E2F Site Activates Transcription in Fission Yeast Schizosaccharomyces pombe and Binds to a 30-kDa Transcription Factor*

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The mammalian transcription factor E2F binds to several cellular proteins including Rb, p107, cyclin A, cyclin E, and p33cdk2 protein kinase in a stage-specific manner during cell cycle. Its recognition sequence, TTTCCGGC, is present in two of the human adenovirus early promoters and in several promoters of cellular genes whose products are implicated in the control of cell proliferation. These observations suggest that E2F may play an important role in cell-cycle regulation and prompted us to ask whether E2F-like activities are present in yeast. We found that the E2F motif can function as an activating sequence in Schizosaccharomyces pombe when cloned upstream of a reporter gene. Consistent with this, the expression of adenovirus E2 promoter in S. pombe was dependent on both E2F motifs of this promoter. A protein, spE2F, that binds to the E2F site was partially purified from S. pombe using DNA-affinity chromatography. The binding specificity of this protein was compared to that of human E2F using a number of mutant E2F sites as competitors. These studies showed that spE2F recognizes a sequence closely related to the E2F site. Ultraviolet cross-linking and Southwestern blot studies indicated that the molecular size of spE2F is 30 kDa. Previous studies have shown that a cis-acting element, ACGCGTNA, also called MluI cell cycle box, or MCB, is critical for the regulated expression of cell cycle related genes both in fission and budding yeast. In S. pombe, the cdc10 gene product binds to this element and controls the cell cycle related genes. Electrophoretic mobility shift assays and molecular size determination studies indicated that spE2F is different from that encoded by cdc10. Thus, our studies suggest that spE2F is a novel transcription factor. We discuss these results in light of recent observations about the periodically expressed genes involved in the cell cycle progression in yeast.

E2F is a cellular transcription factor first identified in studies of the human adenovirus E2-early promoter (1). The E2F recognition sequence TTTCCGGC, or a closely related sequence is found in two E1A-responsive adenovirus early promoters, E1A and E2-early (referred to as E2 promoter in this paper) and several cellular promoters such as dihydrofolate reductase and c-myc (reviewed in Refs. 2 and 3). The activity of E2F is regulated by a number of factors. In virus-infected cells, DNA binding activity of E2F is increased considerably by the 17-kDa polypeptide encoded by the adenovirus early region 4 and also by the viral pre-early gene E1A (2, 3). In mammalian cells, the tumor suppressor gene product Rb physically interacts with E2F (4-6) or another transcription factor, DRTF1, which is related to E2F (7, 8); this interaction may influence the activity of these transcription factors during cell cycle progression. Other studies have shown that E2F is present as a complex with cyclin A, cyclin E, p107, and p33cdk2 protein kinase, indicating the existence of other mechanisms to control the E2F activity (9-15). In addition, E2F complexes are regulated during the differentiation of rapidly growing teratocarcinoma stem cells into postmitotic differentiated cells (16). Thus, E2F may play an important role in the control of proliferation of mammalian cells.

Regulation of transcription of genes involved in cell cycle control has been reported both in budding and fission yeast. In Saccharomyces cerevisiae, a sequence motif ACGCGTNA, also known as the MluI cell-cycle box or MCB, has been identified as an upstream activator element in a number of promoters of genes that are coordinately regulated in the G1/S phase of the cell cycle (17-19). Cell cycle-regulated transcription of these genes is controlled by a transcription complex called DNA synthesis complex or DSC-1 (19). Two transcription factors, p120 and SWI, are believed to be a part of this transcription complex (20-24). Currently, the relationship between p120 and another transcription factor, SWI4, is not clear. Biochemical and genetic studies suggest that the SWI6 protein of the DSC-I complex binds to the MCB sequence (21, 22). Recent studies indicate that the MluI system is conserved in the distantly related fission yeast Schizosaccharomyces pombe. The cdc22 that encodes the large subunit of the ribonucleotide reductase is expressed at the G1/S phase boundary in S. pombe (25). The promoter of this gene contains 7 MCB sequences (26). A DSC-1-like activity

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The abbreviations used are: MCB, MluI cell-cycle box; DSC, DNA synthesis complex; nt, nucleotide(s); PIPES, 1,4-piperazinediethanesulfonic acid; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; Chaps, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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was detected in S. pombe that recognizes the MCB sequences of the cdc22 promoter; the cdc10 protein required for the start and entry into S phase in S. pombe is a part of this activity (26). Another protein, Sct1, has also been shown to be a part of the DSC-1-like activity in S. pombe indicating that additional proteins may be present in the DSC-1 complex in fission yeast (7). Sct1 may exist as a complex with cdc10 protein (27).

