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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, TTTCGCGC, 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 *Mlu*I 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 TTTCGCGC, 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 post-mitotic 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 *Mlu*I cell-cycle box or MCB,[1] has been identified as an upstream activator element in a number of promoters of genes that are coordinately regulated in the G_1/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 SWI6, 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 *Mlu*I 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 G_1/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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[1] The abbreviations used are: MCB, *Mlu*I 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.

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 (27). 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-dl8* (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 E2 promoter fused to a reporter gene, chloramphenicol acetyltransferase (CAT) (pE2WTCAT), and the linkerscan (LS) mutant derivatives which individually mutate the proximal (LS −31/−41) and the distal (LS −55/−66) E2F sites and the ATF site (LS −74/−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 pCEH contains the TATA motif of the human α-chorionic gonadotropin gene promoter, which drives transcription of the CAT gene (33). The vector also contains the autonomously replicating yeast sequence *ars1* and the *URA3* gene for selection.

Extract Preparation and CAT Assay—*S. pombe* cells (approximately 1.5×10^6) grown in Edinburgh minimal medium (supplied by BIO 101; catalog No. 10700) were transformed with pCEH vectors which contained the WT and the mutant E2F sites according to the method of Okazaki *et al.* (34) and plated on Edinburgh minimal medium-agar plates (BIO 101; catalog No. 10701) for 5 days at 30 °C. About 20,000 transformants were pooled from the plates with water, harvested and washed with water twice, and suspended in 1 ml of 1 M sorbitol which contained 0.1 M EDTA and 14 mM 2-mercaptoethanol. The cells were converted to spheroplasts by treating with lyticase (Sigma, 0.1 unit per μl) for 1 h at 30 °C. The spheroplasts were then harvested by centrifugation at 3000 rpm for 10 min at 4 °C, resuspended in 1.2 ml of an extraction buffer which contained 92 mM Tris-HCl (pH 7.4) and 42 mM EDTA, and sonicated on ice for 1 min by a Branson sonifier 450 (output control 2.5). Protein concentrations in the extracts were determined according to Bradford (35) using the Bio-Rad protein assay kit (catalog No. 500-0006). CAT activity in the lysates was determined by an enzyme immunoassay using the CAT-ELISA kit of Boehringer Mannheim Corp. (catalog No. 1362-727) under conditions exactly as recommended by the vendor. Equal quantities of protein were used in the CAT assays (36).

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 5×10^6/ml. Poly(A$^+$) 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 +1 cap site) was 5′-end-labeled with T4 polynucleotide kinase and gel-purified; 10^5 cpm of this primer was annealed overnight at 30 °C to 10 μg of the poly(A$^+$) RNA in 30 μl of hybridization buffer which contained 80% formamide, 40 mM PIPES, pH 6.4, 400 mM NaCl, and 1 mM EDTA. The nucleic acids were precipitated and resuspended in a buffer containing 50 mM Tris-HCl, pH 6.0, 40 mM KCl, 0.5 mM EDTA, 5.0 mM $MgCl_2$, 5.0 mM DTT, and 0.25 mM dNTPs. Twenty 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 cells used for the primer extension analysis according to Durkacz *et al.* (38). Equal quantities of DNA were immobilized on 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 ^{32}P-labeled random-primed 1.6-kilobase DNA fragment coding for the CAT gene, generated by digesting pU4E2CAT with *Xba*I and *Bgl*II. The signals in the autoradiogram 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 (38). The copy numbers of the pU4E2CAT in the transformants were normalized with respect to cdc2 gene.

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 $MgCl_2$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 500 units/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 1 μg/ml tosylphenylalanyl 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 and centrifuged for 10 min at 2,500 rpm. The pellet from this step was resuspended in the above lysis buffer containing 2 M NaCl and incubated at 4 °C for 60 min with constant shaking. The lysate was then spun at 2,500 rpm for 15 min, and the supernatant (nuclear extract) was dialyzed in a buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 10% glycerol, and 1 mM DTT (buffer A). All buffers used in the purification protocol contained the above-mentioned protease inhibitors. The nuclear extracts were then loaded on a heparin-agarose column previously equilibrated with buffer A. The column was then sequentially washed with buffer A and with buffer A containing 0.3 M NaCl. DNA-binding proteins were eluted with buffer A containing 1 M NaCl. The 1 M NaCl eluate of the heparin-agarose column was dialyzed in a buffer containing 25 mM Hepes, pH 7.5, 12.5 mM $MgCl_2$, 20% glycerol, 0.1% Nonidet P-40, 1 mM DTT, and 100 mM KCl (buffer B). The dialyzed fraction was mixed with 20 *A* units of salmon sperm DNA and incubated on ice for 10 min. The sample was then applied to a DNA-affinity column that contained oligomerized E2F sites (ACTAGTTTCGCGCCCTTTCT) as described by Kadonaga and Tjian (39). The column was washed with 5 column volumes of buffer B and then eluted with buffer B containing 1 M KCl. Protein concentrations were estimated by the method of Bradford (35).

