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An E2F site in the 5′-promoter region contributes to serum-dependent up-regulation of the human proliferating cell nuclear antigen gene

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Abstract The synthesis of proliferating cell nuclear antigen (PCNA) is strictly regulated during the cell cycle. To investigate the contribution of the promoter region to the up-regulation of human PCNA expression at the onset of S phase, we have examined 17 putative elements with reporter assays in quiescent L-O2 cells and following serum stimulation. The E2F-like sequence 5′-TTCCCCGCAA-3′ located at −84 to −75 is required for the serum-induced transactivation. In electrophoretic mobility shift assays, nuclear extracts from asynchronous L-O2 cells exhibit two binding activities toward the −75 E2F oligonucleotide, and the minor band, whose formation could be interfered with by E2F-1 antibody, represents an S phase-specific complex. This is the first demonstration of the E2F site in the human PCNA 5′ promoter as a serum-responsive element.

Key words: Human proliferating cell nuclear antigen; E2F; Cell cycle

1. Introduction

The proliferating cell nuclear antigen (PCNA) was first described by Miyachi et al. [1] as a nuclear antigen restricted to proliferating cells that reacts with sera from some patients with the autoimmune disorder systemic lupus erythematosus. It has been well described that PCNA forms a sliding homotrimeric clamp around DNA and functions as a DNA polymerase processivity factor during replication [2] and repair [3]. Through its multiple protein–protein interactions, PCNA coordinates events in replication, epigenetic inheritance, repair, and cell cycle control [4]. Therefore it is important to understand the regulation of PCNA expression and the functions of PCNA within the cell cycle.

PCNA synthesis is strictly regulated during the cell cycle, with a clear increase at the G_1/S transition. It is evident that PCNA represents a key protein necessary for the transition of cells from quiescence to S phase [5–7]. The full activity of the human PCNA (hPCNA) promoter was shown to be approximately within the 210 bp upstream of the transcription start site [8], and the ATF/CRE site (nucleotide −52 to −45) is crucial to both basal transcription and E1A-driven transactivation [9,10]. It had been reported that the 5′ promoter may not be involved in the serum-dependent growth regulation of the hPCNA gene in murine 3T3 cells [11]. Thus, subsequent investigations of the cell cycle regulatory elements focused on the introns [12,13], and the E2F site located in the first intron at position +583 was found to play a positive role in hPCNA transactivation at the onset of S phase [14].

In our previous work, to achieve a complete overview of the hPCNA promoter, we searched the region from −538 to +60 relative to the transcription start site for potential *cis*-elements, and recognized 17 putative targets (summarized in Table 1). Transient expression assays in different cell lines demonstrated that besides the ATF/CRE site detected by Morris and Mathews [9], an E2F site (nucleotides −86 to −75), a CTF site (nucleotides −99 to −95), and an SP1 site (nucleotides −191 to −186) are likely general *cis*-elements [15]. In this study, to comprehensively assess the individual contribution of every putative element to serum responsiveness, we used transient expression assays in synchronous L-O2 cells to analyze the activities of 17 mutant hPCNA promoters, each bearing an 8 bp substitution in one of the 17 putative elements. We found that the E2F site (nucleotide −86 to −75) is required not only for optimal hPCNA promoter activity, but also for the responsiveness to serum stimulation in L-O2 cells. We further characterized this regulatory element and suggest a functional role of an E2F-1-containing complex as an S phase-specific positive regulator of hPCNA expression.

2. Materials and methods

2.1. Cell culture, synchronization and FACS analysis

The normal human hepatic cell line L-O2 was maintained in high glucose Dulbecco's modified Eagle's medium (25 mM glucose; Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL), 100 U/ml penicillin, and 50 μg/ml streptomycin under 5% (v/v) CO_2 at 37°C.

