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Identification of a Yeast Protein with Properties Similar to Those of the Immunoglobulin Heavy-Chain Enhancer-Binding Protein NF-μE3

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We demonstrate that *Saccharomyces cerevisiae* cells possess a 33-41-kilodalton protein with DNA-binding properties remarkably similar to those of the immunoglobulin enhancer-binding protein NF-μE3. We further show that the μE3-binding site functions as an upstream activating sequence in yeast cells, stimulating transcription from a truncated *CYC1* promoter. These data suggest that the yeast protein, designated YEB-3, and NF-μE3 are functionally related and perhaps evolutionarily conserved.

Many of the molecular details of transcription activation in eucaryotic cells have come from studies of yeasts (16, 17, 49). In fact, the general mechanisms that govern transcription in yeasts may be quite similar to those of higher eucaryotes. This principle first emerged from experiments that demonstrated functional reciprocity between many of the transcription factors of yeasts and higher eucaryotes (11, 25, 33, 34, 52). In addition, in vitro experiments have demonstrated similarities between certain yeast and mammalian DNA-binding proteins (5, 7, 9, 19–21, 24, 31, 32, 50).

One of the more thoroughly studied eucaryotic transcription elements is the immunoglobulin heavy-chain (IgH) enhancer (2, 15, 37). Enhancer activity has been shown to correlate well with the presence of at least six protein-binding sites within the enhancer (26, 30, 39, 51). Four of these, μE1, μE2, μE3, and μE4, were defined initially by in vivo footprinting (10). Two others, octa (47, 48) and μEBP-E (41), were first defined by using in vitro binding assays. Even though activity of the enhancer is essentially B-cell specific (with the exception of some T-cell lines, only very low levels of enhancer activity have been observed in other cell types), only one of these sites (octa) is known to bind a B-cell-specific protein (NF-A2, OTF-2 [14, 27, 44, 48]) in addition to a ubiquitous protein (NF-A1, OTF-1, OBP100, NFIII [3, 12]). With the ultimate goal of understanding the mechanism of B-cell-specific expression of the IgH enhancer and to take advantage of the genetic tools offered by yeast cells, we have undertaken a systematic search for yeast proteins that are functionally homologous to mammalian IgH enhancer-binding proteins.

We first assayed the ability of yeast nuclear extracts to bind synthetic double-stranded oligonucleotides in mobility shift assays (13, 47). Extracts were made from diploid *Saccharomyces cerevisiae* YPH49 (obtained from Phil Hieter, The Johns Hopkins University, Baltimore, Md.) by a scaled-down modification of the method of Wiederrecht et al. (53) in which KPO_4 was used in place of KCl in all buffers. We tested six 29-base duplex oligonucleotides corresponding to the μE1, μE2, μE3, μE4, and octa motifs of the IgH enhancer and the NF-κB-binding site of the kappa enhancer. Although we observed at least some weak binding with several of the oligonucleotides, the only specific binding