Preparation of Clarified Extracts from S. pombe—Clarified cell extracts were prepared according to the method of Barker *et al.* (40). Briefly, logarithmically growing *S. pombe* cells (5×10^6/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 (500 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 clarified by centrifugation at $30,000 \times g$

for 20 min at 4 °C, and the supernatants were used for electrophoretic mobility shift assays (EMSA) (22).

Preparation of HeLa Cell Nuclear Extracts—HeLa cells were grown in suspension minimum essential medium containing 7% donor calf serum to a density of 6×10^5 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 $MgCl_2$, 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 3 ml of buffer C (20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) per 10^9 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 ng 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 DTT, 1 mM EDTA, 5% glycerol, 1 μg of BSA, and 1 to 2 μg of affinity-purified protein in a total volume of 30 μl. 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. EMSAs with the clarified extracts were carried out exactly as described (22). EMSAs of human E2F were carried out as described previously with salmon sperm DNA as the nonspecific competitor DNA (42).

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 buffer (blocking buffer) which contained 5% nonfat dry milk, 10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT. The membrane was then hybridized overnight at 4 °C with a blocking buffer that contained 0.2 to 0.4 μg of 5′-end-labeled E2F probe and 200 ng/ml salmon sperm DNA. The filters were washed twice after hybridization with blocking buffer without NaCl, dried, and autoradiographed (44).

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 upstream of the TATA motif that drives transcription of a reporter gene in an autonomously replicating shuttle vector. The vector used in this study, pCEH, contains the TATA motif fused to coding sequences for CAT and also the autonomously replicating yeast sequence, *ars1*, and the *URA3* gene for selection (33) (Fig. 1*A*). The pCEH vector also contains a polyadenylation sequence upstream of the TATA motif to prevent possible readthrough transcription from cryptic promoters that may be present in the vector portion of the plasmid into CAT coding sequences (33). The E2F site was cloned between the polyadenylation sequence and the TATA sequence, as shown in Fig. 1*A*. For a comparison, four additional plasmids with mutant E2F sites cloned in the same

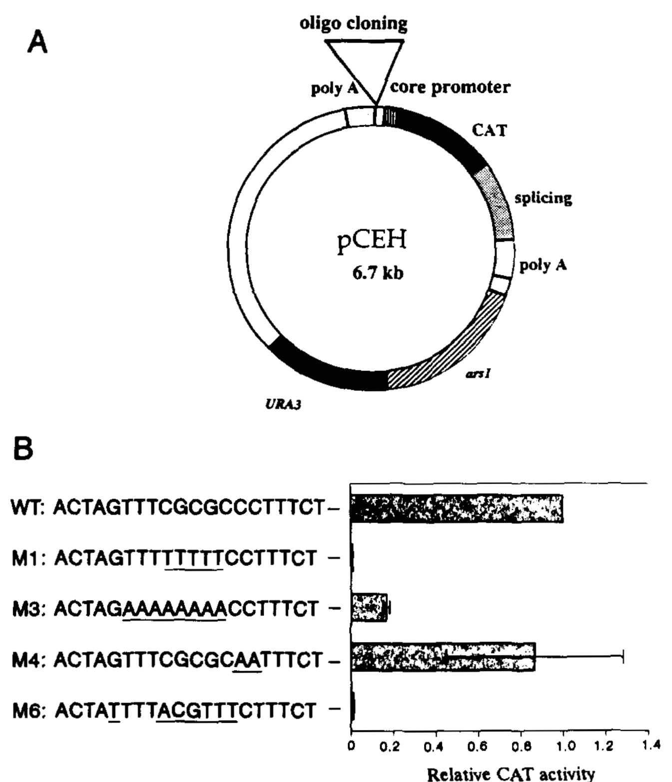

FIG. 1. **Transcriptional activity of pCEH core vector (33) containing E2F binding sequences.** *A*, diagram of the pCEH vector containing the WT or the mutant E2F sites. Oligonucleotides containing WT or mutant E2F sites were inserted at the polylinker site upstream of the TATA motif of pCEH, such that E2F binding sequences are in the same orientation as that of transcription. See "Materials and Methods" for the description of the vector. *B*, CAT activity of the pCEH variants which contain the WT and the mutant E2F sites and the sequences of the WT and mutant E2F oligonucleotides, *M1* to *M6*, used in this study. *S. pombe* cells were transformed with pCEH variants as described (34) and lysed, and the concentration of the protein in the lysates was determined by the method of Bradford (35). CAT activity was then measured with equal quantities of protein using the CAT-ELISA kit from Boehringer Mannheim under conditions recommended by the vendor. The *bar diagram* shows the average activities for the mutants relative to WT, obtained from three independent experiments.

