhardt's solution: 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin) and 0.2% SDS at 65°C overnight. As a probe a 480-bp *Xba*I–*Pvu*II fragment in the coding region of *TIP1* gene (11) and a 1.2-kb fragment of *ACT1* gene (13) were used. The probes were labeled with the Megaprime DNA labeling system (Amersham) and [α-^{32}P]dCTP (3000 Ci/ml Amersham). After hybridization, the filters were washed twice for 1 h each in 1×SSC, 0.1%SDS at 65°C and exposed to an X-ray film. The intensity of bands was measured by the computing densitometer (model 200A, Molecular Dynamics).

Preparation of crude extracts at 10°C and 30°C

S.cerevisiae S288C was grown overnight at 30°C while shaking in YPD medium. The culture was diluted to 7 Klett units into two flasks containing 100 ml of fresh YPD medium each. When the cell density reached approximately 40 Klett units the cells of one flask were collected by centrifugation at room temperature to prepare crude extracts at 30°C, and the other flask was transferred to a 10°C water bath and incubated with shaking for 2 h. The cells from the 10°C culture were collected by centrifugation at 10°C and all the following steps were carried out at either 4°C or on ice. The cells of both 30°C and 10°C cultures were washed with 2 ml of extraction buffer (100 mM Tris–HCl [pH 8.0], 1 mM EDTA, 10% glycerol, 0.4 M ammonium sulfate, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride [PMSF], 2 mM benzamidine, 1μg/ml leupeptin and 1 μg/ml pepstatin A). The cells were then resuspended in 1 ml of extraction buffer and glass beads were added to the meniscus. The suspension was vortexed for 10 min in 1-min bursts with 1 min intervals on ice. Cell debris was removed by centrifugation, the supernatant was collected and the glass beads were washed twice with 2 ml of extraction buffer. The crude extracts were then centrifuged at 100,000×g for 1 h to remove the membrane fraction. Proteins were precipitated from the supernatant by adding an equal volume of 100% ammonium sulfate prepared in 50 mM Tris–HCl (pH 8.0) and 0.5 mM EDTA. After 30 min on ice the precipitate was obtained by centrifugation at 8,000 rpm for 10 min and dissolved in 200 μl of dialysis buffer (50 mM Tris–HCl [pH 8.0], 20% glycerol, 50 mM NaCl, 0.1 mM PMSF, 0.2 mM EDTA, 10 mM 2-mercaptoethanol, 1 μg/ml leupeptin and 1 μg/ml pepstatin A). Dialysis was carried out overnight at 4°C against the same buffer. Protein concentration was estimated by the BioRad protein assay.

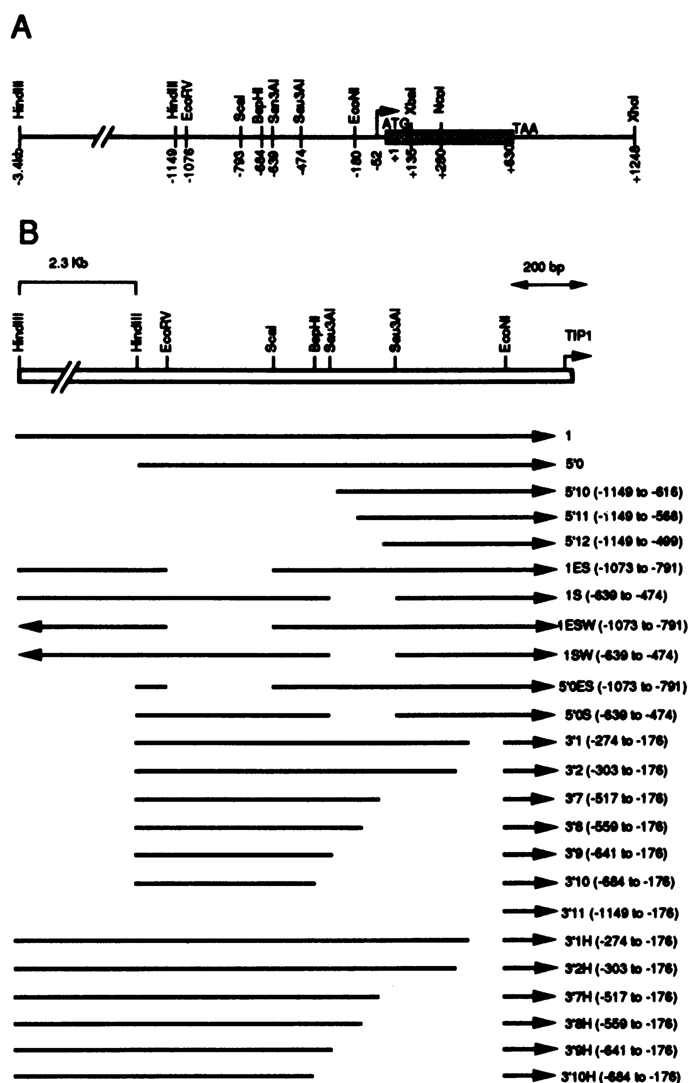

Figure 1. (A). Schematic diagram of the yeast *TIP1* gene showing the locations of the restriction endonuclease cleavage sites used in the construction of various plasmid. The major transcriptional start site (11) is indicated by an arrow. **(B)** Schematic diagram of the plasmids used in the study. Lines indicate the portions of DNA contained in each plasmid. Deleted sequences are represented by open areas and the endpoints are indicated in parentheses. Plasmids 1ESW and 1SW are identical to 1ES and 1S, respectively, but the 2.3-kb *Hind*III fragment has been inserted in the wrong orientation; an arrow on the left direction has been drawn in these two plasmids to indicate this fact. Plamids 3'1H, 3'2H, 3'7H, 3'8H, 3'9H and 3'10H have the same size of deletion as plasmids 3'1, 3'2, 3'7, 3'8, 3'9 and 3'10, respectively, but they contain the 2.3 kb-*Hind* III fragment.

