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Cloning of the gene encoding the yeast protein BTF1Y, which can substitute for the human TATA box-binding factor

(transcription initiation *in vitro*/RNA polymerase B/HeLa cells/initiation factors)

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ABSTRACT **An activity (designated BTF1Y) in extracts of *Saccharomyces cerevisiae* can substitute for the human TATA box-binding factor BTF1 in a reconstituted transcription system containing the adenovirus 2 major late promoter, RNA polymerase B (II), and the basic transcription factors BTF2, BTF3, and STF. We have purified BTF1Y to homogeneity, using as assays reconstitution of *in vitro* transcription and DNase I footprinting on the TATA element. Both activities copurified with a 27-kDa polypeptide as determined by SDS/PAGE. Gel filtration indicated a molecular mass of 28 ± 5 kDa under nondenaturing conditions, suggesting that the native BTF1Y protein is a monomer. BTF1Y was enzymatically cleaved, several peptides were sequenced, and appropriate oligonucleotide probes were synthesized to clone the BTF1Y gene from a yeast genomic library. The BTF1Y gene contains a 720-base-pair open reading frame encoding a protein of 27,003 Da. The recombinant protein expressed in HeLa cells exhibited the same chromatographic characteristics and *in vitro* transcriptional activity as BTF1Y prepared from yeast extracts, confirming the identity of the gene. Gene-disruption experiments indicated that the yeast BTF1Y gene is a single-copy essential gene.**

Accurate and specific initiation of transcription *in vitro* from minimal promoter elements of protein-coding genes requires at least four transcription factors in addition to RNA polymerase B (II) (1–5). The first step in transcription initiation corresponds to the binding of the factor BTF1 (the TATA box-binding factor, also known as TFIID and DB) to the TATA box element (6–8). This binding step is crucial in the initiation pathway, since it results in the formation of a stable "committed" complex onto which the other factors and RNA polymerase B assemble to form a multiprotein complex at the promoter proximal site (3, 4, 9–11).

Although numerous attempts have been made to purify mammalian BTF1, notably from human HeLa cell extracts, purification was only partial (e.g., ref. 8; our unpublished results). However, recent studies have shown that yeast trans-activators can stimulate transcription in mammalian cells (12, 13) and, conversely, that mammalian regulatory factors (e.g., the estrogen receptor) can function in yeast (14–16.) These results indicated a surprising evolutionary conservation of function from yeast to man, demonstrating that trans-activators can interact productively with the basic transcriptional machinery of widely distant species. This conclusion was further supported by the demonstration that the yeast *Saccharomyces cerevisiae* contains an activity (BTF1Y) that can complement an *in vitro* HeLa cell-based transcription system lacking the human BTF1 factor and which, therefore, participates in the formation of a functional initiation complex with the other basal factors of mammalian cells (17, 18). Since further study of the yeast TATA box-binding factor BTF1Y is of obvious interest, we have purified this protein and cloned the corresponding gene.†