location as that of the WT E2F site were constructed. These plasmids are identical with the pCEH, which harbors a single E2F site, except for the mutations within or outside the E2F recognition sequence (Fig. 1*B*; bases mutated are *underlined*). Mutants M-1, M-3, and M-6 contain alterations within the E2F recognition sequence and are not expected to function, whereas one mutant, M-4, which contains alterations outside the E2F recognition sequence is expected to function at WT levels (Fig. 1*B*). *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. 1*B*. The parent plasmid, pCEH, did not show any CAT activity, whereas pCEH, 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. 1*B*). 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 above results showed that the E2F sites can function in *S. pombe*, we determined whether the 2 E2F sites of the adenovirus E2 promoter are critical for its expression in *S. pombe*. We and others have shown that this is a dual, overlapping promoter in which transcription starts at +1, a major cap site, and −26, a minor cap site. These 2 start sites are controlled by two separate TATA motifs (30, 45, 46). Transcription from the major +1 cap site in human cells is dependent on 2 E2F sites and an ATF site (29, 30, 42, 46–50). Fig. 2 shows the nucleotide sequence of the E2 promoter and the +1 (major) and −26 (minor) cap sites for transcription in human cells. Binding sites for transcription factors ATF and E2F and the TATA motifs for the +1 and the −26 promoters are also shown. Previous studies have demonstrated that in human cells the +1 promoter is the major promoter, which is transcribed about 20-fold more efficiently than the −26 start site, which is a minor promoter in human cells (30, 45, 46). We recently expressed a chimeric gene in which the E2 promoter is placed in front of the CAT coding sequences after cloning into an autonomously replicating yeast shuttle vector, pURA4. Our studies showed that in *S. pombe* the E2-early promoter is transcribed faithfully. However, the relative ratio of transcription from +1 to −26 promoter is reversed; the −26 promoter is transcribed about 20-fold more efficiently than the +1 promoter (51). Nucleotide sequences of the previously characterized linker-scan mutant promoters that alter the ATF (LS −74/−85), the proximal (LS −31/−41), and the distal (LS −55/−66) E2F sites are shown below the WT promoter sequence (Fig. 2) (29, 30). These mutants were used in the present investigation. Note that mutations in LS −55/−66 affect the TATA motif of the −26 promoter; the TAAATT sequence is changed to CCGGGT. We previously showed that in human 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).

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                                                                         -26          +1
                     ATF     E2F  -26 TATA         E2F        +1 TATA
WT      GTGGCCGCTG GAGATGACGT AGTTTTCGCG CTTAAATTTG AGAAAGGGCG CGAAACTAGT CCTTAAGAGT CAGCGCGCAG TATTTACTGA AGAGA
                   -80                   -60                   -40                   -20

-31/-41 .......... .......... .......... .......... .........C ..G.T.CG.G .......... .......... .......... .....
-55/-66 .......... .......... ....CC..GA TCCGGG.... .......... .......... .......... .......... .......... .....
-74/-85 ......C.G. ATCCG.G... .......... .......... .......... .......... .......... .......... .......... .....
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FIG. 2. **Nucleotide sequences of the WT and the LS mutants of the adenovirus E2 promoter used in the promoter analysis (29, 30).** Nucleotides altered in the mutant promoters are shown in *small letters* and *underlined*. Binding sites for transcription factors ATF and E2F and TBP (referred to as +1 TATA and −26 TATA) are shown. *Thick arrows* indicate the orientation of the E2F sites. *Thin arrows* indicate transcriptional start sites.

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 as 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. Linkerscan mutants −31/−41 and −55/−66 mutate 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 ATF 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