G_0/G_1 synchronization was obtained by inducing proliferation arrest after serum starvation for 48 h; subsequent re-entry into the cell division cycle was induced by adding fresh serum (15% final concentration). Synchronous progression through S phase was obtained by arresting the cells at the G_1/S transition in the presence of hydroxyurea (1 mM final concentration for 24 h) and subsequently transferring the cells to hydroxyurea-free medium. Finally, cells were synchronized in G_2 in the presence of 0.2 μg/ml nocodazole for 10 h; the block was released by transferring the cells to nocodazole-free medium. All three types of synchronization were monitored by fluorescence-activated cell sorting (FACS) analysis. At regular intervals after the block release, cell samples were harvested in phosphate-buffered saline, fixed in 70% (v/v) ethanol, and incubated with RNase A (500 μg/ml) for 1 h at 37°C. Propidium iodide (100 μg/ml cell sample) was added and the DNA contents of cell samples were analyzed in a

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Abbreviations: PCNA, proliferating cell nuclear antigen; hPCNA, human PCNA

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FACStar Plus cytofluorometer (Becton Dickinson) with the Multicycle software (10 000 events per sample).

2.2. Plasmids, transfection and luciferase assay

The plasmid pGL_2-hPCNA carries the wild-type hPCNA promoter spanning nucleotides −538 to +60 relative to the transcription start site, followed by the firefly luciferase reporter sequence. Plasmids P−509, P−498, P−342, P−330, P−289, P−216, P−199, P−186, P−139, P−122, P−94, P−75, P−64, P−44, P−18, P+10 and P+38, constructed in our previous work [15], each have one of 17 different 8 bp substitutional mutations in potential promoter elements (Table 1). The control promoter construct pGL_2-SV40 carries the cell cycle-independent promoter of the SV40 early gene upstream of the *Renilla* luciferase coding sequence. L-O2 cells were transfected with plasmids using LipofectAMINE reagent (Invitrogen) according to the standard protocol. Transfection experiments were performed on duplicate sets of cultures, which were subjected to determination of luciferase activity and to FACS monitoring of the cycle progression. Luciferase assays were performed using the Promega Luciferase Assay Kit following the manufacturer's instructions. Activities of firefly (experimental) and *Renilla* (control) luciferases were measured in a luminometer BG-P (MGM). Promoter strengths were quantified by calculating the ratio of firefly/*Renilla* luciferase activity of the same lysate sample.

2.3. Preparation of nuclear extracts

Nuclear extracts were prepared from asynchronous and synchronous L-O2 cultures as described previously with some modification [16]. $0.5–1\times10^8$ cells were collected, and washed several times with phosphate-buffered saline. Then the cell pellet was resuspended in five volumes of ice-cold cell homogenization buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) by gentle pipetting. The cells were allowed to swell on ice for 10 min, and pelleted by centrifugation at $250\times g$ for 10 min. Then the pellet was resuspended in three volumes of ice-cold cell homogenization buffer containing 0.05% NP-40, and homogenized with 20 strokes of a tight-fitting homogenizer. The homogenate was centrifuged at $250\times g$ for 10 min. The nuclear pellet was resuspended in 1 ml of ice-cold cell resuspension buffer (40 mM HEPES pH 7.9, 0.4 M KCl, 10% glycerol, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin), and 5 M NaCl was accurately added to a final concentration of 300 mM. After the cells were placed on ice for 30 min, the nuclear extract was centrifuged at $46\,000\times g$ at 4°C for 20 min and the supernatant was frozen in aliquots at −70°C. Protein concentration was quantitated using the Bradford method.

2.4. Electrophoretic mobility shift assay

Briefly, binding reactions were set up with 3–15 fmol of 5′-^{32}P-labeled oligonucleotide and 2–10 μg of nuclear extract in 20 μl of reaction mixture containing 1 mM dithiothreitol, 1 mM EDTA, 0.2% Tween 20, 0.05 μg/μl poly(dI-dC), and 0.05 μg/μl poly-L-lysine. The reaction was incubated at room temperature for 25 min. Competition experiments were performed routinely. In interference experiments, the nuclear extract was pre-incubated with E2F-1 antibody (KH95, from BD Pharmingen) (0.1 μg/μl reaction mixture) on ice for 1 h before the addition of the oligonucleotide probe. The binding complexes were separated by 6% polyacrylamide gel electrophoresis at 4°C in 0.5×TBE at 200 V. Gels were then dried and autoradiographed.