Gel retardation assay

Three different fragments were used for gel retardation assay, the wild-type 165-bp *Sau*3AI fragment, the 99-bp *Sau*3AI obtained from deletion 5'11 (one *Sau*3AI site was provided by the *Bam*HI site of the polylinker and the other by the upstream region of *TIP1*) and the 105-bp *Sau*3AI–*Bsp*1286I fragment (see Fig. 3). The fragments were labeled with [α-^{32}P]dGTP (3000 Ci/ml Amersham) using Klenow at room temperature for 10 min. Free nucleotides were removed by ethanol precipitation and DNA was resuspended in TE buffer (10 mM Tris–HCl [pH 7.5], 1 mM EDTA) at a final concentration of 1 ng/μl. For the reaction, 10 μl of dialysis buffer (see above), 1 μl of 60 mM $MgCl_2$, 1 μl of 1M NaCl and 1 μl of 3 μg/μl poly(dI·3dC)·3poly(dI·3dC) were incubated with 10 μg of protein on ice for 10 min in a final volume of 19 μl. In some experiments, competitors were added at this stage at 100-fold molar excess of the labeled fragment. Finally, 1 μl of radiolabeled probe was added and the tubes were incubated at either 30°C or 10°C for 15 min. After the addition of 5 μl of tracking dye (0.2% bromophenol blue, 0.2% xylene cyanol FF and 25% glycerol), the whole sample was loaded immediately on a 5% polyacrylamide gel which had been prerun at 150 volts for 2 h at room temperature. The gel was electrophoresed at 150 volts at room temperature until the bromophenol blue reached the bottom. The gel was then dried and subjected to autoradiography.

RESULTS

Analysis of the series of 5′ end, 3′ end and internal deletions

TIP1 is a *S.cerevisiae* gene that is expressed at a low level when the cells are incubated at 30°C. Upon a temperature shift from 30°C to 10°C its level of expression increases about 6−8-fold (11). In order to investigate the *cis*-elements involved in the expression and cold shock induction of this gene, a series of 5′ end, 3′ end and internal deletions were created (Fig. 1B) and the levels of *TIP1* mRNA before and after cold shock were analyzed for each deletion by Northern blot hybridization. A restriction map of the region containing the *TIP1* gene as well as the location and size of the deletions used in this study are shown in Fig. 1. The *TIP1* genes bearing the deletions were cloned in the centromere plasmid pRS315 (18) and transformed into *tip1* deletion strain KN052, in which the open reading frame of *TIP1* gene downstream of the *Xba*I site (see Fig. 1A) is replaced with the plasmid sequence used for the disruption (11). KN052 harboring the plasmids bearing the deletions shown in Fig. 1 was subjected to cold shock and RNA was prepared from cultures before and after the temperature shift as described in Materials and Methods. The results obtained after Northern blot hybridization analysis of all the samples are shown in Fig. 2. Plasmid 1, which possesses a 3.45-kb upstream region, seems to contain all of the sequences required for the expression and cold shock induction of *TIP1*. The expression of this gene increases about 6-fold after cold shock (compare lanes 1 and 2 in Fig. 2), a level of induction similar to that reported for the wild-type strain (11). When the 2.3-kb *Hin*dIII fragment is removed and only 1.15-kb upstream region remains (plasmid 5′0), the amount of mRNA at both 30°C and 10°C increases considerably compared with that from plasmid 1 (compare lanes 1 with 3 and 2 with 4). However, the cold shock induction obtained from plasmid 5′0 is only 2−3-fold (compare lanes 3 and 4). This result indicates that the 2.3-kb *Hin*dIII fragment represses the expression of *TIP1* at 30°C and 10°C. Since the 1.15-kb upstream region is still able to induce the expression of *TIP1* severalfold after cold shock we further investigated the regions within this fragment which are involved in the cold shock response. A series of nested deletions originating from the 5′end of plasmid 5′0 were created as described in Materials and Methods. When the expression of the *TIP1* gene was analyzed from all of the deletions it was found that the region from −1149 to −615 bases upstream of the initiation codon ATG could be deleted with no significant effect in the expression of this gene (compare lanes 3 and 4 with 5 and 6). The levels of transcript detected when deletions 5′1 through 5′9 were used are not shown since the expression pattern was similar to those from plasmid 5′0 and 5′10. However, when 48 bp more are deleted (plasmid 5′11) a reduction in the level of mRNA can be observed compared with deletion 5′10 (compare lanes 5 and 7) indicating that the sequence contained between −615 and −567 is able to activate transcription, although cold shock induction is still observed (compare lanes 7 and 8). Deletion of the next 69 bp (plasmid 5′12) causes a decrease in cold shock induction (compare lanes 9 and 10). Note that the amount of the actin transcript in lane 10 is greater than in lane 9. A comparison of the amount of *TIP1* mRNA at 30°C and 10°C in several gels after normalization to the amount of actin transcript has revealed that the cold shock induction obtained with deletion 5′12 is about 1−1.5-fold, while the induction observed with plasmid 5′11 was 2−4-fold. This result indicates that the region encompassed