MATERIALS AND METHODS

Purification of BTF1Y Protein. S100 extract (34 mg of protein per ml) was prepared from frozen *S. cerevisiae* cells as described (19). After overnight dialysis against buffer DB [10 mM Tris·HCl, pH 7.9/5 mM $MgCl_2$/0.5 mM dithiothreitol/17.4% (vol/vol) glycerol/0.2 mM phenylmethanesulfonyl fluoride (PMSF)] containing 0.05 M KCl, 125 ml of 2-fold-diluted S100 was loaded onto a 450-ml heparin-Ultrogel (IBF) column equilibrated in buffer DB containing 0.1 M KCl. After washing, proteins were eluted stepwise with buffer DB containing 0.15 M KCl, 0.37 M KCl (HEPO.37 fraction), and 0.6 M KCl. BTF1Y activity was found in the HEPO.37 fraction. After dialysis in buffer B (50 mM Tris·HCl, pH 7.9/8.7% glycerol/0.1 mM EDTA/0.5 mM dithiothreitol/0.1 mM PMSF) containing 0.08 M KCl, 2 liters of HEPO.37 fraction was loaded onto a 500-ml DEAE-Sephacel column. The flow-through fraction (DEFT80), which contained the BTF1Y activity, was made 50 mM potassium phosphate (pH 6.3) and loaded onto a sulfopropyl-5PW (Toyo Soda, Tokyo) HPLC column equilibrated in the same buffer. Proteins were eluted with a linear 0.08–0.60 M KCl gradient. Active BTF1Y fractions, which eluted at 0.4–0.5 M KCl (SPO.4 fraction), were dialyzed against buffer B (without PMSF) containing 0.05 M KCl, and 25 ml was loaded onto a heparin-5PW (Toyo Soda) HPLC column. Proteins were eluted with a linear 0.05–0.60 M KCl gradient, with BTF1Y activity recovered at ≈0.5 M KCl (HEPO.5 fraction). Ten milliliters of the HEPO.5 fraction was dialyzed against buffer C (50 mM Tris·HCl, pH 7.9/17.4% glycerol/0.1 mM EDTA/0.5 mM dithiothreitol/5 mM $MgCl_2$) containing 0.1 M KCl and loaded onto a 400 μl/heparin-Ultrogel column, equilibrated in the same buffer. Proteins were eluted with 50 mM steps from 0.1 to 0.6 M KCl in buffer C. Active BTF1Y fractions (see Fig. 1*B*) were dialyzed against buffer C (without $MgCl_2$) and loaded onto a G-3000-SW (Toyo Soda) HPLC column that was equilibrated in buffer B containing 0.3 M KCl and 0.1% Brij-58 and was calibrated with a Pharmacia calibration kit. BTF1Y activity was recovered in the 30-kDa region of the elution profile.

BTF1Y Protein Sequencing. Aliquots of HEPO.5 fractions were concentrated using a Speedvac microcentrifuge (Savant) and electrophoresed in an SDS/14% polyacrylamide gel. Proteins were then transferred to a poly(vinylidene difluoride) membrane (Immobilon, Millipore) and stained

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Abbreviations: Ad2MLP, adenovirus type 2 major late promoter; ORF, open reading frame.
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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M29459).

with Ponceau S (20, 21). The region of the membrane containing the 27-kDa polypeptide (≈100 pmol) was excised and treated with 1.25 μg of endo-proteinase lysine-C (Wako Biochemicals, Osaka) for 24 hr at 25°C in 25 mM Tris·HCl, pH 9.3/10 mM EDTA/0.1% hydrogenated Triton X-100/10% acetonitrile. Peptides were separated by reverse-phase HPLC (Brownlee Aquapore RP300, 7μm; 1 × 25 mm column; gradient elution with 10–60% acetonitrile in 0.1% trifluoroacetic acid in 120 min; flow rate 0.05 ml/min). Three peptides (underlined in Fig. 2A) were sequenced with an Applied Biosystems model 477A protein sequencer.

