Identification of an Essential Cis-Element Near the Transcription Start Site for Transcriptional Activation of the Proliferating Cell Nuclear Antigen Gene*

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Dan Yang Huang† and Michael B. Prystowsky‡

From the †Graduate group of Molecular Biology, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19104 and the ‡Department of Pathology, Albert Einstein College of Medicine, Bronx, New York 10461-2490

Interleukin 2 (IL-2) stimulates T lymphocyte proliferation and induces the expression of proliferating cell nuclear antigen (PCNA), a processivity factor for DNA polymerase δ. Previously, deletion analysis suggested cis-element(s) in the proximal region of the PCNA promoter (−40 to +143) are required for IL-2 induction in cloned T lymphocytes. The sequence 5′-TTGCGGGC-3′ located at +10 to +17 is similar to the E2F consensus binding site and is required for optimal PCNA promoter activity. In IL-2-stimulated T cells, nuclear proteins are induced to bind to this sequence as demonstrated using electrophoretic mobility shift assay (EMSA), competition EMSA, and methylation interference analysis. A 180-kDa polypeptide was detected by UV cross-linking to bind specifically to the PCNA E2F-like sequence. Our data indicate that the protein bound to the PCNA E2F-like site is not one of the transcription factor E2F proteins. Our results demonstrate that the E2F-like sequence and the protein(s) binding to it are required for optimal PCNA promoter activity and IL-2 induction of PCNA expression.

PCNA was first described by Miyachi et al. (1978) as a nuclear antigen restricted to proliferating cells that reacts with sera from some patients with the autoimmune disorder systemic lupus erythematosus, hence the name proliferating cell nuclear antigen. PCNA is an auxiliary factor for DNA polymerase δ. Previously, deletion analysis suggested cis-element(s) in the proximal region of the PCNA promoter (−40 to +143) are required for IL-2 induction in cloned T lymphocytes. The sequence 5′-TTGCGGGC-3′ located at +10 to +17 is similar to the E2F consensus binding site and is required for optimal PCNA promoter activity. In IL-2-stimulated T cells, nuclear proteins are induced to bind to this sequence as demonstrated using electrophoretic mobility shift assay (EMSA), competition EMSA, and methylation interference analysis. A 180-kDa polypeptide was detected by UV cross-linking to bind specifically to the PCNA E2F-like sequence. Our data indicate that the protein bound to the PCNA E2F-like site is not one of the transcription factor E2F proteins. Our results demonstrate that the E2F-like sequence and the protein(s) binding to it are required for optimal PCNA promoter activity and IL-2 induction of PCNA expression.

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† Recipient of an American Cancer Society Faculty Research Award. To whom correspondence should be addressed. Tel.: 718-920-2456; Fax: 718-882-8461.
‡ The abbreviations used are: PCNA, proliferating cell nuclear antigen; ConA, concanavalin A; CRE, cAMP response element; DHFR, dihydrofolate reductase; EMSA, electrophoretic mobility shift assay; IL-2, interleukin 2; Inr, initiator element; nt, nucleotide.
al., 1991). One hypothesis for the mechanism of coordinate regulation of DNA synthesis genes in mammalian cells is that the transcription factor E2F plays a central role in activating DNA synthesis genes during growth stimulation (Nevins, 1992). E2F represents a group of related transcription factors with similar DNA-binding specificity that interacts with PRB-related pocket proteins (La Thangue, 1994). Sequence comparison indicates that an E2F-like binding site is present between +10 and +17 of the murine PCNA promoter. In addition to the tandem CRE binding site, our previous results suggested that the proximal PCNA promoter containing the E2F-like site is critical for IL-2-induced transcriptional activation (Huang et al., 1994). Thus, we analyzed the requirement of this E2F-like site for optimal promoter activity and for protein-DNA interactions in the PCNA promoter proximal region and demonstrate a functional role for the E2F-like site in PCNA transcriptional regulation.