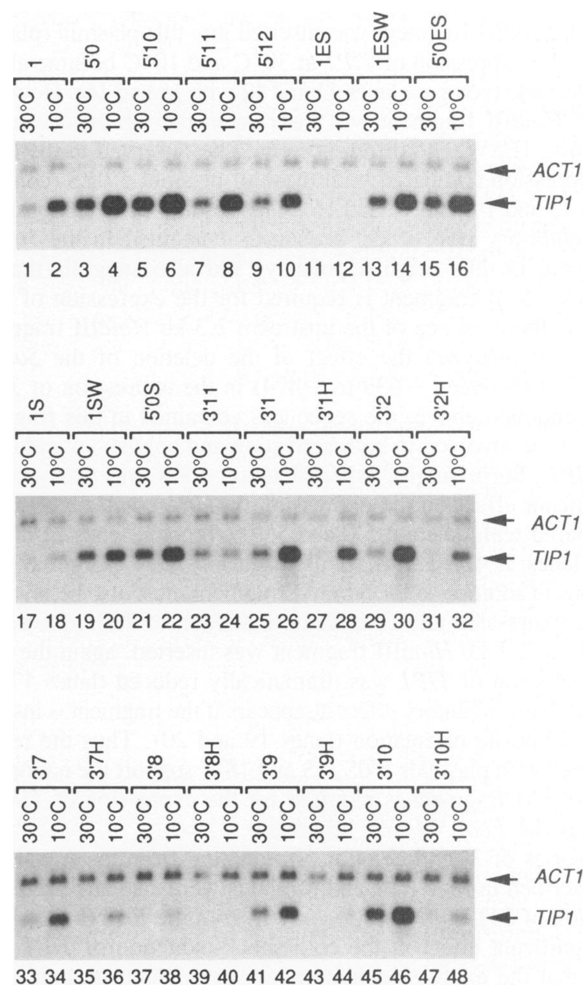

Figure 2. Northern blot hybridization analysis of the series of 5′ end, 3′ end, and internal deletions. RNA was prepared from *S.cerevisiae* KN052, harboring the plasmids indicated on the top of each panel before and after cold shock. 10 μg of total RNA was loaded on each lane. The bands corresponding to *ACT1* and *TIP1* transcripts are indicated with arrows.

between deletions 5′11 and 5′12 (between −567 and −499) is involved in the cold shock induction of *TIP1*. The analysis of the 5′ end deletions thus showed the existence of 3 regions involved in the expression and cold shock induction of the *TIP1* gene. One located in the 2.3-kb *Hin*dIII fragment involved in general repression of transcription; another one located in the 48-bp fragment encompassed between deletions 5′10 and 5′11 (between −615 and −567) involved in activation of transcription; and a third one involved in cold shock located in the 69-bp fragment encompassed by deletions 5′11 and 5′12 (between −567 and −499). The latter two regions reside in a 165-bp-*Sau*3AI fragment (see Fig. 1).

In order to investigate whether these three regions are the only ones involved in the expression of *TIP1* and also to further clarify their roles, a few internal deletions were created. When the *Eco*RV−*Sca*I fragment was deleted (plasmid 5′0ES; see Fig. 1) the levels of expression at both 30°C and 10°C were almost identical to those found with plasmids 5′0 and 5′10 (compare lanes 15 and 16 with lanes 3, 4, 5 and 6 in Fig. 2). This result was expected because the DNA sequences of plasmid 5′0ES are

quite similar to those of plasmid 5'10 (see Fig. 1). When the 2.3-kb *Hin*dIII fragment was inserted into this plasmid (plasmid 1ES), the expression of *TIP1* at 30°C and 10°C became almost undetectable (compare lanes 11 and 12 with 15 and 16). However, if the *Hin*dIII fragment was inserted in the wrong orientation (plasmid 1ESW), no difference could be observed in the levels of expression compared with those of plasmid 5'0ES (compare lanes 13 and 14 with 15 and 16). This result clearly demonstrates the inhibitory role of the sequences contained in the *Hin*dIII fragment in thus original polarity, and also suggests that the *Eco*RV−*Sca*I fragment is required for the expression of *TIP1* gene in the presence of the upstream 2.3-kb *Hin*dIII fragment. Next, we analyzed the effect of the deletion of the *Sau*3AI fragment (between −639 to −474) in the expression of *TIP1*. As mentioned above, the sequences contained in this fragment seem to be involved in both activation and cold shock induction of *TIP1*. Surprisingly, when deletion 5'0S was analyzed no significant effect on the expression of *TIP1* could be observed compared with deletion 5'0 and 5'10 (compare lanes 21 and 22 with lanes 3, 4, 5 and 6). This result indicates that some other regions in addition to the *Sau*3AI fragment must also be involved in the expression and cold shock induction of *TIP1*. However, when the 2.3-kb *Hin*dIII fragment was inserted, again the level of expression of *TIP1* was dramatically reduced (lanes 17 and 18), and this inhibitory effect disappears if the fragment is inserted in the opposite orientation (lanes 19 and 20). Thus the results obtained with plasmids 5'0S, 1S and 1SW support the notion that the *Sau*3AI fragment is required for the expression of *TIP1* as well as the *Eco*RV−*Sca*I fragment.