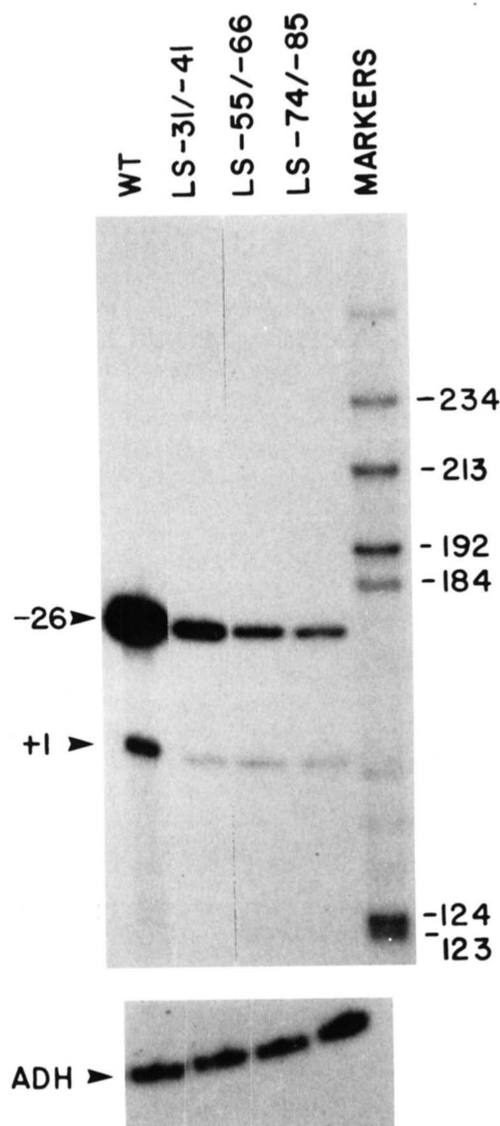

FIG. 3. **Primer extension analysis to determine the role of E2F and ATF sites in the E2 promoter activation.** *S. pombe* cells were stably transformed with pU4E2CAT plasmids containing the WT or the mutant derivatives of E2 promoter fused to CAT coding sequences. Poly(A+) RNAs were prepared from transformed *S. pombe* cells and annealed to a CAT primer (+110 to +136 with respect to +1 cap site) or alcohol dehydrogenase primer (+86 to +109) (37). The primers were then extended by reverse transcriptase and electrophoresed on a 6% DNA sequencing gel and autoradiographed. Markers, a 5′-end-labeled *Hae*III digest of pBR322 DNA. Only relevant portions of the autoradiogram are shown. Primer-extended products generated for the two mRNAs from the CAT coding sequences and the alcohol dehydrogenase mRNA are shown by *arrows*.

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).

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

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.

Mutants	Promoters	
	+1	−26
WT	100	100
LS −31/−42	17.0	16.6
LS −55/−66	27.0	7.6
LS −74/−85	18	3.9

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. 5*A*, *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. 5*A*, *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. 5*A*, *lanes 11–13*). These results indicate that the protein spE2F that we identified in

	E2F SITE (+1 to +8)	Binding S. POMBE	Binding HELA
WT	AAGGACTAG<u>TTTCGCGC</u>CCTTTCT	+	+
M-1*	GACTAGTTT[TTTTT]CCTTTCT	-	ND
M-2*	G[TGATCAAA]CGCGCCCTTTCT	-	ND
M-3*	GACTAG[AAAAAAAA]CCTTTCT	-	ND
M-4*	GACTAGTTTCGCGC[AA]TTTCT	+	ND
M-5*	GA[GTTC]TTTCGCGCCCTTTCT	+	ND
M-6*	GACTA[T]TTT[ACGTTT]CTTTCT	-	ND
M-7	ACTAGTTT[A]GCGCCCTTTCT	+	+/-
M-8	ACTAGT[A]TCG[A]GCCCTTTCT	-	-
M-9	AAGGACTAG[A]TTCGCGCCCTTTCT	+	+
M-10	AAGGACTAGT[AA]CGCGCCCTTTCT	+	+/-
M-11	AAGGACTAGTTTC[T]CGCCCTTTCT	+/-	-
M-12	AAGGACTAGTTTCG[TT]CCCTTTCT	-	-
M-13	AAGGACTAGTTTCGCG[T]CCTTTCT	+	-
M-14	AAGGACT[TC]TTTCGCGCCCTTTCT	+	+
M-15	AAGGACTAG[C]TTCGCGCCCTTTCT	+/-	+/-
M-16	AAGGACTAGT[C]TCGCGCCCTTTCT	+/-	+/-
M-17	AAGGACTAGTT[C]CGCGCCCTTTCT	-	+/-
M-18	AAGGACTAGTTT[G]GCGCCCTTTCT	+	+/-
M-19	AAGGACTAGTTTC[A]CGCCCTTTCT	+/-	-
M-20	AAGGACTAGTTTCG[T]GCCCTTTCT	-	-
M-21	AAGGACTAGTTTCGC[T]CCCTTTCT	-	-
M-22	AAGGACTAGTTTCGCG[A]CCTTTCT	+	-
M-23	AAGGACTAG[G]TTCGCGCCCTTTCT	+/-	+/-
M-24	AAGGACTAGTTTCGCG[G]CCTTTCT	+/-	+/-
AP1	TCGAGCTGCAGCTAGTGA*TGAGTCA*GCCGGATCA	-	ND
CREB/ATF	GATCCCTCCTTGGC*TGACGTCA*GAGAGAGA	-	ND
NFkB	GATCCAGA*GGGGACTTTCC*GAGA	-	ND

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 NFκB 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 NFκB are shown in *italics*. Binding refers to the ability to compete in EMSA with WT E2F site at 100-fold molar excess. The binding data shown here are obtained from the experiments shown in Fig. 5. *, M-1 to M-6 oligonucleotides contain *Sal*I and *Pst*I sites at the 5′ and 3′ ends, respectively. *ND*, not determined.