MATERIALS AND METHODS

Plasmids—A 1.8-kilobase PCNA genomic sequence was isolated previously from a Balb/c liver genomic library and subcloned into the pBluescript vector (Shipman-Appasamy et al., 1990). The pPCNAS-LUC vector was constructed by inserting polymerase chain reaction amplified DNA fragments containing the PCNA promoter (−182 to +143) into a HindIII site of the promoterless pSVOA vector (De Wet et al., 1987; Huang et al., 1994). Cells—The cloned murine T helper cell line, L2, was maintained as described previously (Prystowsky, 1989; Huang et al., 1994). Eight days following exposure to irradiated allogenic spleen cells and 10–20 units/ml IL-2, L2 cells were purified by Ficoll-Hypaque density centrifugation through Ficoll-Hypaque (1.090 g/ml). Splenocytes at a concentration of 6 × 10^7 cells/ml were transfected transiently into L2 cells by the DEAE-dextran method (Ausubel et al., 1994). Resting cell lines were washed with Dulbecco’s modified Eagle’s medium with additives containing 500 units/ml IL-2, L2 cells were cultured for 24 h in Dulbecco’s modified Eagle’s medium with additives containing 0.01 ng of endotoxin/3.6 × 10^8 units of IL-2; specific activity was evaluated by the manufacturer.

Freshly prepared splenocytes were from CBA/J mice, separated from erythrocytes using density gradient centrifugation through Ficoll-Hypaque (1.090 g/ml). Splenocytes at a concentration of 6 × 10^7/ml were transfected into L2 cells by the DEAE-dextran method (Ausubel et al., 1994). To increase the transfection efficiency, L2 cells were stimulated with 100–150 units/ml IL-2 and 10–20 units/ml ConA were incubated for 72 h prior to extraction of proteins. The procedures for UV cross-linking was essentially as described previously (Chodin et al., 1986). Partially methylated PCNA ST probes were 32P-labeled at 5'-end and incubated with L2 cell nuclear extract and poly(dI-dC). After gel electrophoresis (PAGE) at 4°C in containing 25 mM Tris, 190 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol. Either poly(dI-dC) or salmon sperm DNA was used as nonspecific competitor DNA. The gel was dried and autoradiographed. Then the gel was stained with ethidium bromide for 5 min. The pellet was resuspended and washed in 1 ml of a 10% solution of Nonidet P-40 was added and the tube was vigorously rocked at 4°C for 15 min on a shaking platform. The nuclear extract was centrifuged for 5 min in a microcentrifuge at maximum speed at 4°C and the supernatant was frozen in aliquots at −70°C. Protein concentration was quantitated using the Bradford reagent (Bio-Rad).

Electrophoretic Mobility Shift Assay (EMSA)—Electrophoretic mobility shift assay was performed as described previously (Garner et al., 1989). Briefly, 5,000–15,000 cpm of 32P-labeled duplex oligonucleotide probe (Fig. 1) and 3–5 μg of cell extract were mixed at room temperature for 15 min in 20 μl of binding buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol. Either poly(dI-dC) or salmon sperm DNA was used as nonspecific competitor DNA. The binding complexes were separated by a polyacrylamide gel electrophoresis (PAGE) at 4°C in containing 25 mM Tris, 190 mM NaCl, 1 mM EDTA at 25 mA. Gels were then dried and autoradiographed. The sequence of oligonucleotides (sense strand) used for EMSA are listed below with numbers indicating the positions of start and end bases of the oligonucleotides within the PCNA promoter. The binding sites for PE3A, Inr, and E2F are underlign as described previously (Garner et al., 1989). 0.5–1 μg of either a nonspecific oligonucleotide or PCNA promoters was determined to insure that the desired product was prepared and to confirm that the desired DNA fragment is in the correct orientation in the vector.