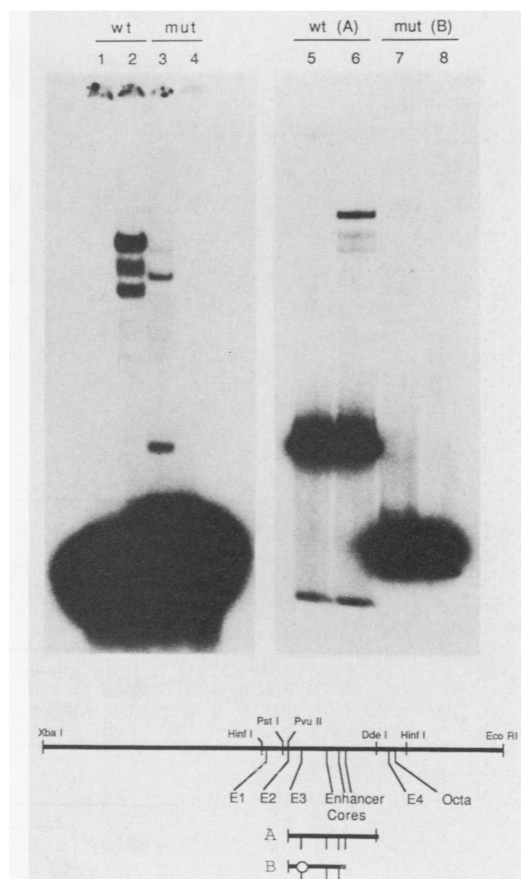

FIG. 1. Binding of yeast nuclear proteins to wild-type (wt) and mutant (mut) μE3-containing oligonucleotides and restriction fragments. Lanes: 1, 4, 5, and 8, labeled DNAs only (25,000 cpm/2 fmol of oligonucleotide or 10,000 cpm/1.3 fmol of restriction fragment per 15 μl of reaction mixture); 2, 3, 6, and 7, labeled DNAs plus 1 μg of yeast nuclear extract. DNAs used in each binding reaction are indicated above each gel. The sequence of the normal μE3 oligonucleotide is 5′-GATCTGGTCATGTGGCAAGGCTATTTGGC-3′ / 3′-ACCAGTACACCGTTCCGATAAACCCCTAG-5′. The base changes in the mutant μE3 motif are shown in Fig. 2.

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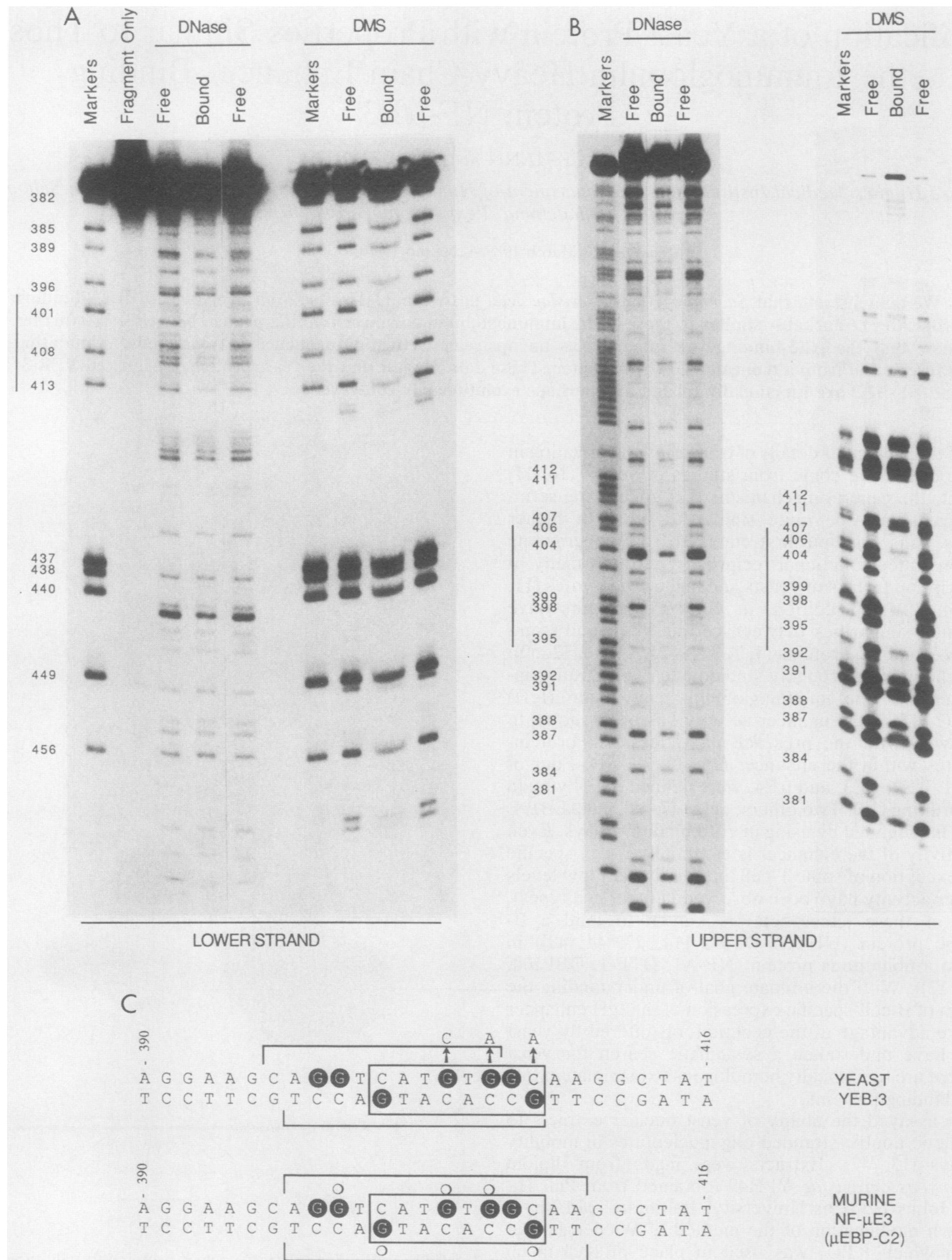