A series of 3' end deletions originating from the *Eco*NI site as described in Materials and Methods were also analyzed. The deletion of 341 bp (−517 to −176) from the *Eco*NI site shows no significant effect in the cold shock-induction of *TIP1* gene such that the expression patterns observed with plasmids 3'1, 3'2 and 3'7 (lanes 25, 26, and 29, 30, and 33, 34) are almost identical to each other and similar to that obtained with plasmid 5'0 (lanes 3 and 4). The expression patterns observed with these plasmids containing the 2.3-kb *Hin*dIII fragment (3'1H, 3'2H, 3'7H and 1) are also similar with each other (lanes 1, 2, and 27, 28, and 31, 32, and 35, 36 in Fig. 2). With the plasmids 3'7 and 3'7H, a slight decrease in the level of mRNA was observed, however no great effect could be seen on cold shock-induction. Note that the deletion contained on plasmid 3'7 is up to 43 bp upstream to the *Sau*3AI site at −474. As seen with plasmid 3'8, the deletion of 42 bp causes barely detectable basal level of *TIP1* mRNA and decreased cold shock induction (lanes 37 and 38) When the 2.3-kb *Hin*dIII fragment is inserted (plasmid 3'8H; lanes 39 and 40) induction is barely detectable at 30 or 10°C. It is noteworthy that the position of deletion 3'8 (−559) is almost identical to the position of deletion 5'11 (−567). Therefore the region involved in cold shock that was predicted by the 5' end deletions is also indicated as being involved in cold shock by deletion 3'8. In deletion 3'9 the entire *Sau*3AI fragment has been removed and therefore not only the region involved in cold shock but also the region involved in activation of transcription were removed in this plasmid (see Fig. 1B). In this case, expression of the *TIP1* gene and cold shock induction can be clearly detected again (lanes 41 and 42). However, the introduction of the *Hin*dIII fragment again makes the detection of the mRNA almost undetectable (lanes 43 and 44). The behavior of this deletion is quite similar to that of plasmid 5'0S and 1S (compare lanes 41 and 42 with 21 and 22, and lanes 43 and 44 with 17 and 18). Deletion of another 43 bp (plasmid 3'10) allows complete recovery of the levels of expression detected with plasmid 5'0 (compare lanes 45 and 46 with lanes 3 and 4). However, the insertion of the *Hin*dIII fragment almost completely blocks the expression of *TIP1* at both 30°C and 10°C (compare plasmid 3'10H, lanes 47 and 48 with plasmid 1, lanes 1 and 2). Therefore, these results demonstrate again the inhibitory effect of the *Hin*dIII fragment and the role of the *Sau*3AI fragment in the activation of the expression of *TIP1*. However, since deletions 3'9 and 3'10 still allow cold shock-induction, some regions upstream of the position of this deletion must also be involved in cold shock. The fact that the deletion of the *Eco*RV−*Sca*I fragment abolishes the expression of *TIP1* when the *Hin*dIII fragment is present strongly suggests that the *Eco*RV−*Sca*I fragment is also involved in activation and cold shock-induction of *TIP1*. A plasmid containing the upstream region only up to the −176 *Eco*NI site (plasmid 3'11) shows no cold shock-induction at all, however the mRNA is clearly detectable (lanes 23 and 24), probably by transcription through the TATA box which is still present in this plasmid.

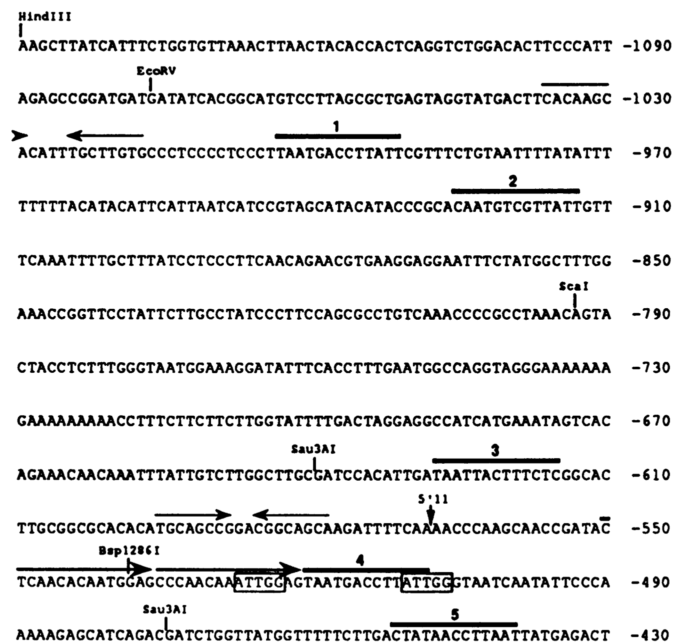

Figure 3. DNA sequence of the upstream region of the *TIP1* gene. The positions of the nucleotides shown here are relative to the translational start site. The major transcriptional start site is located at position −52 (11). The DNA sequence downstream of the second *Sau*3AI site has been already published (11). Several features have been indicated in the sequence. Thick bars (numbered 1−5) indicate a repetitive sequence. Two sets of thin arrows in opposite orientation indicate palindromic sequences. And two consecutive arrows with the same orientation indicate a direct repeat sequence. The sequences ATTGG recognized by Y-box binding factors and CspA, an *E.coli* cold shock-inducible protein, which appears twice in this region are boxed. The position corresponding to the 5' end of deletion 5'11 is marked with a vertical arrow.