Methylation Interference—The methylation interference assay was performed essentially as described previously (Huang et al., 1994). Partially methylated PCNA ST probes were 32P-labeled at 5'-ends and incubated with L2 cell nuclear extract and poly(dI-dC). After gel electrophoresis, free DNA and protein bound DNA were cut and electrophoretically transferred to a DEAE membrane (Schleicher and Schuell) and eluted according to the supplier’s protocol. After cleavage with piperidine, the various DNA fragments were analyzed by urea-PAGE (15%) electrophoresis and autoradiography (Ausubel et al., 1992). Methylation and Gilbert DNA sequencing reactions were performed as described previously (Ausubel et al., 1992) and G. C, A, and T C reactions were carried using a commercial Maxam and Gilbert DNA sequencing kit (Du Pont, Westwood, MA) according to the manufacturer’s instructions.

UV Cross-linking—The procedures for UV cross-linking were essentially as described by Chodin et al. (1986). PCNA ST was labeled with both [32P]dCTP and bromodeoxyuridine triphosphate (Sigma). The binding reactions were scaled up 5-fold and 90 μg of nuclear extracts were used for each reaction. Initial studies showed maximal binding by 30 min. The reactions were irradiated, the products were mixed with SDS-PAGE gel loading buffer, and separated on a 10% SDS-polyacrylamide gel. The specificity of the photoaffinity labeling reaction was established by including 50,000 cpm of a nonlabeled oligonucleotide or PCNA ST. Only the latter dinucleotide should eliminate specific binding of PCNA-specific binding. We used an oligonucleotide containing a CCAAT box motif derived from the PCNA promoter (−99 to −70) as a nonspecific competitor (5'-CCAGGGTACGATTGGTCTCTGGAGAGG-3').

RESULTS

Fine Mapping of cis-Acting Elements in the PCNA Promoter Proximal Region—Sequence analysis of the region around the PCNA transcription start site (+1) suggests the presence of putative transcription factor binding sites, including a PE3A site (−5′-AGGAGG-3′) between −14 and −8, a sequence sharing homology with the TATA box (Smale and Baltimore, 1989) initiator element (Inr) around +1 (CATT), and an E2F-like site
To determine the complexity of the protein(s) interacting with these sequences, a 36-base pair double-stranded oligonucleotide (ST) spanning all three sites (PCNA -14 to +22) was radiolabeled and used in an EMSA (Fig. 1). A series of DNA-protein binding complexes were formed when this probe was incubated with nuclear extracts from the cloned T lymphocyte L2 and mouse splenocytes (Fig. 2). Notably, IL-2 and ConA induce the formation of several complexes with ST in stimulated L2 cells (Fig. 2B, lanes 1 and 2) and in splenocytes (Fig. 2A), respectively. In contrast to PCNA ST, when the same extracts from L2 cells were used with a probe containing a USF binding motif (Gregor et al., 1990; Huang et al., 1994), USF binding activity did not change significantly during IL-2 stimulation (Fig. 2B, lanes 3 and 4). The binding pattern of these induced complexes is very similar in IL-2 stimulated L2 cells and ConA-stimulated splenocytes (Fig. 2A). Initial competition experiments using unlabeled ST as well as a non-related oligonucleotide as competitors of complex formation indicated that complexes I, II, and III are specific for PCNA ST (data not shown). Among them, complex I and II were consistently present in all nuclear extracts we prepared but complex III binding activity varied with different nuclear extract preparations. In addition, three fast migrating bands (Fig. 2B) are not detected reproducibly in other experiments.

To determine where the various IL-2-inducible nuclear proteins bind to the PCNA promoter, a series of short oligonucleotides (Fig. 1) each containing only one of the three putative protein binding sites was made: ST-1 (PEA3), ST-2 (Inr), and ST-3 (E2F). Complex formation with radiolabeled ST-1, ST-2, ST-3, and ST were compared by EMSA using a 24-h IL-2-stimulated L2 cell nuclear extract (Fig. 3). Results in Fig. 3A demonstrated that only ST-3 retains the ability to form all three complexes (complexes I, II, and III). The fast migrating band present in this analysis was not found reproducibly in other experiments.