Cloning and Sequencing of the Yeast BTF1Y Gene. Two degenerate oligodeoxynucleotide probes (A and B) were synthesized based on the amino acid sequences of peptides 1 and 2 (amino acids 219–228 and 84–95, respectively, in Fig. 2A): probe A, 5′-AA(A or C)G(A or G)GAAGAAAT(T or C)TA(T or C)CAAGC(T or A)TT(T or C)GAA-3′; probe B, 5′-GTTGCT(T or C)T(G or A)CATGCT(C or A)G(A or G)AATGCTGAATA(T or C)AAT-3′. A yeast genomic DNA library, constructed by ligation of a partial *Sau*3A digest of FL100 DNA [average fragment size, 2.5 kilobases (kb)] into the single *Bam*HI site of plasmid pFL1 (22), was screened by hybridization on nitrocellulose filters with both ^{32}P-end-labeled probes A and B in 0.9 M NaCl/0.09 M sodium citrate, pH 7/0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin/0.05% sodium pyrophosphate/0.1% SDS containing denatured salmon sperm DNA at 100 μg/ml. The final washes were in 0.9 M NaCl/0.09 M sodium citrate, pH 7/0.05% sodium pyrophosphate at either 72°C (probe A) or 67°C (probe B). After three rounds of screening, several clones that hybridized to both probes were isolated. Two independent clones with insert sizes of 4.6 kb (pL1) and 3.8 kb (pL2) were further analyzed. The *Sma* I–*Sal* I fragments of pL1 and pL2 containing the genomic inserts were subcloned into the *Sma* I and *Sal* I sites of pTZ18U and pTZ19U (United States Biochemical), yielding pL1-18U, pL1-19U, pL2-18U, and pL2-19U, which were used to generate single-stranded DNA. Approximately 1.3 kb of insert was then sequenced on each plasmid, using first probe A as a primer and pL1-18U and pL2-18U DNAs. Additional primers were synthesized for further sequencing, which yielded identical sequences for pL1 and pL2 (Fig. 2A).

Expression of BTF1Y Protein in HeLa Cells. The synthetic oligodeoxynucleotide 5′-TTCCTCATCGGCCATGGTG-GAAAAGAATTCTCTTGATACACC-3′ was used to generate an *Eco*RI site and a consensus Kozak sequence (23) at positions −10 and −5 (with respect to the initiator codon) of the BTF1Y sequence present in pL2-18U, yielding plasmid pEV1. The 1.1-kb *Eco*RI–*Bam*HI fragment of pEV1 containing the complete BTF1Y open reading frame (ORF) was inserted into the *Eco*RI–*Bam*HI sites of the expression vector pSG513 [a derivative of pSG5 (24) with a larger polylinker], giving rise to pEV2. HeLa cells (3×10^6 per 150-mm tissue culture dish; 50% confluence) were transfected with 20 μg of pEV2 (or pSG513 as control) DNA as described (13). Forty hours later, whole-cell extracts (0.8 ml; 1.2 mg/ml) were prepared (3) from six dishes of pEV2- or pSG513-transfected HeLa cells and loaded on 500-μl heparin-Ultrogel columns equilibrated in buffer DB containing 0.1 M KCl (see above). Proteins were eluted with buffer DB containing KCl in 50 mM steps (Fig. 3 *A* and *B*). After dialysis against buffer B containing 80 mM KCl, the 350–450 mM KCl fractions from the heparin-Ultrogel columns were pooled, and aliquots (450 μl) were loaded onto 400-μl DEAE-Sephacel columns equilibrated in buffer B containing 80 mM KCl. The columns were washed twice with buffer B containing 80 mM KCl (FT-2 and FT-3 in Fig. 3 *C* and *D*), and the absorbed proteins were eluted stepwise with KCl.

Disruption of the BTF1Y Gene. The 2.4-kb *Eco*RI–*Eco*RI fragment of pL2 containing the complete BTF1Y ORF was cloned into the *Eco*RI site of pTZ19U. The *Xba* I site present in the pTZ19U polylinker was then destroyed and the 1.17-kb *Hin*dIII–*Hin*dIII fragment containing the yeast *URA*3 gene from pFL1 (22) was inserted by blunt-end ligation into the *Xba* I site located within the BTF1Y ORF, creating pDIS (Fig. 4A). pDIS cleaved with *Eco*RI was used to transform two yeast diploid strains, Δura3 trp 1-4 (constructed by F. Lacroute, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France) and YNN290 (25). Ura$^+$ colonies were selected and the structure of several transformants was checked by Southern blotting. Several positive clones were induced to sporulate and the dissected tetrads were grown on rich medium (YPED). The Ura phenotype of growing clones was determined by replica plating onto minimal medium (YNB) with and without uracil.