FIG. 2. Properties of yeast μE3-binding proteins as revealed by DNase I footprint and methylation interference assays and comparison with those of murine NF-μE3. The lower strand (A) and upper strand (B) of fragment A (see Fig. 1) or fragment A with additional 5′ sequences (to the *Hin*fI site) were analyzed by DNase I footprint and dimethyl sulfate interference assays. The lower and upper strands were analyzed on 5 and 8% denaturing polyacrylamide gels, respectively. Free and bound complexes are indicated. G-cleavage reactions or G-plus-A-cleavage reactions were used as markers. Cleaved guanidine residues are numbered by the system of Gillies et al. (15). Relevant regions of protection are indicated by vertical lines. (C) Summary of the results for the yeast protein (YEB-3) and murine NF-μE3/μEBP-C2. The methylguanines that interfere with binding in vitro are encircled; the region protected from DNase I cleavage is bracketed. Open circles indicate guanines identified by in vivo studies (10); the conventional μE3 motif is boxed; arrows mark altered bases in the mutant μE3 motif (see Fig. 1).

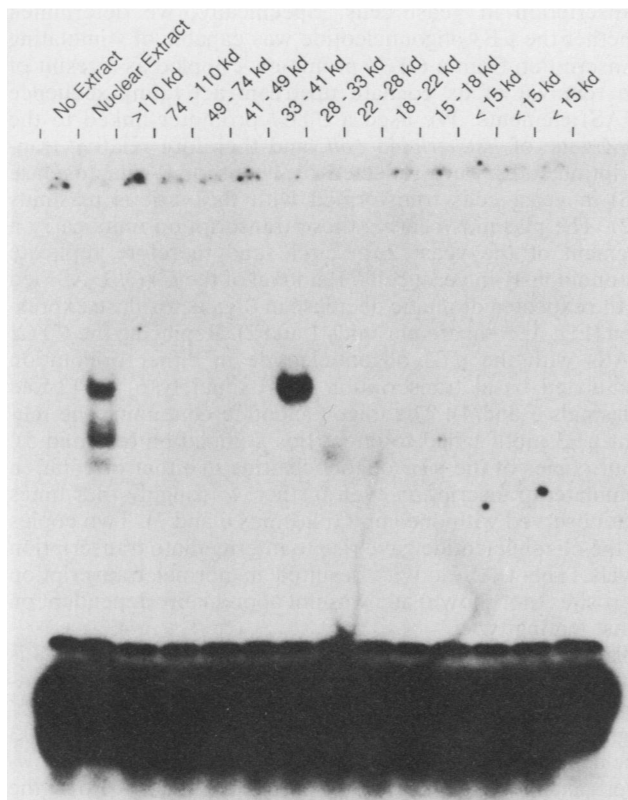

FIG. 3. Determination of the apparent molecular size of yE3BP. Yeast nuclear proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; gel slices were processed (see text) and analyzed in mobility shift assays by using the μE3 oligonucleotide. The first lane contained labeled oligonucleotide only; the second lane contained oligonucleotide plus unfractionated yeast nuclear extract. The remaining lanes contained labeled oligonucleotide plus proteins eluted from various gel slices, with the approximate protein molecular size range indicated for each gel slice. kd, Kilodaltons.

was to the μE3 and octa oligonucleotides, with specificity being defined by the ability of homologous unlabeled oligonucleotides to uniquely compete for the binding (not shown).