To determine whether the putative binding sites are required for complex formation, a series of binding site mutations (see "Materials and Methods") was made in these oligonucleotides based on previously defined consensus sequences of PEA, Inr, and E2F sites and on mutation studies (Yoo et al., 1991; Smale and Baltimore, 1989; Mudryj et al., 1990). Fig. 3B shows a competition binding experiment in which ST was 32P-labeled and incubated with IL-2-stimulated L2 cell extract. Excess amounts of unlabeled oligonucleotides containing the non-mutated or mutated binding site were used as competitors of complex formation. Among them, ST-3 was the most efficient competitor. In contrast, ST-3m1, an oligonucleotide with a mutation in the E2F-like site (see Fig. 3B) failed to inhibit any of the binding activities. This result indicates that the E2F-like site is essential for complex formation.

It is interesting that both ST-1 and ST-2 show some dose-de-
Fig. 3. Nuclear factors binding to PCNA ST.

A. EMSA of nuclear factors binding to PCNA promoter oligonucleotides. ST-1, ST-2, and ST-3 containing PEA3, Inr, and E2F-like sequence, respectively, were 32P-labeled and incubated with IL-2-stimulated L2 cell nuclear extract. B. EMSA competition experiment was performed as described under “Materials and Methods.” ST was 32P-labeled and incubated with IL-2-stimulated L2 cell nuclear extract. ST-1, ST-2, and ST-3 and their corresponding binding site mutations (ST-1m, ST-2m, and ST-3m) were used as competitors. Increasing amounts of each competitor oligonucleotide (5 or 20 ng) were used. The three complexes are indicated as I, II, and III. Free probe (free) runs at the bottom of the gel.

pendent inhibition of the binding activities (Fig. 3B). Since there is no obvious common sequence among ST-1, ST-2, and ST-3, this result suggests that the binding protein(s) may have multiple contact sites along the PCNA promoter sequence. It is more striking that ST-2m which has a mutated Inr element failed to inhibit complex formation, suggesting that the protein complexes may also contact the Inr element. To reconcile these data with Fig. 3A where there was no detectable binding to radiolabeled ST-1 and ST-2, we propose that the interaction of the three complexes with sequences from ST-1 and ST-2 (includ- ing the Inr element) is of low enough affinity that the interactions can only be inferred in the presence of high concentrations of oligonucleotides used during competition. On the basis of the data presented in Fig. 3, we conclude that the E2F-like site is required for initiating complex formation and the protein complexes may contact other sequences within the PCNA promoter.

Nuclear Proteins Interact Specifically with the PCNA E2F-like Site—We noted that a sequence (5'-GGGCGG-3') between +14 and +19, which overlaps with the E2F-like site, has only one mismatch of 6 bases when compared to the SP1 consensus sequence (5'-GGGCGG-3'). To determine the contribution of the E2F-like site and the SP1 site to complex formation, one additional E2F-like site mutation ST-3m2 (see “Materials and Methods”), was made. Unlike ST-3m1, ST-3m2 should only interfere with E2F binding but not the binding to the putative SP1 site. Results in Fig. 4A showed that both ST-3m1 and ST-3m2 abolished the formation of all three complexes, suggesting that the overlapping SP1 site is not required for complex formation. In addition to the three bands, some faster migrating bands appear variably in different experiments. A fast migrating band may represent an SP1-binding complex because this complex apparently still forms with the ST-3m2 probe in which the SP-1 site is intact, but not with the ST-3m1 probe.