RESULTS

Purification of BTF1Y. BTF1Y activity was monitored both by DNase I footprinting on the adenovirus 2 major late promoter (Ad2MLP) TATA element and by *in vitro* transcription of a template containing the Ad2MLP (pM34, 309-nucleotide run-off transcript; see ref. 26) in a reconstituted HeLa cell-based transcription system lacking BTF1 (17). As shown by the protein profile of the BTF1Y fractions from six chromatographic steps (Fig. 1A), heparin-5PW HPLC followed by concentration–rechromatography of the active fractions on heparin-Ultrogel resulted in a remarkable purification. The run-off transcription activity (Fig. 1*B Upper*) and the footprinting activity on the Ad2MLP TATA box (Fig. 1*B Lower*, −21 to −31 protection) were coeluted from the heparin-Ultrogel column, and the pooled active fractions contained a single major polypeptide of ≈27 kDa (Fig. 1*A*, lane 7). That this protein corresponded to BTF1Y was supported by the result of gel filtration with G-3000 resin, which resulted in BTF1Y-dependent transcription activity only in those fractions that contained the 27-kDa polypeptide (Fig. 1*C*). Moreover, BTF1Y behaved as a monomer in the G-3000 chromatography conditions, since its activity was eluted with an apparent molecular mass of 28 ± 5 kDa.

Cloning and Sequencing of the BTF1Y Gene. Transcriptionally active BTF1Y fractions from a heparin-5PW HPLC column were fractionated by SDS/PAGE and electroblotted. After *in situ* lysine-C endoproteinase hydrolysis of the electro-blotted 27-kDa polypeptide, three peptides were purified and microsequenced. Two sets of degenerate oligonucleotide probes were synthesized and used for screening a yeast genomic library. Two clones, pL1 and pL2, that hybridized to both oligonucleotide probes were subcloned in pTZ18U and pTZ19U and sequenced on both strands. A 1265-bp sequence present in both pL1 and pL2 (Fig. 2A) contained a 720-nucleotide ORF (positions 375–1094) that translated into a polypeptide of 240 residues, with a calculated size of 27,003 Da, in agreement with the expected molecular mass of BTF1Y (see above). As expected, the three sequenced peptides, each preceded by a lysine residue, were found within the translated sequence (starting at residues 16, 84, and 219, underlined in Fig. 2A). In a search of the EMBL (release 18) and Swiss-Prot (release 10) data bases, no significant homology was found between the BTF1Y DNA sequence and published DNA sequences or between the deduced protein sequence and those of known proteins.

Except for a single TATA box-like sequence (5′-ATATAAAA-3′, starting at position 112 in Fig. 2A), no significant homology to any of the eukaryotic regulatory sequences compiled by Wingender (27) was found within the 374 bp immediately 5′ of the initiation codon. Interestingly, an internal homology search revealed the presence of two homologous domains with two blocks of almost perfect homology (QNIV, positions 68 and 158; LIF-SGK-V-TGAK,