S. pombe cells recognizes the E2F binding sequence TTTCGCGC or a sequence that closely resembles this motif.

Comparison of the Binding Specificity of spE2F and E2F—To better understand the binding specificity of spE2F, EMSA was carried out in the presence of a number of single-base substitution mutations of the E2F recognition sequence as competitors, and the results were compared with that of human E2F. A strict comparison of these results is difficult because the protein preparations in these 2 cases are not of the same quality. The yeast protein is purified on a DNA-affinity column, whereas HeLa cell crude nuclear extracts were used as a source of human E2F. Nonetheless, with a few exceptions, in general there is a good correlation between binding specificity of spE2F and human E2F. There is no strict requirement for a specific base in the +1 position for both human and *S. pombe* E2Fs (the bases in the TTTCGCGC sequence are numbered +1 to +8, with the first T as +1; this numbering is unrelated to cap-site numbering). spE2F binding was not impaired when T is changed to A (Fig. 5*A*, *mutant M-9*, *lane 19*), and partial binding was observed when T was changed to C or G (Fig. 5*A*, *mutants M-15* and *M-23*, *lanes 28* and *38*) (see Fig. 4 for a summary of binding results). The nucleotide at +8 also seems to be less critical, although G at this position is not preferred (Fig. 5*A*, *mutant M-24*, *lane 39*). In contrast, human E2F did not bind when this base was mutated to T or A (Fig. 5*B*, *mutants M-13* and *M-22*, *lanes 8* and *21*); changing this base to G resulted in partial binding (mutant M-24, lane 23). Several other minor differences were also noted. Specifically, changing G at +5 to A prevented mammalian protein binding (Fig. 5*B*, *mutant M-19*, *lane 18*), whereas this mutation did not abolish binding to spE2F (Fig. 5*A*, *lane 32*). Further, changing T at +3 to C (M-17) prevented spE2F binding (Fig. 5*A*, *mutant M-17*, *lane 30*), while a weak but detectable binding to E2F was observed (Fig. 5*B*, *lane 16*). The mutations in M-10, in which 2 consecutive Ts at positions +2 and +3 were changed to A residues, did not affect binding of spE2F (Fig. 5*A*, *lane 20*), whereas this mutation resulted in partial binding to E2F (Fig. 5*B*, *lane 5*). In summary, although both the yeast and mammalian proteins recognize the E2F motif, the two proteins differ in their binding specificities in some respects.

Relationship between spE2F and cdc10 Protein—As indicated previously, the E2F binding sequence has a weak homology with the MCB element which is recognized by the

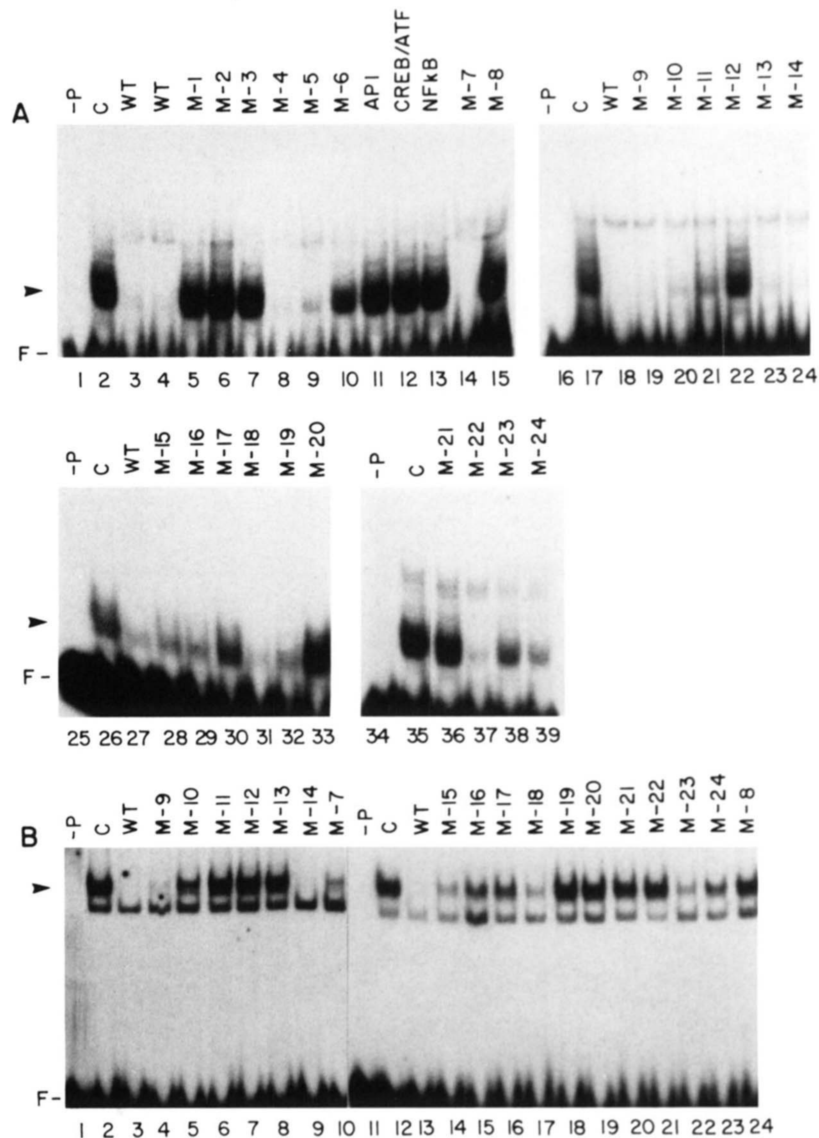

FIG. 5. **Competition experiments to show the binding specificity of spE2F and E2F in EMSAs.** A duplex oligonucleotide which contains the E2F site closer to the +1 cap site (WT sequence shown in Fig. 4) of the E2 promoter was 5′-end-labeled and incubated either with the *S. pombe* protein partially purified by a DNA-affinity column or crude HeLa cell nuclear extracts and with 100-fold molar excess of unlabeled oligonucleotides shown in Fig. 4. The DNA-protein complexes were resolved on 5% native PAGE, and the gel was dried and autoradiographed. *A*, EMSA with spE2F. *B*, EMSA with crude HeLa cell nuclear extracts. *−P*, without protein. *C*, sample without sequence-specific competitor. *F*, free probe. DNA-protein complexes are shown by *arrows*. The band below the E2F band in *B* is nonspecific.