The results of a competition binding experiment are shown in Fig. 4B. Consistent with the results from Fig. 4A, mutations in the E2F-like site, both ST-3m1 and ST-3m2, reduce the inhibition of complex formation with the wild type ST-3 probe. The relative binding affinity for the wild type and mutated E2F-like sequences is: ST-3 > ST-3m2 > ST-3m1. In addition to complexes I, II, and III, a series of fast migrating complexes could be detected with the radiolabeled ST-3 probe. The two bands immediately below complex II (a1 and a2) and the fastest migrating band (a3) show a competition pattern similar to complex II suggesting that they may also form on the E2F-like site. However, the nature of these binding complexes is not ascertained because of their low abundance and because they are only present in some of our nuclear extract preparations. The relatively abundant fast migrating bands (b and c) were judged to be nonspecific for the E2F-like site, band c might represent an SP-1 related binding factor since band c is competed by ST-3 m2 but only very poorly by ST-3m1. The functional relevance of these binding activities is unknown.

Functional Role of the Interactions between Nuclear Factors and the PCNA E2F-like Site—To determine the contribution of this E2F-like site to PCNA promoter activity, two PCNA mutants (M-1 and M-2) corresponding to the mutations in ST-3m1 and ST-3m2 were prepared in the context of the pPCNA5-LUC construct (–182 to +143) as described previously (Huang et al., 1994). In these constructs, PCNA genomic sequences were inserted into a HindIII site of a promoterless vector pSVOA. These constructs were transiently transfected into IL-2-stimulated L2 cells by the DEAE-dextran method. Because of the difficulties in transfecting quiescent L2 cells, we stimulated L2 cells with IL-2 for 22 h prior to transfection, as described under “Materials and Methods.”

Fig. 5 shows that mutations in the E2F-like site (M-1 and M-2) cause partial reduction in PCNA promoter activity. The relative promoter strengths of the wild type promoter and the two promoter mutants are consistent with the results of the relative binding affinity determined by EMSA (Fig. 4C), wt > M-2 > M-1. These data demonstrate that the E2F-like binding activity is required for optimal PCNA promoter activity and...
Fig. 4. Specificity of nuclear factors binding to PCNA E2F-like site. A, EMSA of nuclear factors binding to PCNA E2F-like site. PCNA ST-3 (wt), ST-3m1 (m1), and ST-3m2 (m2) were 32P-labeled and incubated with L2 cell nuclear extract. B, 32P-labeled ST-3 was incubated with 24 h IL-2-stimulated L2 cell nuclear extract. Unlabeled oligonucleotides ST-3, ST-3m1, and ST-3m2 were used as competitors. Increasing amounts of each competitor (0, 1, 5, and 20 ng) were used in the binding reactions. Complex I and II are the most prominent bands. Low amounts of complex III can also be found. The faster migrating bands (a1, a2, a3, b, and c) were variable in repeated experiments. Free probe (free) runs at the bottom of the gel.

Fig. 5. Effect of mutations at the E2F-like site on PCNA promoter activity in L2 cells. pPCNA5-LUC construct containing -182 to +143 genomic sequence of the mouse PCNA gene and two E2F-like site mutation constructs (M-1 and M-2) were transiently transfected into L2 cells. The promoterless vector pSVOA was also transfected as a negative control. To assure efficient transfection, L2 cells were stimulated with IL-2 (10–20 h) prior to transfection. Luciferase activities are presented as the percentage of luciferase activity derived from transfection of the wild type PCNA construct, pPCNA5-LUC (pPCNA5). All results were normalized to luciferase DNA content as measured by DNA slot blotting. Results represent the average of duplicates. The experiment was repeated three times with similar results. Standard deviations of luciferase activity are indicated.