The following oligonucleotides were annealed with their complementary strands, and then used in electrophoretic mobility shift assays: −75 E2F, 5′-gatcAAGTCTTCCCCGCAAGGCgatc-3′; −75 E2F-mut, 5′-gatcAAGTCctgatcaaAAGGCgatc-3′; the high affinity E2F binding oligonucleotide [17], 5′-CTAGATTTCCCGCGGATC-3′; TATA, 5′-GCAGAGCATATAAGGTGAGGTAGGA-3′. Oligonucleotides −75 E2F and −75 E2F-mut contained 18 bp of hPCNA promoter sequence from −89 to −72 (uppercase letters) with the potential E2F sites (underlined) or their 8 bp-substituted derivatives (lowercase letters within the promoter sequence) and 8 bp of unrelated flanking DNA (lowercase letters).

3. Results

3.1. The activity of the hPCNA promoter is cell cycle-dependent in L-O2 cells

PCNA synthesis is induced in quiescent cells by stimulation with serum, growth factors, and adenovirus infection. It fluctuates during the cell cycle [18]. To find out whether the hPCNA promoter region functions in the cell cycle-related transcriptional regulation, we analyzed the reporter expression directed by wild hPCNA promoter (from −538 to +60) in several cell lines, which were synchronously progressing through the cell cycle, including HeLa, HepG2, MCF-7, and L-O2, and finally discovered that in L-O2 cultures the activity of exogenous hPCNA promoter was cell cycle-dependent.

pGL_2-hPCNA and pGL_2-SV40 constructs were transfected in asynchronously cycling L-O2 cells. After transfection, the cells were first synchronized in G_0/G_1 by serum starvation for 48 h and subsequently restimulated to enter the cell division cycle by raising the serum concentration. Cell samples were harvested at regular intervals after restimulation. Cell cycle block and re-entry were monitored by FACS analysis. As shown in Fig. 1A, the ratios between hPCNA promoter activity (firefly luciferase) and SV40 early promoter activity (*Renilla* luciferase) were efficiently elevated following serum stimulation, and the highest level was observed 12–15 h after

Table 1
Mutation sites in the hPCNA promoter

Vector	Mutation sites	Replaced sequence for CTGATCAA	Possible binding factor	Putative binding sequence of factors
P−509	−516 to −509	gcggGGGAATGTtaag	NF-κB	GGGA(A/C)TnYCCC
P−498	−505 to −498	ttaaGAGGATGAtagg	Ets-1	R(G/C)CGGAAGTY
P−342	−349 to −342	cttgATTTGCATttca	Oct-1[a]	ATGCAAAT
P−330	−337 to −330	ttcaCTTTCACTttcg	PRDI[a]	AGTGAAAGTG
P−289	−296 to −289	gtggGAGGCTGAggag	Sp1	GGCGGG
P−216	−223 to −216	cggaCTTGTTCTgcgg	p53[a]	(RRRC(A/T)(T/A)GTTT)X2
P−199	−206 to −199	cgggTTCAGGAGtcaa	RA	AGGTCAn$_{2\ or\ 5}$AGGTCA
P−186	−193 to −186	caaaGAGGCGGGgaga	Sp1[a]	GGCGGG
P−139	−146 to −139	ttccTCCAATGTatgc	CTF[a]	CCAAT
P−122	−129 to −122	ctagGGGGCGGGcctc	Sp1[a]	GGCGGG
P−94	−101 to −94	gacaCGATTGGCccta	CTF[a]	CCAAT
P−75	−84 to −77	agtcTTCCCCGCaagg	E2F	TTTTC(G/C)CG(G/C)
P−64	−71 to −64	aggcCGTGGGCTggac	AP2	CCC(A/C)n(G/C)(G/C)(G/C)
P−44	−51 to −44	tggtGACGTCGCaacg	ATF[a]	TGACGY(C/R)R
P−18	−25 to −18	tgagAGCGCGCGcttg	–	–
P+10	+10 to +17	acggTTGCAGGCgtag	E2F	TTTTC(G/C)CG(G/C)
P+38	+38 to +45	tgtcTTTCTAGGtctc	E2F	TTTTC(G/C)CG(G/C)

[a]Factor-related sequence in the hPCNA promoter is equal to its putative binding sequence.