Thus, it appears that there are striking similarities between the yeast and mammalian systems with regard to the regulation of the expression of genes involved in cell cycle control. In addition, it is now clear that, from yeast to man, many transcription factors are conserved throughout evolution. These observations prompted us to ask whether the E2F motif would activate transcription in S. pombe and whether S. pombe cells contain proteins that would bind to E2F motifs. In this paper we show that a single E2F site can activate transcription of a reporter gene in S. pombe when placed upstream of a minimal promoter. By using a DNA-affinity column which contains the E2F recognition sequence, we have partially purified a 30-kDa protein from S. pombe, designated S. pombe E2F (spE2F), and compared the binding specificity of this protein with that of human E2F. spE2F recognizes a sequence closely resembling the E2F site, and this transcription factor is different from that encoded by the cdc10 gene. We discuss these results in light of recent results of the regulation of the periodically expressed yeast genes during cell cycle.

MATERIALS AND METHODS

Yeast Strains and Plasmids—S. pombe strain h+, ade6-704, leu1-32, his3-237, ura4-d88 (28) was used for promoter expression studies. For protein purification, a protease-deficient strain, TM011 (h-, leu1-32) (a gift from Jo Ann Wise, Urbana-Champaign) was used. Plasmids which contain a wild type adenovirus E2F promoter fused to a reporter gene, chloramphenicol acetyltransferase (CAT) (pE2F1CAT), and the linkerless (LS) mutant derivatives which individually mutate the proximal (LS31-41) and the distal (LS55-66) E2F sites and the ATF site (LS74-85) of the +1 promoter have been described earlier (29, 30). The wild type (WT) and mutant promoters fused to CAT coding sequences were cloned into an autonomously replicating yeast shuttle vector, pURA4 (31), using standard recombinant DNA procedures described elsewhere (32). Plasmid pCPE contains the TATA motif of the human a-chlorionic gonadotropin gene promoter, which drives transcription of the CAT gene (33). The vector also contains the autonomously replicating yeast sequence ami1 and the URA3 gene for selection.

Partial Purification of a Polypeptide from S. pombe That Binds to the E2F Site—S. pombe cells (protease-deficient strain TM011) grown in YEL media as described above were harvested from logarithmically growing culture (10 liters). The cells were suspended in a lysis buffer containing 10 mM Tris-HCl, pH 7.6, 5 mM KCl, 1.5 mM MgCl2, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 500 units/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml N-p-tosyl-l-lysine chloromethyl ketone (TLCK), 1 μg/ml tosylphenylalanil chloromethyl ketone (TPCK), 1 μg/ml antipain, and 1 μg/ml pepstatin A and broken by vortexing with glass beads (500 microns) 6 times for 30 s at 0 to 4 °C. The cell extracts were clarified by centrifugation at 30,000 g for 20 min, and the supernatant (nuclear extract) was dialyzed in a buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM KCl, 1.5 mM MgCl2, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 500 units/ml aprotinin, 1 μg/ml leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride.

RNA Analysis—Yeast cells were transformed with pURA4, which contains WT E2 CAT construct (pU4E2CAT) and the mutant derivatives, according to the method of Beach and Nurse (28). Transformed colonies containing stably replicating plasmid were grown in YEL media (0.5% yeast extract and 3% glucose supplemented with 0.1 mg/ml L-leucine, 0.1 mg/ml adenine, and 0.1 mg/ml uracil) at 28-30 °C to a density of 5 × 10⁶/ml. Polyclonal RNA was prepared from transformed yeast cells in the midexponential phase (31) and used for primer extension analysis (30). Briefly, a 27-nucleotide (nt)-long CAT primer corresponding to nucleotides +110 to +136 (with reference to the E2F site) was 5'-end-labeled with 32P-dATP (2.5 μCi/ml) and 0.25 μCi/ml [α-32P]dATP. Ten units of avian myeloblastosis virus reverse transcriptase were then added, and the sample was incubated for 3 h at 42 °C. The reaction was stopped by adding RNase A (40 μg/ml) and EDTA (20 mM) and incubated at 37 °C for 30 min; the products were analyzed on a 6% DNA sequencing gel. Alcohol dehydrogenase mRNA was quantitated under identical conditions using 5 μg of poly(A) RNA with an appropriate primer (37).

Assay for Copy Number of E2 Plasmids—To determine the copy number of the WT and mutant derivatives of the pU4E2CAT in S. pombe transformants, total DNA was prepared from a portion of the E2CAT transformants for the primer extension analysis. The assay for the pU4E2CAT copy number was performed as described by Durkacz et al. (38). Equal quantities of DNA were immobilized onto a nitrocellulose filter using the Bio-Rad slot blot apparatus as described (32) along with different copy numbers of purified pU4E2CAT DNA. The filter was then hybridized with [32P]-labeled random-paired 1.6-kilobase DNA fragment coding for the CAT gene, generated by digesting the pU4E2CAT plasmid with Xba I and Bst EII. The signals were quantitated by a densitometer, and the copy numbers were determined by comparing the signals with that of pU4E2CAT DNA. The filter was then reprobed with a DNA fragment coding for the single-copy cdc2 gene (39). The copy numbers of the pU4E2CAT in these transformants were normalized with respect to cdc2 gene.