The DNA sequence up to the *Hin*dIII site at −1149 containing those regions which are involved in the regulation of the expression of *TIP1* is shown in Fig. 3. The sequence downstream from the second *Sau*3AI site (−474) has already been published (11). A computer aided search for homology between the sequence of this 1.15-kb fragment and the upstream regions of the other two cold shock inducible genes whose sequences are

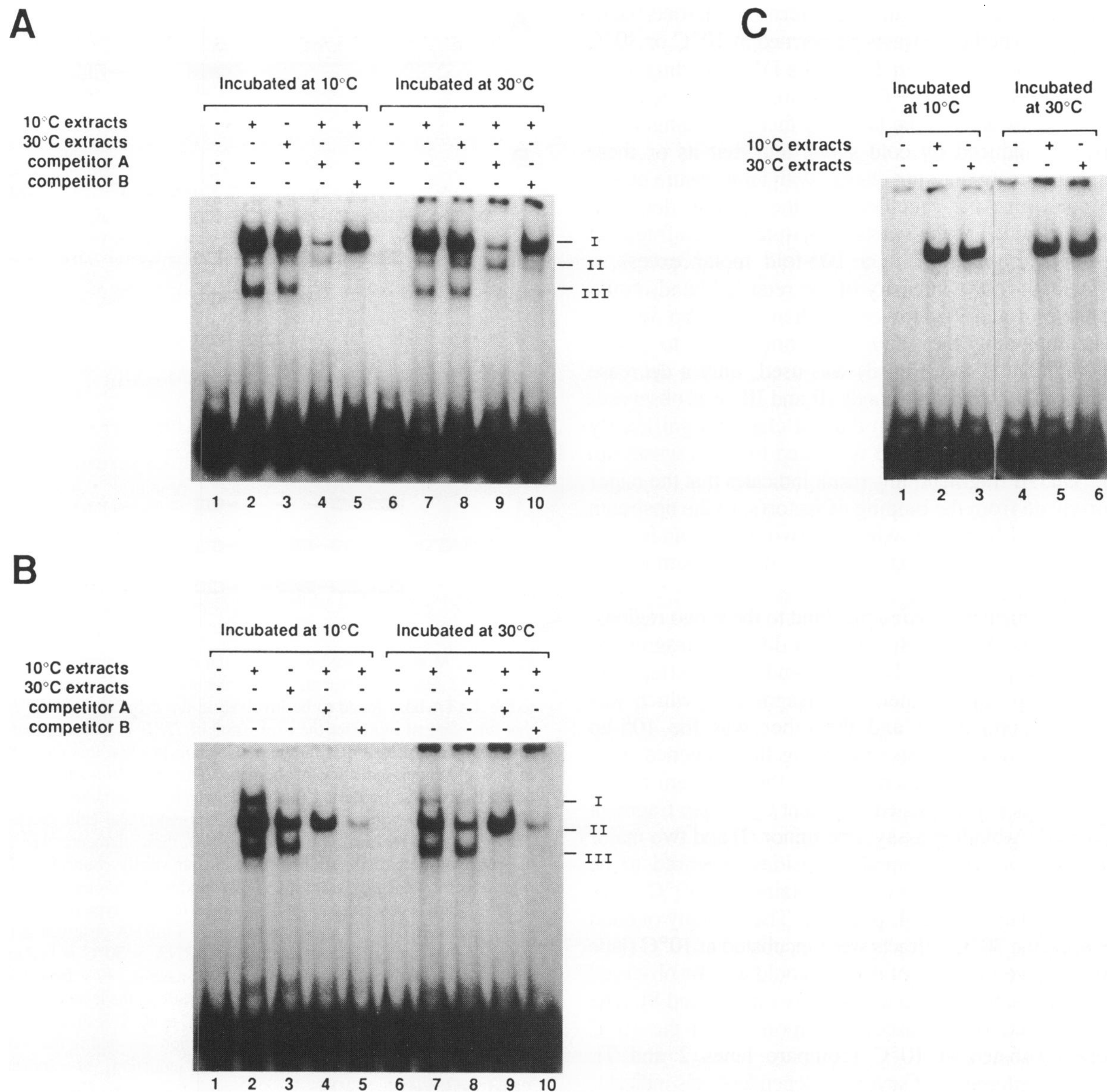

Figure 4. Detection of the factors that bind to the *Sau*3AI fragment by gel retardation assay. (**A**) The entire *Sau*3AI fragment (−639 to −474, fragment A) was used as a probe for detection of binding factor(s). Extracts prepared at either 10°C or 30°C were used in the reaction mixture (indicated by + or −) and incubated at either 10°C (left panel) or 30°C (right panel) as described in Materials and Methods. In some reactions, competitors were used (also indicated by + and −). After incubation the samples were loaded on a 5% polyacrylamide gel. The gel was then dried and subjected to autoradiography. (**B**) Gel retardation assay using the 99-bp *Sau*3AI fragment obtained from plasmid 5′11 (fragment B). The same competitors were used as in the experiment represented in panel A. (**C**) Gel retardation assay using the 105-bp *Sau*3AI−*Bsp*1286I fragment (fragment C) as a probe.

known, *NSR1* and *TIR1* (21, 13, 14), did not reveal in any significant similarity among these three genes, suggesting that they may be regulated in a different manner. The upstream region of *TIP1* gene also did not show significant homology with any other sequences deposited at GenBank. However, several interesting features can be observed in this region, most of them located in the *Sau*3AI fragment, which will be discussed below.