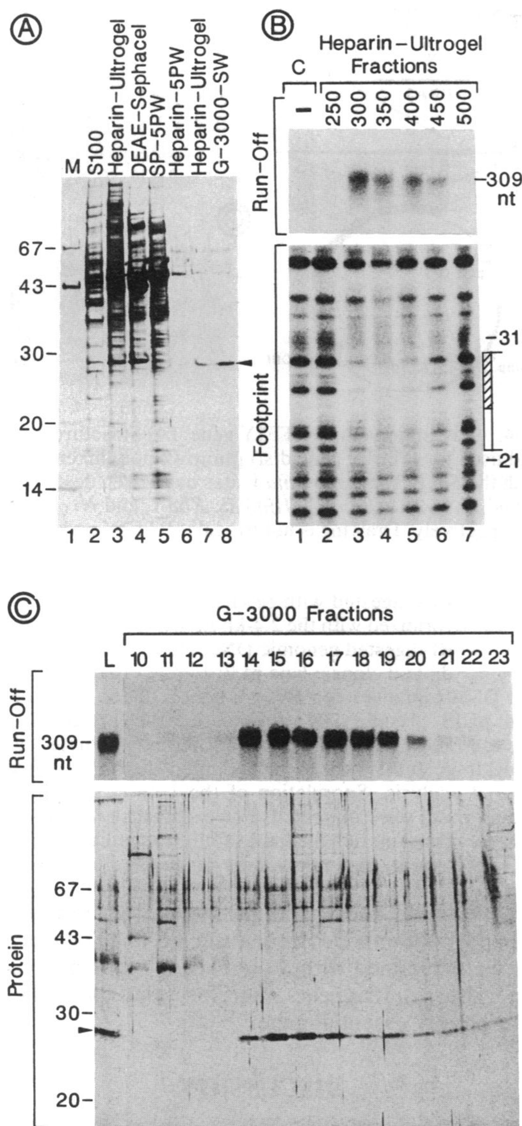

FIG. 1. Purification of BTF1Y. (*A*) SDS/PAGE analysis of purification. Samples (≈10 μg of protein in lanes 2–5; ≈1 μg in lanes 6–8) of the chromatographic fractions were electrophoresed and silver-stained. Arrowhead, BTF1Y polypeptide (27 kDa). Lane M, markers [molecular masses (kDa) at left]. (*B*) BTF1Y transcription and DNA-binding activities of the heparin-Ultrogel fractions. Transcription activity (*Upper*) was measured by run-off RNA synthesis with 3 μl of each heparin-Ultrogel fraction (250–500 mM KCl, lanes 2–7 as indicated), an Ad2MLP template, and a HeLa *in vitro* transcription system lacking the BTF1 factor (17). The specific Ad2MLP run-off transcript is 309 nucleotides (nt) long. Lane C (control), no fraction added. DNA-binding activity (*Lower*) was detected by DNase I footprinting on the coding strand of the Ad2MLP sequence of pM97 (26). Lanes 2–7, 6 fmol (≈15,000 cpm) of the 294-base-pair (bp) *Bam*HI–*Sac* II fragment from pM97 (5′-end-labeled at the *Bam*HI site) and 3 μl of the indicated heparin-Ultrogel fractions were incubated in 20 μl of 4 mM Tris·HCl, pH 8.0/60 mM KCl/5 mM $MgCl_2$/17.4% glycerol/0.1% Brij-58 with poly(dG·dC) at 4 ng/μl; after a 40-min preincubation at 20°C, 25 ng of DNase I was added for 30 sec. After phenol/chloroform extraction, DNA was electrophoresed as described (26). Lane 1, naked DNA. Protected region (−21 to −31) is indicated; hatched area corresponds to the TATAAA sequence. (*C*) G-3000 gel filtration. An aliquot (500 μl) of the active fractions of the second heparin-Ultrogel column was fractionated on a G3000-SW HPLC column (0.75 × 60 cm; flow rate, 0.3 ml/min; 0.6-ml fractions). Aliquots (8 μl) of fractions 10–23 were tested for transcription activity (*Upper*) as described for *B*. Aliquots of the same fractions (100 μl) were analyzed by SDS/14% PAGE with silver staining (*Lower*). Arrowhead, 27-kDa polypeptide. Lanes L, aliquots of the loaded heparin-Ultrogel fraction.