Preparation of Clarified Extracts from S. pombe—Clariﬁed cell extracts were prepared according to the protocol of Barker et al. (34). Briefly, logographically growing S. pombe cells (5 × 10⁶/ml) were harvested and washed in 0.9% saline, and the cell pellet was suspended in 5 ml of a buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.3 M KCl, 0.2% Nonidet P-40, 10% glycerol, 2 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride. Cells were then mixed with an equal volume of glass beads (150 microns) and disrupted by vigorous vortexing 4 times for 30 s. All steps were carried out at 0 to 4 °C. The cell extracts were centrifuged by centrifugation at 30,000 × g.
Preparation of HeLa Cell Nuclear Extracts—HeLa cells were grown in suspension minimum essential medium containing 7% donor calf serum to a density of 6 × 10⁶ cells/ml and harvested, and the nuclear extract was prepared exactly as described by Dignam et al. (41). Briefly, the cells were washed in phosphate-buffered saline and resuspended in 2 packed cell volumes of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM DTT) and lysed by 10 strokes in a Kontes Dounce glass homogenizer with a B type pestle. The nuclei were pelleted by centrifuging the homogenate at 25,000 × g for 20 min. The nuclear pellet was then suspended in 5 ml of buffer C (20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) per 10⁶ cells and homogenized with type B pestle (10 strokes), and this suspension was stirred gently with a magnetic stirring bar for 30 min at 4 °C. It was then centrifuged at 25,000 × g for 30 min, and the supernatant (nuclear extract) was dialyzed against buffer D (20 mM HEPES, pH 7.9, 20% (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) and stored at -80 °C. Protein concentration of the nuclear extract was determined by the method of Bradford (35).

EMSA—EMSA of the partially purified spE2F was carried out as follows. Approximately 0.3 µg of the 5'-end-labeled oligonucleotide which contained the E2F binding site was mixed with 100 ng of salmon sperm DNA in a buffer containing 10 mM Tris-HCl, pH 7.5, 30 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% glycerol, 1 µg of BSA, and 1 to 2 µg of affinity-purified protein in a total volume of 30 µl. The sample was incubated for 30 min at 4 °C, mixed with 3 µl of 50% (v/v) glycerol that contained 0.1% marker dyes and 1% Chaps (Sigma). The DNA-protein complexes were resolved on 5% native polyacrylamide gels. Electrophoresis was carried out at 4 °C using Tris-acetate-EDTA buffer (0.7 mM Tris-HCl, pH 7.5, 3.3 mM sodium acetate, and 1 mM EDTA). The gels were dried before autoradiography.

UV Cross-linking—Photochemical cross-linking of the protein to the DNA was carried out as described by Chodosh et al. (43), with minor modifications. An aliquot of the EMSA was placed on parafilm and irradiated for 4 min at a distance of 4.5 cm from a UV source (300 nm filter, Fotodyne). Photoadducts were analyzed on a 12% SDS-PAGE. The gel was dried before autoradiography.

Southwestern Blotting—Fifty µg of protein obtained by 1 M salt elution of a heparin-agarose column was separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond C, Amersham) using a mini Trans-Blot apparatus. The membrane was preincubated for 1 h with a blocking buffer (20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) and stored at -80 °C. Protein concentration of the nuclear extract was determined by the method of Bradford (35). The samples were incubated for 30 min at 4 °C, mixed with 3 µl of 50% (v/v) glycerol that contained 0.1% marker dyes and 1% Chaps (Sigma). The DNA-protein complexes were resolved on 5% native polyacrylamide gels. Electrophoresis was carried out at 4 °C using Tris-acetate-EDTA buffer (0.7 mM Tris-HCl, pH 7.5, 3.3 mM sodium acetate, and 1 mM EDTA). The gels were dried before autoradiography.

RESULTS

Activation of a Reporter Gene by an E2F Site in S. pombe—To determine whether an E2F site can function as an upstream activator element in S. pombe, we cloned 1 and 2 copies of an oligonucleotide containing the E2F recognition sequence of the WT or mutant E2F sites were inserted at the polylinker location as that of the WT E2F site, except for the mutations within or outside the E2F recognition sequence (Fig. 1B; bases mutated are underlined). WT, M1, M2, and M-6 contain alterations within the E2F recognition sequence and are not expected to function, whereas mutant, M-4, which contains alterations outside the E2F recognition sequence is expected to function at WT levels (Fig. 1B). S. pombe cells were transformed with these plasmids, and about 20,000 transformants in each case were pooled from the plate, washed, and lysed, and protein concentrations were determined as described under "Materials and Methods." 0.5 µg of protein from each cell lysate was used for CAT assays. The experiment was repeated at least three times for each plasmid in which the CAT assays were carried out from cell lysates prepared from newly transformed S. pombe cells. In this approach, which was also used by others (33, 36), it is not necessary to determine the copy number of the plasmids as equal quantities of protein derived from nearly 20,000 pooled transformants were used for comparing the CAT protein. By pooling a large number of transformants, we eliminate interclonal variations in the copy number. Any error
that may arise in quantitating the activity of the promoter constructs due to variations in the copy number in the transformants is negligible. Average CAT activities obtained from three independent experiments are shown in Fig. 1B. The parent plasmid, pCEH, did not show any CAT activity, whereas pCEHc, with a single copy of the E2F site, displayed high levels of CAT activity, indicating that the E2F site can function as an upstream activator in S. pombe (Fig. 1B). CAT activity was negligible for mutants M-1 and M-6 in which the E2F site was mutated with multiple base changes. Mutant M-3, in which the E2F site was replaced by contiguous A residues, was also defective for transcription; the CAT activity was reduced 5-fold in this case. In contrast, mutant M-4, whose mutations are outside the E2F recognition site, activated transcription to near WT levels. These results show that transcriptional activation of the reporter gene in pCEH results from the E2F recognition sequence and not the presence of fortuitous cis-acting elements in the oligonucleotides. When 2 copies of the E2F site were cloned in tandem, CAT activity increased 2-fold (data not shown). Thus, we conclude that the E2F site can function as an upstream activator element in S. pombe.