Identification of the factors that bind to the *Sau*3AI fragment

The Northern blot hybridization analysis of the series of deletions originated in the upstream region of *TIP1* gene indicated that two different regions located in the 165-bp *Sau*3AI fragment are involved in the activation and cold shock induction of this gene; the region located upstream is predicted to be involved in the activation of transcription, and the one located downstream is predicted to play a role in the cold shock induction of the gene. In order to identify the putative factors that may bind to these two regions, gel retardation assays were carried out using three different DNA fragments as probes. The results obtained are shown in Fig. 4. In panel A the entire 165-bp *Sau*3AI fragment containing the sequence from −639 to −474 (designated fragment A) was used for the DNA-binding assay. One major (I) and two minor bands (II and III) appeared to be retarded in the gel when the DNA was incubated at 30°C with extracts prepared from 30°C cultures (lane 8). No retarded band could be observed in the control sample where no extracts were added

(lanes 1 and 6). No difference in the pattern of retarded bands could be observed whether extracts performed at 10°C or 30°C were used or whether incubation during the DNA-binding assay was carried out at 10°C or 30°C (compare lanes 2, 3, 7 and 8). These results indicate that the factor or factors binding to this fragment are not induced by cold shock and that its or these affinity for this fragment does not change with temperature either. In order to determine the specificity of the binding detected, competitors were added to the reaction mixture. When fragment A was used as a competitor in a 100-fold molar excess, a significant decrease in the intensity of the retarded bands could be detected (lanes 4 and 9). However, when the 99-bp *Sau*3AI fragment encompassing the sequence from −567 to −474 (designated fragment B; see Fig. 3) was used, only a decrease in the intensity of the two minor bands (II and III) was observed; the intensity of the major band (I) did not change significantly (lanes 5 and 10). Since fragment B is located in the downstream region of the *Sau*3AI fragment, this result indicates that the major band must originate from the binding of factor(s) to the upstream region of the *Sau*3AI fragment, while the two minor bands must originate from the binding of factor(s) to the downstream region of this fragment.

In order to distinguish the factors that bind to these two regions, the 165-bp fragment A was split into two different fragments; both fragments are approximately 100-bp and they overlap with each other by 32 bp. One of them was fragment B which was used above as a competitor; and the other was the 105-bp *Sau*3AI−*Bsp*1286I fragment encompassing the sequence from −639 to −534 (see Fig. 3) which contains the upstream region of the *Sau*3AI fragment (designated fragment C). When fragment B was used for DNA-binding assay, one minor (I) and two major bands (II and III) of almost equal intensities appeared to be retarded in the gel when the extracts obtained at 30°C were incubated at 30°C (lane 8; Fig. 4, panel B). The intensity of band II increased when the 30°C extracts were incubated at 10°C (lane 3). An increase in the intensity of band II could also be observed when the 10°C extracts were used (compare lanes 7 and 8). The intensity of the band II increased even more when the 10°C extracts were incubated at 10°C (compare lanes 2 and 7). Quantitation by densitometry of several independent gels indicates that the band intensity obtained with extracts prepared from 10°C cultures and incubated at 10°C was 2−3-fold stronger than with extracts prepared from 30°C cultures and incubated at 30°C. The intensity of band I also increased when the incubation temperature used was 10°C or the extracts were obtained from 10°C cultures. No significant difference was observed with band III (compare lanes 2, 3, 7 and 8). These results indicate that the amount of the factor(s) binding to this region as well as its (their) affinity for the DNA increase at lower temperatures. When fragment A and fragment B were used as competitors it was found that fragment B competed with all three bands, as it was expected. However, fragment A competed well with bands I and III, but not with band II, which is the major band seen in the 10°C extracts that had been incubated at 10°C. The fact that the major retarded bands that appeared with fragment A (band I, panel A) and fragment B (band II, panel B) do not decrease in intensity when the other fragment is used as competitor indicates that the factors that cause these two bands are different and therefore bind to different positions on fragment A. Since all of fragment B is included in fragment A, the major band detected when fragment A was used as a probe (band I, panel A) must be due to factor(s) that bind to upstream of fragment B. However band II in panel