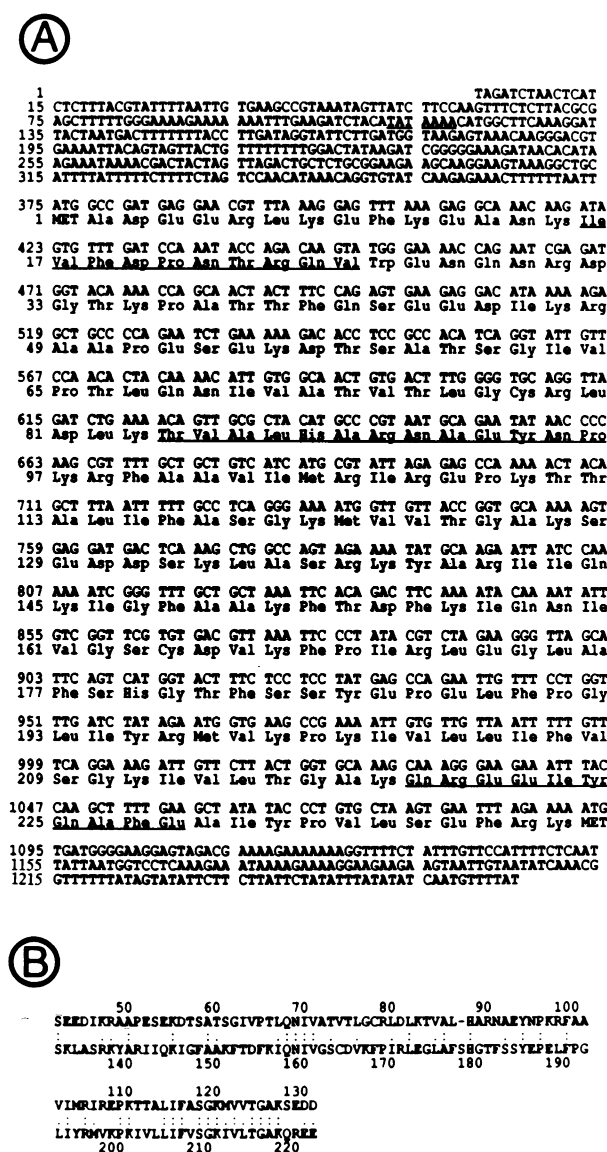

FIG. 2. (*A*) Sequence of the BTF1Y gene. The 1265-base sequence encompassing the 720-base ORF is shown. The three BTF1Y peptides that were microsequenced are underlined, as is the putative 5′ flanking TATA box. (*B*) Sequence homology within the BTF1Y protein. Amino acids are marked relative to position 1 of the ORF. Identical amino acids are indicated by two dots, and single dots indicate similar amino acids. The overall homology is 33% when only identical amino acids are taken into account (standard deviations above the control mean were 5).

positions 114 and 205; see Fig. 2*B*). Moreover, the hydropathicity profiles of the 42–131 and 132–222 sequences were very similar (data not shown).

Transcriptional Activity of the BTF1Y Gene Expressed in HeLa Cells. To demonstrate that the cloned gene encodes a functional BTF1Y protein, the cloned ORF was expressed in HeLa cells. Cells were transfected with either the parental expression vector pSG513 (used as a control) or pEV2 (pSG513 containing the BTF1Y ORF). Whole-cell extracts were then prepared, loaded on a heparin-Ultrogel column, and eluted with 50 mM KCl steps (Fig. 3). Aliquots were assayed for BTF1Y activity in an *in vitro* HeLa cell-based transcription system lacking BTF1 (see above). As expected (see Fig. 1*B*), BTF1-like activity was found in three of the fractions derived from pEV2-transfected cells (350–450 mM KCl, Fig. 3*B*). With identically prepared extracts of HeLa cells transfected with the parental vector pSG513, the en-