Requirement of E2F Sites for Transcription of the Adenovirus E2 Promoter in S. pombe—Because the promoter sequence from LS -55/-66 affects the TATA motif of the -26 promoter; the TAAATT sequence is changed to CCGGTT. We previously showed that the E2F sites are critical for the expression of the +1 promoter activity in mammalian cells, within the context of the viral chromosome, the TTAAGA sequence around -30, the 2 E2F sites present in an inverted orientation and the ATF site are all critical for the expression of the +1 promoter (30).

To determine the role of E2F sites in E2 promoter function in S. pombe, the LS mutants described above (Fig. 2) were rebuilt into pURA4, and expression of the mutant promoters was quantitated by determining the mRNA levels by the primer extension approach. Poly(A)* RNA was prepared from yeast cells transformed with pURA4 containing the WT and mutant promoters and annealed with a 5*-end-labeled 27-nt-long primer complementary to +110 to +136 (with respect to the +1 start site) of the CAT gene. The primer was then extended with reverse transcriptase, and the extended products were resolved on a DNA sequencing gel. This strategy can distinguish between the mRNAs transcribed from the 2 overlapping promoters. The mRNAs transcribed from the +1 promoter should generate a 136-nt-long product, whereas mRNAs transcribed from the -26 promoter should generate a product 162 nt long. As an internal control, expression of the chromosomal alcohol dehydrogenase gene (37) was also quantitated using an appropriate primer. The radioactive bands in the autoradiogram were quantitated by a laser densitometer. Unlike the experiment described above, these experiments were performed by growing individual transformants in large scale cultures. It was therefore necessary to determine the copy number of the pU4E2CAT in the transformants. Total DNA from each transformant was prepared and hybridized to a DNA fragment that encodes CAT coding sequences using the slot blot hybridization approach described under “Materials and Methods.” The copy number of pU4E2CAT in the total DNA was determined by including a known amount of pU4E2CAT DNA in each experiment. The mRNA levels were then normalized to copy number of pU4E2CAT in the transformants. Linker scan mutants -31/-41 and -55/-66 mutually activate the proximal and distal E2F sites, respectively, and, in human cells, these mutants transcribe with much reduced efficiency (30). In S. pombe, transcription from LS -31/-41 and -55/-66 was reduced approximately 4- to 6-fold (Fig. 3 and Table I) indicating that these 2 E2F motifs are critical for the E2 promoter activity in S. pombe. The requirement of the 2 E2F sites for the activity of this promoter in S. pombe is not surprising. This promoter has a unique architecture in that the two E2F sites are positioned in an inverted orientation and form a part of a large imperfect palindrome. Proper spacing and orientation of these 2 E2F sites are critical for the promoter activity in mammalian cells, and E2F binds to these two sites cooperatively (52). Mutations in any one of the two E2F sites abolishes its activity (52). As in mammalian cells (29, 30, 42, 47-50), the CAT site appears to be critical for the E2 promoter activity in S. pombe. A yeast homolog of mammalian ATF has been reported both in S. cerevisiae (53, 54) and in S. pombe (54, 55). Transcription from the -26 cap site was also reduced for the 3 LS mutants used in this study (Fig. 3). As stated above, LS -55/-66 mutates the TATA motif of the -26 promoter, which may be a reason for the reduced activity of this mutant with respect to the -26 promoter. It is also conceivable that the cis-acting motifs of the +1 promoter also control the -26 promoter. This aspect was not investigated further.