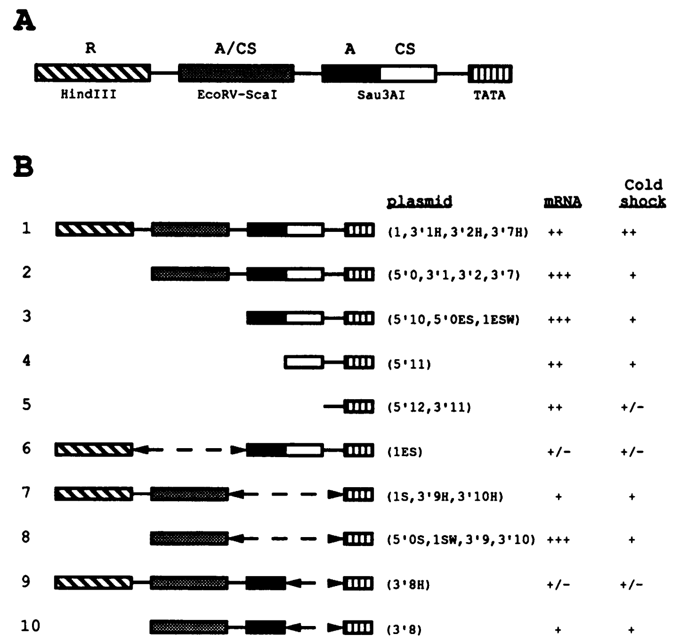

Figure 5. Model proposed to explain the expression and cold shock-induction of the *TIP1* gene. (A) Schematic representaion of the 4 different regions (in addition to the TATA box) found to be involved in the expression of *TIP1*. R indicates that this element represses the expression of *TIP1*. A indicates that this element activates the expression of the gene. CS indicates that this element is involved in the cold shock-induction of the gene. The restriction sites shown under the boxes indicate the bordering sites inside which the elements have been mapped. (B) Diagram showing the effect in the expression and cold shock-induction of *TIP1* gene after removal of one or several of these elements. The fragments on the right and left of the dashed lines are physically connected. The plasmids represented by each diagram are shown under the column plasmid. The level of *TIP1* mRNA observed at 30°C is shown for each type of construction under the column mRNA: + + indicates the level of mRNA observed in the wild-type strain, + + + indicates that the level of mRNA at 30°C is higher than in the wild-type strain, + indicates a lower level of mRNA, +/− indicates that mRNA is undetectable or barely detectable. The increase in the level of mRNA observed after shifting the temperature down from 30°C to 10°C is shown in the cold shock column: + + indicates the cold shock induction detected in the wild-type strain (6−8-fold induction), + indicates 2−4-fold induction, +/− indicates less than 2-fold induction.

B was not competed out by fragment A even though fragment B is contained in fragment A. These results can be explained if the factor(s) that bind to the upstream region of fragment A shows more affinity for this fragment than the ones that bind to the downstream portion and the binding of the upstream factor(s) somehow prevents the binding of the downstream factor(s). Note that the bands that originate from binding to the downstream portion (bands II and III, panel A) are weaker than the one originated by binding to the upstream portion (band I, panel A).

Next, we used fragment C for a DNA-binding assay in order to identify more directly the factor(s) that bind to the upstream region of the *Sau*3AI fragment. The results obtained are shown in the panel C of Fig. 4. In this case only one band appeared to be retarded. The intensity of which did not change by temperature, such that no significant difference could be observed in intensity between the assay carried out with 10°C extracts incubated at 10°C and the assay with 30°C extracts incubated at 30°C (compare lanes 2, 3, 5 and 6). This result is similar to that obtained with the major band retarded by fragment A (band I, panel A). This indicates that we are most likely detecting the

same factor(s) with both fragments A and C. In conclusion, two different regions of the *Sau*3AI fragment are recognized by factors and this leads to the appearance of retarded bands in DNA-binding assays. The factor(s) that bind to the upstream region do not seem to be affected by temperature, while those binding the downstream region are affected by cold shock. These results are in good agreement with the ones obtained with the series of deletions shown above.

DISCUSSION

In this report we have investigated the *cis*- and *trans*-acting elements involved in the regulation of the expression of *TIP1*, a cold shock inducible gene of *S.cerevisiae*. The Northern blot analysis of a series of deletions in the upstream region of this gene has revealed the existence of at least 4 different regions, in addition to the TATA box, required for the proper expression of *TIP1* at 30°C and after cold shock. A model which may explain the results obtained by Northern blot analysis is proposed in Fig. 5. One of the four regions is located in a 2.3-kb *Hin*dIII fragment, and possesses an inhibitory role. Another one is located in a 284-bp *Eco*RV−*Sca*I which seems to be involved both in activation and cold shock induction of this genes. The other two are located in a 165-bp *Sau*3AI fragment; the one located upstream is involved in activation of transcription and the one located downstream is involved in cold shock induction (Fig. 5A). When these three DNA fragments with the four regions are present in the upstream region of the *TIP1* gene, a wild-type level of expression is observed (Fig. 5B, line 1). The deletion of the *Hin*dIII fragment leads to a higher level of mRNA at both 30°C and 10°C, although the difference in the amount of mRNA between these two temperatures is lower than that observed in the wild-type strain. It is possible that the *Hin*dIII fragment is directly involved in the cold shock induction of this gene although, because of the large amount of mRNA already existing at 30°C we cannot rule out the possibility that some other factors may prevent the mRNA from accumulating over a certain limit, reducing the magnitude of the induction after the temperature downshift. Regarding the function of the *Eco*RV−*Sca*I and the *Sau*3AI fragments, in the absence of the *Hin*dIII fragment, one of them can be deleted with no detectable difference in the expression of *TIP1* at 30°C and after cold shock compared with a plasmid containing both of them (Fig. 5B, lines 2, 3 and 8). However, both of them must be present for expression when the *Hin*dIII fragment is inserted in the plasmid; deletion of either of them in this case dramatically reduces the expression of this gene at both 30°C and 10°C (Fig. 5B, lines 6 and 7). Although it is clear that the *Eco*RV−*Sca*I fragment is involved in both activation of transcription and cold shock induction, we have been unable so far to determine whether there is only one region with both functions, two regions or even more regions, each region with a different role. However, in the *Sau*3AI fragment two different elements have been identified, one located in the upstream portion of this fragment which activates transcription, and another one located in the downstream portion, responsible for a 2−3-fold induction after cold shock. Although the deletion of the entire *Sau*3AI fragment shows no effect in the expression of *TIP1* in the absence of the *Hin*dIII fragment and the presence of the *Eco*RV−*Sca*I fragment (line 8), deletion of only the element involved in cold shock significantly reduces the expression of *TIP1* at both 30°C and 10°C (Fig. 5B, line 10).This indicates that the element involved in the activation of transcription detected in the upstream portion of the *Sau*3AI fragment is functional only in combination with the cold shock element and the cold shock factor. Deletion of the cold shock element still allows the activator to bind to the activating sequences (Fig. 4C), but the factor cannot activate transcription (Fig. 5B, line 10). The *Eco*RV−*Sca*I fragment can be functional, but the activator bound downstream of the *Eco*RV−*Sca*I fragment will block transcription. Only after deletion of the DNA sequences recognized by the activator, the region downstream of the *Eco*RV−*Sca*I fragment will be cleared and transcription stimulated from the *Eco*RV−*Sca*I fragment can proceed (Fig. 5B, line 8).