Transfected DNA

Luciferase activity (% of pPCNA5)

- pSVOA - M-1 - M-2

Methylation Interference Analysis of the Interaction of Nu-
clear Proteins with the E2F-like Site—To determine the exact contact bases of these binding complexes within the PCNA promoter, methylation interference analysis was performed. In this experiment, only the two relatively abundant complexes, I and II, were analyzed (Fig. 6). ST was end-labeled and partially methylated by dimethyl sulfate before being subjected to EMSA. Both the protein-bound and free oligonucleotides were recovered from the gel and treated with piperidine, which specifically cleaves all the methylated guanidine (G) residues. Then the cleavage products were resolved on a sequencing gel. Methylation interference analysis for both of the DNA strands is shown in Fig. 6. Maxam and Gilbert DNA sequencing reactions were performed to confirm the positions of the G-residues in the PCNA promoter sequence. Methylation interference analysis of the shifted bands corresponding to complex I and complex II resulted in identical interference patterns centered at the E2F-like site on both strands. The pattern shows specificity for the E2F-like site on both strands with the top (coding) strand results more clearly overlapping with the E2F-like motif. The top strand contains three G-residues within the E2F site that interfere with E2F binding when methylated. On the bottom strand, one of the two G-residues within the E2F-like site shows methylation interference. The second G residue in the center of the E2F-like motif is very poorly cleaved in the free DNA and, thus, it is impossible to determine whether there is methylation interference at this position.

This pattern is consistent with the results obtained from both EMSA and competition experiments (Fig. 4). Notably, the Inr sequence CATT located at -2 to +2 is not protected in the methylation interference analysis suggesting that the nuclear protein is in close contact with DNA at the E2F-like site. Our data show variable and weak methylation interference at the PEA3 site which we believe is inconclusive and requires further experimentation to determine if there is protein binding at this site. Taken together, we conclude that the major binding com-
complexes form at the E2F-like sequence between +10 and +17.

The Nuclear Proteins That Interact with the E2F-like Site Are Distinct from E2F—To determine whether a member of the transcription factor E2F family binds to the PCNA E2F-like site, nuclear proteins binding to oligonucleotides containing the E2F consensus sequence derived from the hamster DHFR promoter were compared with those binding to the PCNA E2F-like site. We concluded on the basis of these data (Fig. 7) that the E2F family members do not bind to the PCNA E2F-like site because: 1) there is no cross-competition between the PCNA E2F-like site and DHFR-E2F with mutation at the E2F site. We concluded on the basis of these data (Fig. 7) that the E2F family members do not bind to the PCNA E2F-like site because: 1) there is no cross-competition between the PCNA E2F-like site and DHFR-E2F with mutation at the E2F motif (E2Fm) were 32P-labeled and incubated with 24-h IL-2-stimulated L2 cell nuclear extract. Poly(dI-dC) or salmon sperm DNA was used as nonspecific competitor DNA. They each gave different binding complexes. In addition to nonspecific DNA, various oligonucleotides (50 ng) containing PCNA ST-3 (PCNA), DHFR-E2F, or DHFR-E2F mutation (DHFR-E2Fm) were used as competitors in the binding reactions. The specific PCNA binding complexes (I and II) and specific E2F binding complex are indicated.

FIG. 6. Methylation interference analysis of nuclear proteins binding to PCNA E2F-like binding site. A, coding strand of the PCNA ST was end-labeled at the 5'-end. The probe DNA was partially methylated with dimethyl sulfate before addition to the binding reaction. Labeled DNA in the free (F) and bound (I and II) bands were recovered, cleaved at sites of methylation, and analyzed by urea-PAGE. The methylated guanine residues that interfere with binding of proteins are depicted at the site (G). Maxam and Gilbert sequencing, G, G + A, T + C, and C reactions were performed and loaded in parallel to confirm the positions of each guanine residue in the PCNA sequence. B, analysis of the non-coding strand of the PCNA probe (5'-end labeled), the same procedure was performed as above. C, sequence of ST and its methylation interference pattern are summarized.