Identification of a Protein in S. pombe Extracts That Binds to the E2F Site—The demonstration that the E2F site can
activate a reporter gene in *S. pombe*, combined with the observation that 2 E2F sites are required for the E2 promoter function in *S. pombe* strongly supports the existence of E2F-like activity in *S. pombe*. We have used biochemical approaches to determine whether the *S. pombe* cells contain a factor that binds to the E2F motif. Nuclear extracts were prepared from a protease-deficient strain of *S. pombe* growing at logarithmic phase and loaded onto a heparin-agarose column. Extreme caution was taken to prevent proteolysis by adding a variety of protease inhibitors in all buffers during purification (see "Materials and Methods"). The column was washed with 0.3 M NaCl and then eluted with 1 M NaCl. The fractions were assayed for E2F binding activity with EMSA with the E2F site closer to the +1 cap site of the E2 promoter as a probe (nucleotide sequence corresponding to -50 to -27; Fig. 2). A distinct complex was detected in EMSA that could be competed with the homologous unlabeled oligonucleotide containing an E2F site. There was also high background in these gels (data not shown). Therefore, we further purified the protein preparation with the DNA-affinity column described by Kadonaga and Tjian (39), using the above-mentioned E2F site DNA. The protein fraction from the heparin-agarose column was loaded onto the DNA-affinity column, which was then washed extensively with a buffer containing 0.1 M KCl and eluted with a buffer containing 1 M KCl. A silver-stained gel showed a prominent band migrating at approximately 30 kDa (see below); based on crude estimates, the protein is not more than 10% of the total protein. We have named this protein as *S. pombe* E2F (spE2F).

The 1 M eluate of the DNA-affinity column was then assayed for E2F binding activity in EMSA. A duplex oligonucleotide that contains the recognition site for the E2F (the same as that used for the DNA-affinity column) was 5'-end-labeled and incubated with the affinity-purified protein; the DNA-protein complexes were resolved in 5% native PAGE. A number of unlabeled oligonucleotides that contain mutations within and outside the E2F recognition sequence TTTCGCGC were used as competitors to establish the specificity of the binding. These included both single-base substitutions and multiple mutations. Many of these mutants were also tested with crude HeLa cell nuclear extracts for comparison. Fig. 4 shows the nucleotide sequence of the WT E2F site used as a probe in these studies, the different mutant oligonucleotides which carry the mutant E2F sites, and also the oligonucleotides which carry the AP1, NFκB, and the CREB/ATF binding sites used in the competition experiments. The ability of these oligonucleotides to compete with the WT E2F site in EMSA (results obtained from the experiments shown in Fig. 5) are also summarized in Fig. 4. As shown in Fig. 5, a DNA-protein complex (Fig. 5A, lanes 2, 17, 26, and 35) was detected that was competed out efficiently with unlabeled homologous oligonucleotide (lanes 3, 4, 18, and 27). Mutant oligonucleotides that contain mutations outside the E2F recognition sequence competed efficiently (M-4, M-5, and M-14, lanes 8, 9, and 24, respectively). Mutant oligonucleotides M-1, M-2, and M-3 contain several bases mutated within the E2F recognition site, whereas mutants M-8, M-10, and M-12 each contain 2 base mutations. All of these mutants failed to compete with the E2F recognition site in EMSA except the mutant M-10 (see below) (Fig. 5A, lanes 5, 6, 7, 15, 20, and 22). Further, oligonucleotides that contain recognition sequences for transcription factors AP1, NFκB, and CREB/ATF (Fig. 4) did not compete (Fig. 5A, lanes 11–13). These results indicate that the protein spE2F that we identified in

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**Table I**

*Normalized values (percentage of WT) for mRNAs transcribed from the +1 and the −26 cap sites of the adenovirus E2 promoter in *S. pombe***

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Promoters</th>
<th>+1</th>
<th>−26</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>LS −31/−42</td>
<td>17.0</td>
<td>16.6</td>
<td></td>
</tr>
<tr>
<td>LS −55/−66</td>
<td>27.0</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>LS −74/−85</td>
<td>18.0</td>
<td>3.9</td>
<td></td>
</tr>
</tbody>
</table>

The radioactive bands from the autoradiogram shown in Fig. 3 were quantitated by a laser densitometer. The copy number of the pU4E2CAT plasmid in the *S. pombe* transformants was determined by slot-blot hybridizations as described under "Materials and Methods," and the signals in the autoradiogram shown in Fig. 3 were normalized to the copy number.
Fig. 4. Nucleotide sequences of the various oligonucleotides which contain mutations in the E2F site, and the oligonucleotides which contain AP1, CREB/ATF, and the NFkB binding sites, used in EMSA as competitors. Nucleotide sequences of only one strand are shown. E2F binding sequences in WT are underlined and numbered from +1 to +8. Mutations are shown in boxes. Recognition sequences for AP1, CREB/ATF, and NFkB are shown in italics. Binding refers to the ability to compete in EMSA with WT E2F at 100-fold molar excess. The binding data shown here are obtained from the experiments shown in Fig. 5.