The use of fragment B which contains the DNA sequences involved in cold shock has allowed the identification of the cold shock factor (CSF) by a DNA-binding assay (Fig. 4B). The CSF appears to be present in cells growing at 30°C, although the amount of DNA-binding activity is less in 30°C-growing cells than in cold shocked cells. CSF is also able to bind DNA at 30°C, but its affinity increases at lower temperatures. The DNA-binding activity observed with extracts prepared from 10°C cultures and incubated at 10°C during the DNA-binding assay is about 2−3-fold higher than that observed with extracts prepared from 30°C cultures incubated at 30°C (Fig. 4B, lanes 2 and 7). An increase of 2−3-fold is also the difference observed in the level of *TIP1* mRNA before and after cold shock when plasmids containing only the regulatory elements contained in the *Sau*3AI fragment in the upstream region of *TIP1* are analyzed by Northern blot hybridization (Fig. 5B, line 3). Therefore, it is possible that the induction of *TIP1* by cold shock can occur due to an increase in the amount and the affinity of CSF after cold shock. However, as we have shown, several other regions in addition to the *Sau*3AI fragment are involved in the expression of the gene, and the level of induction in the wild-type strain is about 6−8-fold. Further work will be required to elucidate the role of each element and the factors that bind to them as well as their interaction with one another in order to understand the exact mechanism of the cold shock induction of *TIP1*.

Comparison between the cold shock factor that binds to the downstream portion of the *Sau*3AI fragment and the yeast heat shock factor (HSF) demonstrates that both CSF and HSF binding of DNA can be detected in extracts from cells growing at 30°C (22, 23). However, the amount of HSF and its affinity to bind DNA does not change with temperature as we have demonstrated for CSF. Activation of transcription of heat shock proteins is caused by phosphorylation of HSF (22,23) while in case of CSF, greater amounts of protein as well as increased binding affinity to DNA may be enough to explain the activation of transcription of *TIP1* at lower temperatures. At this stage we do not know the reason why the affinity of CSF for DNA is higher at lower temperature. It may be due to a conformational change of the protein resulting from the temperature downshift. It is possible that some post-translational modifications are responsible for this change.

The sequence of the 1.15-kb upstream of the initiation codon of *TIP1* gene shows several interesting features (Fig. 3). A sequence of 13 bp is repeated 5 times in this fragment (underlined with thick bars and numbered in Fig. 3). Two of them are located in the *Eco*RV−*Sca*I fragment, another two are in the *Sau*3AI fragment, and the fifth one is just downstream of the second *Sau*3AI site. The sequence of repeat 1 is identical to that of repeat 4 and the similarity of these two with the others is 77%, 69% and 69% with repeats 2, 3 and 5, respectively. Two sets of

palindromic sequences can be observed (indicated with two sets of thin arrows drawn in opposite orientations in Fig. 3), one in the *Eco*RV−*Sca*I fragment and the other in the *Sau*3AI fragment. The two sets of palindromic sequences show no similarity with one another. A third feature is the presence of a direct tandem repeat of 15 bases (underlined with thick arrows in Fig. 3) located in the *Sau*3AI fragment. The identity between these two repeats is 80%. Finally, the sequence ATTGG, which is recognized by the Y-box binding factors (24) and CspA, the major *E.coli* cold shock protein (7), is present twice in the *Sau*3AI fragment and is boxed in Fig. 3. At this point we do not know whether one or more of these features play a role in the regulation of the expression of *TIP1* gene. Further characterization of the *Hin*dIII, *Eco*RV−*Sca*I and *Sau*3AI fragments will be needed in order to identify the factors that bind to them, the sequences that they recognize, and their roles in transcription. In addition, characterization of the induction of other cold shock inducible genes will be required to clarify the mechanism of the cold shock response in yeast and other organisms.

In summary, there are at least four different regions which are involved in the regulation of expression of *TIP1*. In addition, it is possible that elements located in the coding region or in the 3′-untranslated region may be involved in the regulation of expression of *TIP1*. In this regard it should be noted that a transcriptional activating sequence located in the coding region has been reported for *TIR1* (*SRP1*), a cold shock-inducible gene homologous to *TIP1* (25). It is not known, however, whether this sequence plays a role in the cold shock induction of *TIR1*. The large number of regions involved in the expression of *TIP1* indicates that the regulation of the cold shock response may be much more complicated than the expression of heat shock genes where only one heat shock element and one heat shock factor are required to increase the level of mRNA of these genes severalfold after heat shock.

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