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. 7*A*). This band was competed out with unlabeled homologous oligonucleotide at 25- to 50-fold molar excess (Fig. 7*B*). 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. 7*C*). These mutants also did not compete for the spE2F binding in EMSA (Fig. 5*A*, *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.

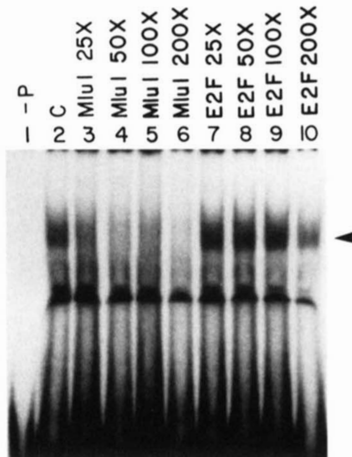

FIG. 6. **EMSA showing differences in binding specificities between the cdc10 and spE2F proteins.** Two μg of protein from clarified extracts prepared from *S. pombe* were incubated with a 5′-end-labeled oligonucleotide containing 3 copies of the MCB sequence, TCGACAACGCGT, cloned in tandem exactly as described by Lowndes *et al.* (22). DNA-protein complexes were separated using a native PAGE with Trisborate buffer, and the gel was dried and autoradiographed. The MCB-specific complex is shown by an *arrow*. *Lanes 3–6*, competition with unlabeled MCB sequence, 3 copies cloned in tandem at 25-, 50-, 100-, and 200-fold molar excess. *Lanes 7–10*, competition with 3 copies of an E2F binding site cloned in tandem at 25-, 50-, 100-, and 200-fold molar excess. −*P*, without protein. *C*, sample without sequence-specific competitor.

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. 8*A*, 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. 8*B*). 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 G_1 phase of the cell cycle, whereas p107, cyclin A, and cdk2 kinase bind to E2F in the S phase of the cell cycle. 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-

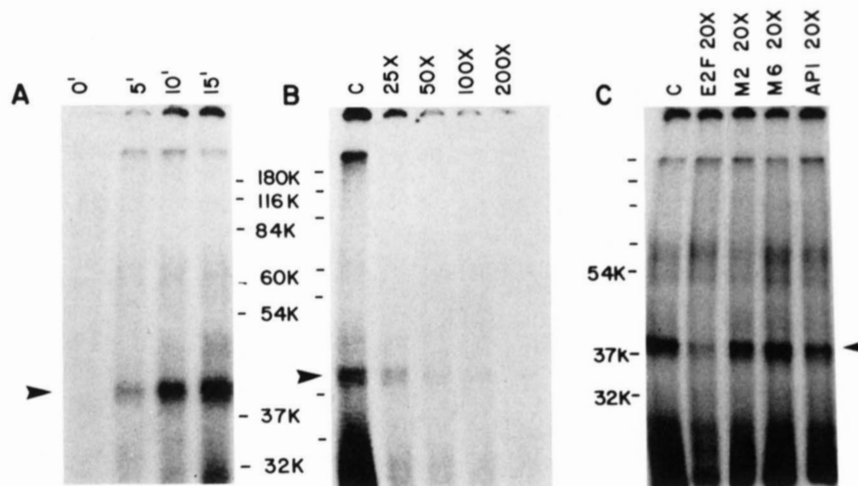

FIG. 7. **Identification of a polypeptide in *S. pombe* specific for the E2F site by UV cross-linking.** Binding reactions contained a 20-base-pair 5′-end-labeled duplex oligonucleotide that contained the WT E2F site (Fig. 4), 100 ng of salmon sperm DNA, and 1 to 2 μg of the affinity-purified spE2F and were irradiated with a UV source. The photoadducts were analyzed on a 12% SDS-PAGE. The gels were dried before autoradiography. *A*, a time course experiment in which the samples were cross-linked for 0, 5, 10, and 15 min before SDS-PAGE. *B*, competition with the unlabeled E2F site. *C*, competition with unlabeled WT E2F site, the mutant derivatives of the E2F site, *M-2* and *M-6*, and the AP1 binding site. For nucleotide sequences of these oligonucleotides see Fig. 4. The *numbers* at the *top* indicate molar excess of the unlabeled oligonucleotides used in the assays. Commercially available prestained protein molecular weight markers were used as standards. The cross-linked complexes are shown by *arrows*.