table

\begin{tabular}{|c|c|c|}
\hline
\textbf{E2F SITE} & \textbf{BINDING S. POMBE} & \textbf{HELA} \\
\hline
\textbf{WT} & AAGGACTAGTTTCGGCCCTTTCT & + \\
\textbf{M-1*} & GAGCTTTTTGTTTTTTTCTCTTCT & - ND \\
\textbf{M-2*} & GTGTCAAAACTGGGTTTTTTCT & - ND \\
\textbf{M-3*} & GAGTTTTTTTTTTTTTTTTTTTTTTTTCT & - ND \\
\textbf{M-4*} & GAGCTTTTTTTTTTTTTTTTTTTTTTTCT & + ND \\
\textbf{M-5*} & GAGTTTTTTTTTTTTTTTTTTTTTTTTCT & + ND \\
\textbf{M-6*} & GAGTTTTTTTTTTTTTTTTTTTTTTTTCT & - ND \\
\textbf{M-7} & ACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & + +/- \\
\textbf{M-8} & ACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & - \\
\textbf{M-9} & AAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & + +/- \\
\textbf{M-10} & AAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & + +/- \\
\textbf{M-11} & AAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & +/- +/- \\
\textbf{M-12} & AAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & - \\
\textbf{M-13} & AAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & +/- +/- \\
\textbf{M-14} & AAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & + \\
\textbf{M-15} & AAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & +/- +/- \\
\textbf{M-16} & AAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & +/- +/- \\
\textbf{M-17} & AAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & +/- +/- \\
\textbf{M-18} & AAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & +/- +/- \\
\textbf{M-19} & AAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & +/- +/- \\
\textbf{M-20} & AAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & - \\
\textbf{M-21} & AAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & - \\
\textbf{M-22} & AAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & + \\
\textbf{M-23} & AAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & +/- +/- \\
\textbf{M-24} & AAGGACTAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTCT & +/- +/- \\
\hline
\textbf{AP1} & TCGAGGTCCGGCTGCTAGTGATGACCCCTCAGTCAGTA & - ND \\
\textbf{CREB/ATF} & GATCCCTCCTTCCGCTGCTACCCCTCAGTCAGTA & - ND \\
\textbf{NFkB} & GATCCAGGGCGATTCCCTCAGTCAGTA & - ND \\
\hline
\end{tabular}
cdc10 protein in *S. pombe*. To determine whether spE2F is related to the cdc10 protein, clarified whole cell extracts were prepared from *S. pombe*, and EMSA was carried out exactly as described (26) with a labeled probe containing 3 copies of the MCB sequence cloned in tandem (see legend to Fig. 6 for the nucleotide sequence). As shown in Fig. 6, a DNA-protein complex was detected that could be competed out efficiently with unlabeled homologous oligonucleotide at 25- to 50-fold molar excess (Fig. 6, lanes 3-6). In contrast, an oligonucleotide which contained 3 copies of E2F site cloned in tandem did not compete with MCB for binding over a range of concentrations tested (Fig. 6, lanes 7-10). To confirm that the DNA-protein complex generated in the experiment shown in Fig. 6 is the result of the cdc10 protein binding to the MCB element, we repeated these experiments using clarified extracts prepared from a ts mutant of cdc10 as described by Lowndes et al. (26). Extracts from both WT and ts mutants were preincubated at 37 °C for 0, 15, 30, and 60 min before assaying for their binding activities in EMSA. A DNA-protein complex specific for the MCB sequence was detected in the case of WT extract even after 60 min of preincubation. However, in the case of the ts mutant, preincubation of the extract even for 15 min resulted in the disappearance of the DNA-protein complex confirming that the complex identified in the experiment shown in Fig. 6 is the result of the cdc10 protein (data not shown).

**Molecular Weight of spE2F**—To determine the molecular weight of spE2F, we used UV cross-linking and Southwestern blot hybridization approaches. The affinity-purified protein was incubated with the end-labeled E2F probe used for EMSA under conditions employed for EMSA and irradiated with a UV source as described by Chodosh et al. (43). The UV-irradiated complexes were resolved on a 12% SDS-PAGE. A major band migrating at the 44-kDa position was observed when the sample was irradiated with a UV lamp for an increasing length of time (Fig. 7A). This band was competed out with unlabeled homologous oligonucleotide at 25- to 50-fold molar excess (Fig. 7B). To establish the specificity of the cross-linking, we incubated the sample in the presence of 2 duplex oligonucleotides which contain mutations in the E2F binding sites (M-2 and M-6) and also the transcription factor AP1 binding site (Fig. 4). These mutants did not compete with the E2F site for UV cross-linking (Fig. 7C). These mutants also did not compete for the spE2F binding in EMSA (Fig. 5A, lanes 6, 10, and 11). The molecular size of the DNA-protein complex identified in the UV cross-linking experiment...
is approximately 44 kDa. After subtracting the molecular size of the probe used for the UV cross-linking, the molecular size of the spE2F is approximately 30 kDa. As a confirmation, we repeated the UV cross-linking experiments with uniformly labeled E2F probe which was substituted with bromodeoxyuridine. After UV cross-linking, the DNA-protein complex was digested with DNase I, and the labeled polypeptides were analyzed on an SDS-polyacrylamide gel. A 30-kDa labeled protein was detected in these experiments, which was competed out with an unlabeled E2F site (data not shown). We conclude that a 30-kDa polypeptide in S. pombe extract specifically recognizes the E2F binding site. The UV cross-linking studies also show a strong but diffuse band below the 26.6-kDa marker which is competed out with unlabeled E2F site but not with mutant E2F sites (Fig. 7, B and C). This could be due to degradation of spE2F during UV cross-linking. Alternatively, this could also be due to the presence of proteins related to spE2F in S. pombe that bind to the E2F site with high affinity. This aspect was not investigated further.