FIG. 7. Comparison of complex formation with the PCNA E2F-like site and DHFR-E2F motif. PCNA ST-3 (PCNA), hamster DHFR-E2F oligonucleotides (E2Fm), and DHFR-E2F with mutation at the E2F motif (E2Fm) were 32P-labeled and incubated with 24-h IL-2-stimulated L2 cell nuclear extract. Poly(dI-dC) or salmon sperm DNA was used as nonspecific competitor DNA. They each gave different binding complexes. In addition to nonspecific DNA, various oligonucleotides (50 ng) containing PCNA ST-3 (PCNA), DHFR-E2F, or DHFR-E2F mutation (DHFR-E2Fm) were used as competitors in the binding reactions. The specific PCNA binding complexes (I and II) and specific E2F binding complex are indicated.

solved in an EMSA and were covalently cross-linked by UV irradiation; subsequently, the cross-linked species were subjected to SDS-polyarylamide gel electrophoresis.

Several labeled bands were obtained with different doses of UV irradiation. The specificity of the photoaffinity labeling was determined by adding either nonspecific or specific DNA to the binding reactions. The labeling of the 180-kDa band was not competed with an excess of nonspecific oligonucleotide in the binding reaction (Fig. 8, lanes 3 and 7) but was efficiently competed with a specific competitor: an oligonucleotide bearing PCNA E2F-like site (Fig. 8, lanes 4 and 8). The binding specificity of the labeled species of 30- and 43-kDa proteins were not clear. These data demonstrated that a 180-kDa protein binds specifically to PCNA E2F-like site. It is distinct from the known E2F-binding proteins because E2F family members have been characterized to be 40-60 kDa.

DISCUSSION

In mammalian cells, stimulation of quiescent cells by serum or growth factors causes a marked increase in the mRNA levels of PCNA, and other genes coding for proteins of the DNA synthesis machinery, such as DHFR, thymidine kinase, DNA polymerase α, and thymidine synthase (Pardee, 1989; Nevins, 1992).

The growth factor-responsive expression of the mouse PCNA gene in L2 cells requires elements within a 180-base pair region immediately upstream of the transcriptional start site (Huang et al., 1994). Previously we have shown that tandem cAMP response element binding protein/ATF binding sites located at nucleotides from −37 to −52 in the PCNA promoter are critical for IL-2-induced PCNA promoter activity (Huang et al.)

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E2F-5 (La Thangue, 1994; Lees, 1993). Many transcription factors including E2F-1, DP-1, E2F-2, E2F-3, E2F-4, and E2F-5 are involved in DNA binding and protein-protein interactions in the regulation of cell cycle. It is apparent that the term E2F represents a family of transcription factors with similar DNA-binding specificity that appear to have distinct functions. Therefore, E2F is an expanding group of related transcription factors.

Figure 8. Biochemical characterization of the E2F-like binding activities. UV cross-linking of proteins to \(^{32}P\)-labeled PCNA ST probe was performed as described under “Materials and Methods.” Samples were UV irradiated for either 30 min (lanes 1-4) or 60 min (lanes 5-8) as indicated. A band of approximately 180 kDa is labeled (-). Competition experiments were performed in the presence of different unlabeled oligonucleotides. Lanes 1 and 5, with no nuclear extract; lanes 2 and 6, with nuclear extracts in the absence of competitor; lanes 3 and 7, with nuclear extracts in the presence of nonspecific-competitor (NS) oligonucleotides (containing a CCAAT box); lanes 4 and 8, with nuclear extracts in the presence of ST as a specific competitor (S).

In this report, we identify an E2F-like motif with the sequence 5'-TTGCGGGC-3' located between +10 and +17 of the murine PCNA promoter that is required for PCNA transcriptional regulation. It is worth noting that in several other genes, including the hamster and murine DHFR promoters, E2F elements are found within a sequence near transcription start sites and are critical for transcriptional regulation (Blake and Azizkhan, 1989; Means et al., 1992).