To demonstrate that the mobility shifts resulted from yeast proteins binding specifically to the μE3 and octa motifs, we examined the binding to similar oligonucleotides containing mutated sites, previously shown to abolish in vivo activity (26). Three base changes were introduced into the μE3 oligonucleotide (see Fig. 2). The mutant octa oligonucleotide replaced the ATTTGCAT octa sequence with ATCTAGAT. The pattern of shifted bands seen with the normal μE3 oligonucleotide (Fig. 1, lane 2) was completely altered when an oligonucleotide containing the mutant μE3 motif was used instead (lane 3). The intensity of the upper band was drastically reduced, the lower two bands disappeared, and two new bands of different mobility appeared (we have not characterized the binding specificity of these new complexes). These results were corroborated by using two larger restriction fragments derived from the IgH enhancer (lanes 5 to 8). Incubation of yeast extracts with enhancer fragment A, containing the wild-type μE3 motif (lane 6), resulted in a triplet pattern of shifted fragments reminiscent of the pattern obtained with the oligonucleotide. Fragment B, carrying the mutant μE3 element, was not shifted at all (lane 7). Taken together, the results indicate that yeast cells possess a protein whose binding specificity overlaps the IgH enhancer μE3 motif. In contrast to these results, the octa mutation had no effect on binding to the octa oligonucleotide, indicating that the specific binding was not directed by the octa motif per se but was likely due to flanking sequences. Consistent with this view, a separate oligonucleotide carrying the octanucleotide with different flanking sequences failed to form any specific complexes with yeast proteins (not shown). These latter results were somewhat unexpected because a yeast octa-binding activity had previously been suggested (35).

To further characterize the binding with the μE3 motif, we carried out DNase I protection and methylation interference studies to delineate the precise DNA contacts of the yeast protein (8). Figure 2 illustrates the results obtained when the upper band of the triplet obtained with fragment A (Fig. 1,

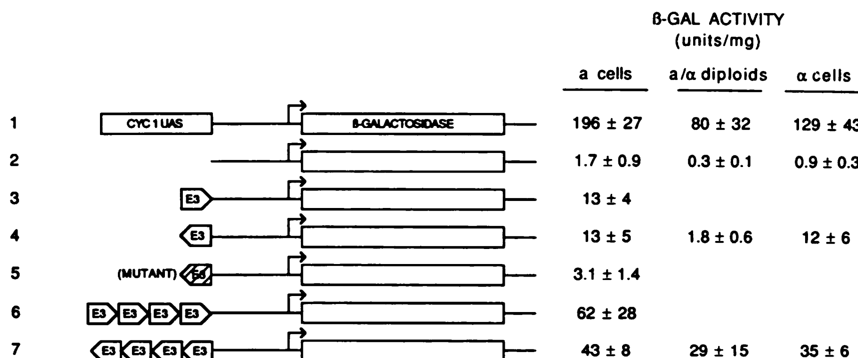

FIG. 4. Effect of the μE3 motif on transcription in yeast cells. All plasmids are derivatives of pLG669-Z, in which β-galactosidase expression is driven by a *CYC1* promoter. Plasmid 1 carries an intact *CYC1* promoter (with its cognate UAS elements). Plasmid 2 carries a *CYC1* promoter deleted of its UAS elements. The remaining plasmids carry μE3 oligonucleotides in place of the *CYC1* UAS elements. Arrows indicate the orientation of the oligonucleotides; arrows pointing to the right correspond to the orientation given in the legend to Fig. 1 (pointing in the direction of transcription of the IgH gene). The hatched arrow indicates on oligonucleotide carrying the mutant μE3 motif (see Fig. 2). Yeast **a** cells (146**a**; *MAT***a** *ura3 his4 leu2 trp1*), α cells (29α; *MATα ura3 his4 leu2 trp1*), and **a**/α cells (YPH49; *MAT***a***/α ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp-1Δ1/*trp-*1Δ1*) were transformed with each of these plasmids, and β-galactosidase levels of at least five individual transformants were determined. Numbers represent the means and standard deviations for these values. Since the diploid cells were not isogenic to the two haploid strains, β-galactosidase levels cannot be directly compared.

lane 6) and analyzed. An examination of free and bound forms of each strand of the fragment clearly identified a region that was both sensitive to dimethyl sulfate and resistant to DNase I. A summary of the results, along with those previously reported for partially purified murine NF-μE3 (from Peterson and Calame [40]; referred to by those authors as μEBP-C2), is also shown in Fig. 2. The affected region lies approximately between nucleotides 395 and 409 of the enhancer (numbering system of Gillies et al. [15]); this is the same region that encompasses the μE3 motif. In fact, a comparison of the DNA contacts made by the yeast protein and murine NF-μE3 shows a striking similarity between the two proteins. Dimethyl sulfate interference assays identified six to seven guanines that were involved in the binding of the yeast protein. Methylation of four and seven of these residues also inhibits binding of human and murine NF-μE3, respectively (1, 10, 40, 45, 46). DNase I sensitivity of the yeast binding activity identified virtually the same region of protection reported for NF-μE3. Similar results were obtained with all three of the complexes obtained with the mobility shift assay (data not shown), and we therefore assume that the additional bands represent either degradation products or different members of a family of related proteins.