In an independent approach, we used Southwestern blotting to identify the proteins from S. pombe that bind to the E2F motif. Heparin-agarose column fractions eluted at 1 M NaCl were fractionated on 10% SDS-PAGE, renatured, transferred to a nitrocellulose membrane, and hybridized with a 5'-end-labeled double-stranded oligonucleotide containing the E2F recognition sequence (44). As shown in Fig. 8A, a single radiolabeled band corresponding to approximately 30 kDa was detected, suggesting that the size of the polypeptide that binds to the E2F site in S. pombe is 30 kDa. Consistent with these results, a major band migrating at the 30-kDa position was detected in silver-stained gels when the affinity-purified sample was fractionated on SDS-PAGE (Fig. 8B). Another major band with equal intensity migrating just below the 30-kDa band is also visible in this gel. This protein could be related to spE2F, or it might be a modified form of the spE2F (phosphorylated form, for example). Several less intense bands migrating faster than the 30-kDa band can also be detected in this gel. These products were not characterized further.

**DISCUSSION**

In mammalian cells, the transcription factor E2F binds to several cellular proteins, including the tumor suppressor gene product Rb (4–8), an Rb-related protein, p107, cyclin A, cyclin E, and the cdk2 kinase (9–15). Rb binds to E2F in G1 phase of the cell cycle, whereas p107, cyclin A, and cdk2 kinase bind to E2F in G2 phase. Currently, the significance of these complexes in the cell cycle is not clear. It is likely that these cellular proteins modulate E2F activity at different stages of the cell cycle. Furthermore, regulation of the E2F complexes has been observed upon retinoic acid...
Lune I, column fraction and 1-2 protein in obtained from the heparin-agarose column chromatography and the samples.

Significantly affect activation. Second, in fractionated on a 12% SDS-PAGE gel for the proteins obtained from the heparin-agarose column chromatography and the DNA-affinity chromatography. 10 μg of protein from heparin-agarose column fraction and 1-2 μg of the affinity-purified protein were fractionated on a 12% SDS-PAGE gel, silver-stained for the proteins obtained from the heparin-agarose column chromatography. Lane 1, heparin-agarose column fraction. Lane 2, affinity-purified fraction. The arrows indicate the 30-kDa polypeptide.

induced tumor cell differentiation (16). Given these observations, it is likely that such a protein would play a fundamental role in the growth of all eukaryotic cells.

Two independent approaches suggest the existence of a protein in S. pombe that binds to the E2F motif. First, an E2F site, when cloned upstream of a minimal promoter, can activate transcription. Only the oligonucleotides in which E2F site, when cloned upstream of a minimal promoter, can activate transcription. Only the oligonucleotides in which E2F sites are mutated fail to activate transcription; mutations outside the E2F binding site do not significantly affect activation. Second, in S. pombe, as in mammalian cells, the activity of the adenovirus E2 +1 promoter is dependent on the 2 E2F sites. A protein fraction from S. pombe nuclear extracts purified using a DNA-affinity column binds to an E2F site of the E2 promoter. Binding is specific to the E2F recognition sequence because mutations outside the binding site have little effect on binding, whereas a number of mutations within the recognition sequence prevent binding. A protein of 30-kDa molecular size cross-links to the E2F site in vitro. This protein is much smaller than the human E2F (56, 57).

Although there are a few differences with respect to the specificity of recognition of the E2F motif by human E2F and spE2F, it is clear that spE2F recognizes a variant of the 8-base-pair E2F binding site. This is evident from the competition studies. For example, both human and S. pombe E2Fs bind to variants of the E2F motif in which the first base is T, A, or C, whereas binding is considerably impaired when T is changed to G (see Fig. 4 for a summary of binding results). The last base (the +8 position) does not seem to be essential for spE2F binding because mutation of this base to other bases does not affect binding, although G at this position is less preferred. In contrast, human E2F fails to bind to a mutant E2F site in which C at the +8 position is changed to T or A. Like the mammalian protein, bases outside the E2F motif are not involved in spE2F binding since 2 of the mutants that contain mutations 5′ to the E2F site and 1 which contains mutations 3′ to the binding site do not affect binding. We have observed other minor differences in binding specificity between S. pombe and human E2Fs. For example, G at the +5 position is required for mammalian protein binding (C in this position was not tested in this study), whereas the yeast protein binds to mutants with A or T in this position. An accurate comparison is difficult at present because a consensus E2F site has not yet been determined. Although our mutational analysis is limited, these results suggest that the binding specificity of the yeast and mammalian proteins is similar but not identical. It is possible that spE2F is an E2F-related protein.