Transcription factor E2F was originally identified as a protein binding to a cis-element needed for the activation of the adenoviral early E2 gene promoter (Kovesdi et al., 1986; Berk, 1986). There are at least six similar but not identical E2F-like sites found in promoters of several cellular genes, with the sequence often being TTTCGCGC or TTTGGCGC (Mudryj et al., 1990; Nevins, 1992). Regulation of E2F activity involves its interactions with multiple cellular proteins including pRB, cdk2, and cdk2 (Nevins, 1992). The retinoblastoma protein pRB as a tumor suppressor forms a specific complex with E2F-protein binding to the PCNA E2F-like site as well as the murine DHFR E2F core sequence is required for complex formation with the PCNA E2F-like site. In contrast, E2F-1 is a 54-kDa nuclear protein previously identified in cross-linking experiments by several groups (Mudryj et al., 1990; Chellappan et al., 1991). The other E2F proteins have molecular mass ranging from 40 to 60 kDa. Therefore, this 180-kDa protein binding to the PCNA E2F-like site is distinct from all known E2F family proteins. However, we cannot rule out the possibility that this 180-kDa protein is related to the E2F family of transcription factors.

RNA polymerase is known to bind to DNA at the transcription start site to form an initiation complex with general transcription factors such as TFIIID. Three forms of RNA polymerase II large subunit have been described, differing in apparent molecular mass, 180, 210, and 240 kDa (Sawadogo and Sentenac, 1990; Young, 1991). Is it possible that the 180-kDa protein binding next to the initiation site represents RNA polymerase? Our results argue that this protein is not RNA polymerase because: first, methylation interference data indicate that the 180-kDa protein contacts a small region (nt +12 to +19) within or adjacent to the E2F-like site, while previous analyses have revealed that RNA polymerase protects a much larger region of DNA on both sides of the initiation site (Sawadogo and Sentenac, 1990); second, the interaction of the 180-kDa protein with DNA requires the E2F-like site, while RNA polymerase is thought to bind to DNA in a nonspecific manner.

A similar situation exists with studies of the DHFR promoter and our findings with the mouse PCNA promoter. E2F proteins have been shown to bind to the E2F site in the murine DHFR promoter between nt –10 and –3 (Slansky et al., 1993) but Means et al. (1992) have shown that HIP1, a 180-kDa protein also binds near this site at –9 to –1. This sequence has been shown to function as the initiator element that controls the start site for murine DHFR transcription (Means and Farnham, 1990). The protein binding to the PCNA E2F-like site that we characterized in this report is similar to HIP1. 1) Both proteins have molecular mass of approximately 180 kDa. 2) Both bind to a sequence at or adjacent to the transcription initiation site. 3) Both proteins contact sequences which share homology to the E2F site. Our data suggest that the 180-kDa protein does not bind to the hamster DHFR E2F site (Fig. 7). We noticed that the murine DHFR E2F motif contains a G as the second base 5' of the E2F site, in contrast, the hamster E2F motif contains an A at the –2 position. Previously it has been shown that the sequence adjacent to the murine DHFR E2F core sequence is required for complex formation of HIP1 (Means et al., 1992). Therefore a difference of the sequences in the –2 position of the DHFR E2F elements may account for the observation that this 180-kDa protein binds to the PCNA E2F-like site as well as the murine DHFR E2F motif but not to the hamster DHFR E2F motif.

Mutations that abolish complex formation on this E2F-like site only reduced PCNA promoter activity moderately, to about 60–80% of the wild type promoter activity. One of the explanations for this is that other cis-elements including the tandem CRE/ATF binding sites in the PCNA promoter also contribute substantially to optimal promoter activity. Further experiments making double or multiple mutations of these cis-elements in the PCNA promoter should help to determine the cooperation between these sites for PCNA promoter activity.

The identification of an E2F-like binding site in the PCNA
promoter and of nuclear proteins interacting with this E2F-like site in this report provides a basis for further study on the mechanism of PCNA transcriptional regulation and contributes to our understanding of the mechanisms by which IL-2 activates T lymphocyte proliferation.

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