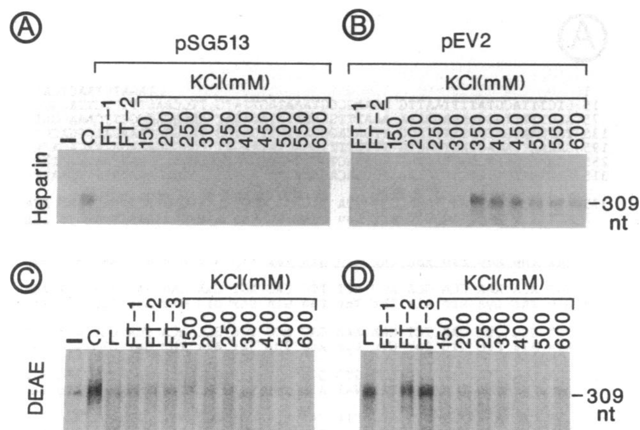

FIG. 3. Transcription activity of the BTF1Y protein produced in HeLa cells. The expression vector pEV2, containing the BTF1Y ORF, and the parental expression vectors pSG513 (as a control) were transfected into HeLa cells. Whole cell extracts were prepared, the proteins were chromatographed on heparin-Ultrogel columns, and aliquots (5 μl) of the recovered fractions were assayed for BTF1 transcription activity by the run-off assay as in Fig. 1. The specific 309-nucleotide (nt) run-off transcript was present in incubations containing the 350–450 mM KCl fractions derived from the pEV2-transfected cells (*B*), but not in those corresponding to the control pSG513-transfected cells (*A*). The active pEV2 fractions were pooled (as well as their corresponding pSG513 fractions) and chromatographed on DEAE-Sephacel columns. The BTF1Y activity was found (5-μl aliquots were assayed) in the flow-through (FT) fractions corresponding to pEV2-transfected cells (*D*), but not in those corresponding to the control cells (*C*). Lane (–) *A* and *C*; no addition of chromatographic aliquots; lane C in *A* and *C*; control run-off transcription with a 5-μl aliquot from an HEP0.5 fraction (see BTF1Y purification in *Materials and Methods*); lane L in *C* and *D*; 5-μl aliquots from the pooled 350–450 mM KCl fractions of the heparin-Ultrogel columns displayed in *A* and *B*, respectively, assayed for run-off transcription.

dogenous BTF1 activity was too low to be detected (Fig. 3*A*). The active fractions from "pEV2" heparin-Ultrogel and the equivalent fractions from "pSG513" heparin-Ultrogel were then loaded on DEAE-Sephacel columns and step-eluted aliquots were tested for BTF1 activity. Aliquots of the flow-through fractions (FT2 and FT3) derived from pEV2-transfected HeLa cells were active (Fig. 3*D*), whereas no BTF1 activity was found in the corresponding fractions derived from pSG513-transfected cells (Fig. 3*C*). Note that the BTF1Y activity extracted from yeast cells was also found in the DEAE-Sephacel flow-through fractions, whereas HeLa cell BTF1 is known to be eluted from DEAE-Sephacel at 250 mM KCl (3). Therefore, the BTF1-like activity detected in pEV2-transfected cells must arise from the expression of the cloned BTF1Y gene.

The BTF1Y Gene Is Essential for Yeast. To test whether the BTF1Y gene is essential for yeast, the chromosomal coding sequence was disrupted. pDIS, an integrative vector containing the BTF1Y gene disrupted by insertion of *URA3* (Fig. 4*A*), was digested with *Eco*RI and used to transform the Δ*ura*3 *trp 1–4* diploid strain. The genomic DNA of several Ura$^+$ transformants was analyzed by Southern blotting. The results obtained with the DNA of four such transformants (C1–C4) and the DNA of the nontransformed diploid strain (A and B) are shown in Fig. 4*B*. Clearly one allele of Ura$^+$ transformants contained a fragment of higher molecular weight due to *URA3* insertion. To determine whether the disruption was lethal in haploid progeny of the diploid transformants, cells were induced to sporulate and the resulting tetrads were dissected onto rich medium to score for spore germination. In 20 tetrads from diploids containing the disruption, only two spores per tetrad formed colonies (Fig. 4*C*). Furthermore, these colonies were Ura$^-$, indicating that they did not contain a *URA3*-disrupted BTF1Y allele. Similar results were obtained with *S. cerevisiae* strain YNN290 (25) (data not shown). It appears, therefore, that the BTF1Y gene is a single-copy essential gene.