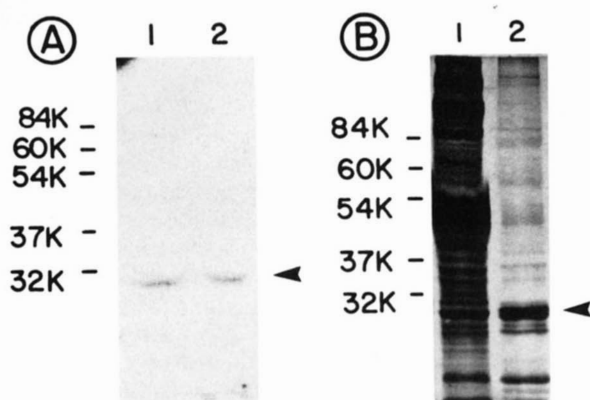

FIG. 8. **Southwestern blot analysis and silver-stained gels of the affinity-purified spE2F show a 30-kDa protein.** *A*, Southwestern blot analysis. Approximately 50 μg of protein from heparin-agarose column fractions were fractionated by SDS-PAGE, renatured, transferred to a nylon membrane, hybridized with ^{32}P-labeled E2F probe, and autoradiographed. *Lanes 1* and *2* are duplicate samples. *Numbers* to the *left* of *lane 1* indicate the position of the molecular weight markers. *B*, silver-stained 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 (62) and stained with silver (63). *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 recognition sequences 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 *Mlu*I cell cycle box or MCB, coordinately regulates a number of genes which encode products required for DNA synthesis at or near the G_1/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 *Mlu*I 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 G_1/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 2 33-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 TTTCGCGC 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 CGCG 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 TTTAGCGC, TTTCTCGC, TTTGGCGC, and TTTCACGC 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 G_1 cyclins and the site-specific endonuclease HO; these genes are expressed at the G_1/S phase boundary (18). The promoters of these genes contain a sequence CACGAAAA which is in part complementary to the E2F motif TTTCGCGC, 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 TTTCGTGC (the complement of CACGAAAA; *mutant M-20*, Fig. 4) fails to bind to spE2F (Fig. 5, *lane 33*). 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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REFERENCES