These binding data provide compelling evidence that yeast cells contain a μE3-binding protein. We have designated this protein YEB-3 (yeast μE3-binding protein).

To determine the molecular weight of YEB-3, we subjected crude yeast nuclear extracts to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, eluted and renatured proteins from individual gel slices, and carried out mobility shift assays by using labeled μE3 oligonucleotides (42). YEB-3 activity (upper band of the triplet) was detected in a gel slice corresponding to a molecular size range of 33 to 41 kilodaltons. This result argues that YEB-3 does not exist as a heterodimeric protein with subunits of disparate molecular sizes and that its binding does not require cofactors present in the yeast nuclear extract. We favor the model in which YEB-3 binds DNA as either a monomer or a homodimer. From a similar experiment, we have come to the same conclusions concerning NF-μE3 but have determined its molecular size to be roughly 25 kilodaltons larger than that of yE3BP (H. Beckmann and T. Kadesch, manuscript in preparation). The middle band of the triplet seen with whole nuclear extract was not recovered in this assay. This result may reflect an inherent inability to denature and renature the protein responsible for this complex or the fact that this binding activity represents a heterodimeric protein.

The existence of a specific DNA-binding protein does not necessarily mean that the protein functions as a transcription factor (23). Ultimately, two tests must be used to prove this relationship. First, mutation or deletion of the DNA sequences that comprise the binding site should result in decreased transcription. Alternatively, linking of the binding site to a reporter gene should activate transcription of that gene in an appropriate manner. Second, the presence or absence of the protein must be reflected by transcription rates. For some DNA-binding proteins, the second criterion has been met by carrying out in vitro transcription reactions using factor-depleted extracts (4, 6, 29, 43). In the case of IgH enhancer-binding proteins, only those that bind to the octa motif have been shown to meet both criteria and can therefore be considered true transcription factors (12, 28, 44).

As a first step toward determining the functional role of YEB-3, we tested the ability of the μE3 motif to stimulate transcription in yeast cells. Specifically, we determined whether the μE3 oligonucleotide was capable of stimulating transcription from a *CYC1* promoter, crippled as a result of the removal of its cognate upstream activating sequence (UAS) elements. We used a *CYC1* promoter linked to the *lacZ* gene of *Escherichia coli*, and therefore relative transcription rates were reflected by levels of β-galactosidase (18) in yeast cells transformed with the various plasmids (22). The plasmids bearing these transcription units carry a segment of the yeast 2μm circle and therefore replicate autonomously in yeast cells. Removal of the *CYC1* UASs led to the expected dramatic decrease in β-galactosidase expression (Fig. 4; compare plasmids 1 and 2). Replacing the *CYC1* UASs with the μE3 oligonucleotide in either orientation stimulated basal transcription approximately 6- to 12-fold (plasmids 3 and 4). The oligonucleotide containing the mutant μE3 motif failed to effect this stimulation (plasmid 5). Four copies of the μE3 oligonucleotide in either orientation stimulated transcription even further, to roughly four times that observed with one copy (plasmids 6 and 7). Two copies of the oligonucleotide gave rise to intermediate transcription levels. The UAS activity resulted in normal transcription start sites (not shown) and was not appreciably dependent on yeast mating type.

The simplest interpretation of our results is that YEB-3 is a transcription factor that acts through its cognate DNA-binding site. Consistent with this, we have noted that the 5′ region of the yeast *GAL80* gene contains a match of 11 of 13 nucleotides to the region footprinted by YEB-3 (38). However, as noted above, these data alone do not prove the validity of this interpretation. Verification will require the generation of YEB-3 mutants or YEB-3-dependent transcription in vitro. Moreover, it remains to be seen in general whether mammalian and yeast transcription factors are homologous at the level of protein sequence and can therefore truly be considered to be evolutionarily conserved. For the case of AP-1, the sequence of the yeast gene (yAP-1) predicts homology with mammalian AP-1 that is restricted to the DNA-binding domain (36).

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