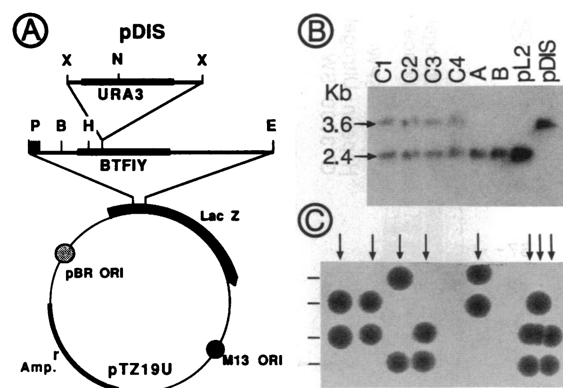

FIG. 4. Disruption of the BTF1Y gene. (*A*) Structure of the pDIS plasmid used for BTF1Y gene disruption. P; polylinker of pTZ19U, in which the *Sal* I, *Xba* I and *Sma* I sites have been destroyed; E, B, H, X, and N, *Eco*RI, *Bam*HI, *Hin*dIII, *Xba* I, and *Nco* I restriction sites, respectively. Drawing is not to scale. BTF1Y and URA3 genes are transcribed leftward and rightward, respectively. (*B*) Southern blot of Ura$^+$ transformants. Lanes C1–C4, DNA (12 μg) of each transformant was digested with *Eco*RI, electrophoresed in an agarose gel, and hybridized with the 2.4-kb *Eco*RI fragment of pL2; lanes A and B, *Eco*RI-digested genomic DNA (12 μg) from two nontransformed Ura$^-$ diploid clones; lane pL2; 15 ng of *Eco*RI-digested pL2 plasmid DNA (nondisrupted BTF1Y gene); pDIS, 15 ng of *Eco*RI-digested pDIS plasmid DNA (disrupted BTF1Y gene). Arrows indicate the *Eco*RI fragment (2.4 kb) of the nondisrupted allele and the *Eco*RI fragment (3.6 kb) of the disrupted allele. (*C*) Viable spores from tetrad analysis. Sporulation of the Ura$^+$ transformants was induced and asci were dissected. For each ascus (arrows) the four spores were placed on rich medium (YPED) as indicated by the bars at left. In each case, only two spores germinated (see text).

DISCUSSION

The yeast protein that can replace the human HeLa cell TATA box factor BTF1 (TFIID) in an *in vitro* reconstituted transcription system (17, 18) has been purified to apparent homogeneity. The purified protein, which binds specifically to the TATA box of the Ad2MLP and can restore accurate and specific transcription from this promoter, has an apparent molecular mass of 27 kDa, in agreement with previous reports (18, 28, 29). The corresponding *S. cerevisiae* gene has been cloned and sequenced. The yeast BTF1Y gene encodes a polypeptide of 240 amino acids with a calculated molecular mass of 27,003 Da and a pI of 9.9, which agrees well with the observed chromatographic behavior of BTF1Y. Only minor differences, resulting notably in a threonine residue instead of an alanine residue at position 50, were found between the present BTF1Y gene sequence and that of the gene isolated in a parallel study by Schmidt *et al.* (30). Whole-cell extracts from HeLa cells transfected with an expression vector containing the BTF1Y sequence were shown to contain a BTF1 transcriptional activity presenting the chromatographic properties specific to the yeast BTF1Y protein, thus confirming the identity of the cloned gene. No such activity was found in cells transfected with the parental expression vector.

No obvious similarity was found between the sequence of the BTF1Y polypeptide and those of proteins in data bases. In particular, there are no sequence similarities to structural motifs known to be associated with DNA-binding proteins and transcriptional trans-activators (ref. 31 and refs. therein). However, the BTF1Y polypeptide presents an internal ho-

mology, which suggests that it has evolved by duplication of an ancestral gene and may contain two homologous functional domains.

Finally, the BTF1Y protein characterized here appears to be essential for yeast as judged from the results of a gene-disruption experiment. Whether this protein mediates the effect of the TATA box elements present in yeast gene promoters remains to be established.

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