Two sequence motifs somewhat related to the E2F recognition motif have been identified as upstream activator elements in several promoters of the genes expressed under cell cycle control in budding yeast. One of these, the MluI cell cycle box or MCB, coordinately regulates a number of genes which encode products required for DNA synthesis at or near the G1/S phase boundary. Synthetic oligonucleotides containing MCBs inserted into the appropriate reporter plasmids are sufficient for the periodic expression of the reporter genes, indicating that MCB elements are responsible for the coordinate regulation and periodic expression of the DNA synthesis genes (17,18). S. cerevisiae cells contain a protein complex called DSC-1, that specifically binds to MCB. Transcription factors p120 and SWI6 are a part of this complex (20-24); genetic studies show that SWI6 is directly or indirectly responsible for the DSC-1 binding to MCB (21,22). In another study, partial purification of S. cerevisiae extracts has led to the identification of a 17-kDa protein, MCBF, that binds to the MCB element (58). Currently, the relationship between MCBF and the SWI6 protein is not clear. The MluI cell cycle box is also conserved in S. pombe. cdc22, which encodes a subunit of the ribonucleotide reductase, is the only gene so far known to be expressed at the G1/S phase boundary in S. pombe (25). The promoter of this gene contains 7 MCBs and, like S. cerevisiae, synthetic MCBs can confer periodicity to reporter genes (26). A DSC-1-like activity has been detected in S. pombe (22), and the cdc10 gene product is part of this complex. The cdc10 gene product appears to be responsible for the regulation of MCB-containing genes in S. pombe because (a) cdc10 mutants down-regulate the expression of the cdc22 gene; (b) cell extracts prepared from cdc10 mutants failed to form a DNA-protein complex in EMSA with MCB probes; (c) by supershift assays, the cdc10 gene product has been shown to be a part of the S. pombe DSC-1-like complex (22); and (d) there is a significant amino acid sequence homology within the 233-amino-acid repeats of transcription factors SWI4, SWI6, and the cdc10 gene product (59). Interestingly, in spite of all these observations, cdc10 does not complement SWI4 and SWI6 gene products in S. cerevisiae (59). For several reasons, we believe that the 30-kDa protein identified in our study is different from the cdc10 gene product. First, the E2F site does not compete with the MCB sequence in EMSA even at 200-fold molar excess (Fig. 6). Second, the molecular size of spE2F is much smaller than that of the cdc10 gene product, which is 85 kDa (60). The molecular size of spE2F was confirmed in numerous experiments by Southwestern blot and UV cross-linking studies. Extreme precautions were taken to avoid possible proteolysis during protein purification by adding a variety of protease inhibitors. Third, the binding specificity of the spE2F is clearly different from that of the S. pombe MCB binding protein. spE2F recognizes at least 7 of the 8 bases of the E2F motif, whereas the MCB consists of a palindromic 6-base pair sequence. Binding of spE2F was unaffected when C at the fourth position of the TTTCCGCC sequence was changed to A or G. Significant spE2F binding also was observed when G at the fifth position was changed to T or A. In contrast, the
central CGCC sequence in the MCB sequence has been strongly conserved. Indeed, a recent survey of 40 MCB sequences from different promoters shows the frequency of 30/32 for A in the first position, 31/32 for C in the second position, 32/32 for G in the third position, 31/32 for C in the fourth position, 32/32 for G in the fifth position, and 32/32 for G in the sixth position (18). That there is efficient or significant binding of spE2F to mutants TTTTCGCC, TTTTGGCC, and TTTTACGC clearly shows that this protein is different from the MCB binding protein. spE2F is also different from the newly reported Sct1 protein; the Sct1 protein probably functions as a heterodimer with cdc10 protein, and its molecular size is 72 kDa (27).

In S. cerevisiae, another complex containing SWI4/SWI6 proteins controls transcription of a set of genes which includes G1 cyclins and the site-specific endonuclease HO; these genes are expressed at the G1/S phase boundary (18). The promoters of these genes contain a sequence CACGAAA which is in part complementary to the E2F motif TTTTCGCC, with the exception of C at +6 of the E2F motif. This motif has not been identified in promoters of fission yeast. Our results show that spE2F does not recognize this sequence. The mutant TTTTCGTC (the complement of CACGAAA; mutant M-20, Fig. 4) fails to bind to spE2F (Fig. 5, lane 3). Our study therefore identifies a novel transcription factor in S. pombe that binds to a sequence closely related to the E2F recognition sequence. An activity that binds to the E2F site was also reported in S. cerevisiae recently (61) indicating the conserved nature of this protein. Currently, our data are insufficient to establish that the S. pombe protein that binds to the E2F site is a true homolog of mammalian E2F. Nonetheless, the presence of such a protein in S. pombe, together with the observation that E2F sites are functional in fission yeast raises the possibility that this protein may perform functions similar to those associated with human E2F. It should now be possible to obtain a cDNA clone for this protein and to determine its role in the growth regulation of S. pombe. These experiments are in progress.

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