1. Kovesdi, I., Reichel, R., and Nevins, J. R. (1986) *Cell* **45**, 219–228
2. Flint, J., and Shenk, T. (1989) *Annu. Rev. Genet.* **23**, 141–161
3. Nevins, J. R. (1992) *Science* **258**, 424–429
4. Chellapan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M., and Nevins, J. R. (1991) *Cell* **65**, 1053–1061
5. Bagchi, S., Weinmann, R., and Raychaudhuri, P. (1991) *Cell* **65**, 1063–1072
6. Chittenden, T., Livingston, D., and Kaelin, W. G., Jr. (1991) *Cell* **65**, 1073–1082
7. Bandara, L. R., and LaThangue, N. B. (1991) *Nature* **351**, 494–497
8. Girling, R., Partridge, J. F., Bandara, L. R., Burden, N., Totty, N. F., Hsuan, J. J., and LaThangue, N. B. (1993) *Nature* **362**, 83–87
9. Mudryj, M., Devoto, S. H., Hiebert, S. W., Hunter, T., Pines, J., and Nevins. J. R. (1991) *Cell* **65**, 1243–1253
10. Devoto, S. H., Mudryj, M., Pines, J., Hunter T., and Nevins, J. R. (1992) *Cell* **68**, 167–176
11. Cao L. (1992) *Nature* **355**, 176–179
12. Shirodkar, S., Ewen, M., Decaprio, J. A., Morgan, J., Livingston, D. M., and Chittenden, T. (1992) *Cell* **68**, 157–166
13. Bandara, L. R., and LaThangue, N. B (1991) *Nature* **352**, 249–251
14. Pagano, M., Draetta, G., and Jansen-Durr, P. (1992) *Science* **255**, 1144–1147
15. Lees, E., Faha, B., Dulic, V., Reed, S. I., and Harlow, E. (1992) *Genes & Dev.* **6**, 1874–1885
16. Reichel, R. (1992) *Gene Exp.* **2**, 259–271
17. Andrews, B. J., and Herskowitz, I. (1990) *J. Biol. Chem.* **265**, 14057–14060
18. Johnston, L. H., and Lowndes, N. F. (1992) *Nucleic Acids Res.* **20**, 2403–2410
19. Andrews, B. J. (1992) *Nature* **355**, 393–394
20. Andrews, B. J., and Herskowitz, I. (1989) *Nature* **342**, 803–833
21. Taba, M. R., Muroff, I., Lydall, D., and Nasmyth, K. (1991) *Genes & Dev.* **5**, 2000–2013
22. Lowndes, N. F., Johnson, A. J., Breeden, L., and Johnston, A. L. (1992) *Nature* **357**, 505–508
23. Dirick, L., Moll, T., Auer, H., and Nasmyth, K. (1992) *Nature* **357**, 508–513
24. Siderova, J., and Breeden, L. (1993) *Mol. Cell. Biol.* **13**, 1069–1077
25. Gordon, C., and Fantes, P. (1986) *EMBO J.* **5**, 2981–2985
26. Lowndes, N. F., McInerny, C. J., Johnson, A. L., Fantes, P. A., and Johnston, L. H. (1992) *Nature* **355**, 449–453
27. Caligiuri, M., and Beach, D. (1993) *Cell* **72**, 607–619
28. Beach, D., and Nurse, P. (1981) *Nature* **200**, 140–142
29. Murthy, S. C. S., Bhat, G. P., and Thimmapaya, B. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 2230–2234
30. Manohar, C. F., Kratochvil, J., and Thimmapaya, B. (1990) *J. Virol.* **64**, 2457–2466
31. Jones, R. H., Moreno, S., Nurse, P., and Jones, N. C. (1988) *Cell* **53**, 659–667
32. Ausubel, F. M., Brent, R., Kingston, R. E., More, D. D., Smith, J. A., Seidman, J. G., and Struhl, K. (1987) *Current Protocols in Molecular Biology*, Greene Publishing Associates and John Wiley and Sons, New York
33. Toyama, R., and Okayama, H. (1990) *FEBS Lett.* **268**, 217–221
34. Okazaki, K., Okazaki, N., Kume, K., Jinno, S., Tanaka, K., and Okayama, H. (1990) *Nucleic Acids Res.* **18**, 6485–6489
35. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
36. Toyama, R., Bende, S. M., and Dhar, R. (1992) *Nucleic Acids Res.* **20**, 2591–2596
37. Russel, P. R., and Hall, B. D. (1983) *J. Biol. Chem.* **258**, 143–149
38. Durkacz, B., Beach, D., Hayles, J., and Nurse, P. (1985) *Mol. & Gen. Genet.* **201**, 543–545
39. Kadonaga, J. T., and Tjian, R. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 5889–5893
40. Barker D. G., Johnson, A. L., and Johnston, L. H. (1985) *Mol. & Gen. Genet.* **200**, 458–462
41. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489
42. SivaRaman, L., and Thimmapaya, B. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 6112–6116
43. Chodosh, L. A., Carthew, R. W., and Sharp, P. A. (1986) *Mol. Cell. Biol.* **6**, 4723–4733
44. Silva, C. M., Tully, C. B., Petch, L. A., Jewell, C. M., and Cidlowski, J. A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 1744–1748
45. Mathis, D. J., Elkaim, R., Kedinger, C., Sassone-Corsi, P., and Chambon, P. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 7383–7387
46. Zajchowski, D. A., Boeuf, H., and Kedinger, C. (1985) *EMBO J.* **4**, 1293–1300
47. SivaRaman, L., Subramanian, S., and Thimmapaya, B. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 5914–5918
48. Yee, A. S., Reichel, R., Kovesdi I., and Nevins, J. R. (1987) *EMBO J.* **6**, 2061–2068
49. Lee, K. A. W., Hai, T.-Y., SivaRaman, L., Thimmapaya, B., Hurst, H. C., Jones, N. C., and Green, M. R. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 8355–8359
50. Jalinot, P., Wintzerith, M., Gaire, M., Hauss, C., Egly J. M., and Kedinger, C. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 2484–2488
51. Swaminathan, S., Malhotra, P., Manohar, C. F., Dhar, R., and Thimmapaya, B. (1993) *Nucleic Acids Res.* **21**, 2737–2746
52. Hardy, S., and Shenk, T. (1989) *Mol. Cell. Biol.* **9**, 4495–4506
53. Lin, Y.-S., and Green, M. R. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 109–113
54. Jones, R. H., and Jones, N. C. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 2176–2180
55. Prentice, H. L., and Kingston, R. E. (1992) *Nucleic Acids Res.* **20**, 3383–3390
56. Kaelin, W. G., Jr., Krek, W., Sellers, W. R., Decaprio, J. A., Ajchanbaum, F., Fuchs, C. S., Chittenden, T., Li, Y., Farnham, P. J., Blanar, M. A., Livingston, D. M., and Flemington, E. K. (1992) *Cell* **70**, 351–364
57. Helin, K., Lees, J. A., Vidal, M., Dyson, N., Harlow, E., and Fattaey, A. (1992) *Cell* **70**, 337–350
58. Verma, R., Patapoutigan, A., Gordon, C., and Campbell, J. L. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 7155–7159
59. Breeden, L., and Nasmyth, K. (1992) *Nature* **329**, 651–654
60. Aves, S. J., Durkacz, B. W., Carr, A., and Nurse, P. (1985) *EMBO J.* **4**, 457–463
61. Mai, B., and Lipp, M. (1993) *FEBS Lett.* **321**, 153–158
62. Laemmli, U. K. (1970) *Nature* **227**, 680–685
63. Wray, W., Boulikas, T., Wray, V. P., and Hancock, R. (1981) *Anal. Biochem.* **118**, 197–203