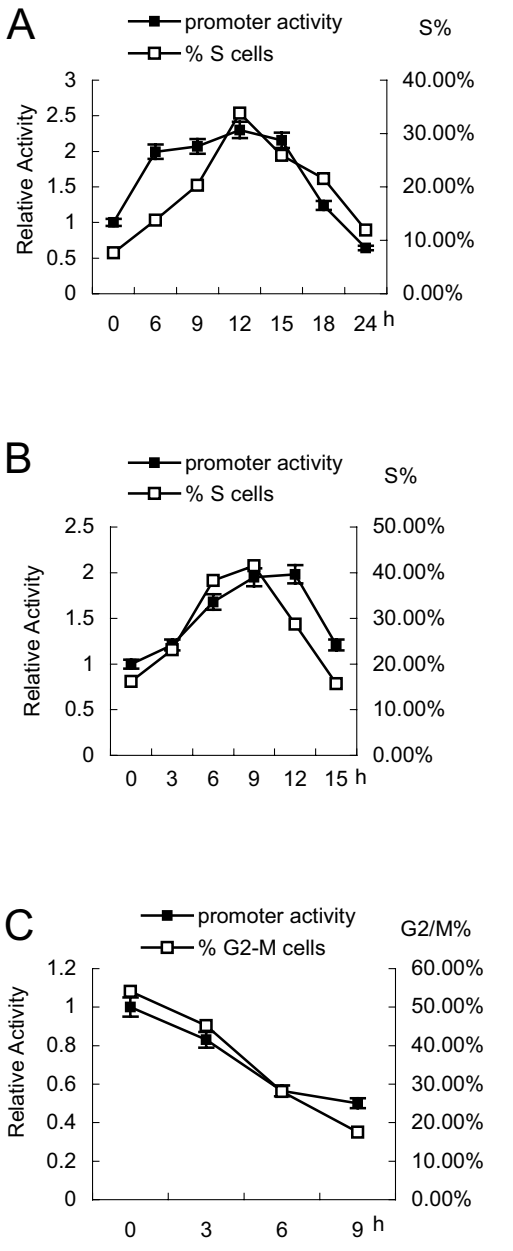

Fig. 1. Transient expression assays of hPCNA promoter activity during the cell cycle. The proportions of the cells in corresponding phases in transfected cultures monitored by FACS are also shown. The promoter strengths were calculated as firefly/*Renilla* activity in each transfected sample. The relative activity at 0 h was taken as 1. Mean and S.D. values were calculated from at least three independent assays. A: The relative activities of the hPCNA promoter in G_0-arrested and restimulated L-O2 cells. B: The relative activities of the hPCNA promoter in G_1/S-arrested and restimulated L-O2 cells. C: The relative activities of the hPCNA promoter in G_2/M-arrested and released L-O2 cells.

release, when cells reached S phase (Fig. 1A). The SV40 promoter activity did not significantly vary in arrested or restimulated cultures (data not shown). Therefore, the up-regulation of the hPCNA promoter upon entry into S phase was specific.

To assess hPCNA promoter activity during S phase progression in more detail, L-O2 cells transfected with reporter constructs were synchronized using the hydroxyurea block/release protocol. The starting point of administration was controlled to make sure that the 0 h sample was collected 36 h after transfection. Hydroxyurea is a powerful inhibitor of DNA replication, and its addition to the culture medium yields cell populations that are arrested at the G_1/S transition. After removal of the drug, S phase progressed more slowly than during an ordinary cell cycle [19]; thus this method of synchronization enabled us to resolve accurately the progression through S phase. Luciferase assay confirmed that the hPCNA promoter showed certain activity as early as G_1/S transition (0 h), and continued to increase throughout S phase (3–12 h after release) (Fig. 1B). Twelve hours after the removal of hydroxyurea when the cells were either in the G_2 phase or entering the mitotic division, hPCNA promoter activity was drastically decreased. Up-regulation of the hPCNA promoter was specific and was not simply a consequence of resumed transcriptional activity during recovery from the toxic effects of the hydroxyurea treatment, because the expression of the pGL_2-SV40 construct did not show any significant variation during the cell cycle progression (data not shown).

Similarly, transfected cells were synchronized in G_2/M by exposure to nocodazole, whose microtubule-depolymerizing activity is well established, for 10 h before harvesting. The nocodazole method enabled us to follow the progression through the mitotic division. The results indicated that the activity of the hPCNA promoter was down-regulated during M phase, and the ratio of hPCNA/SV40 promoter activity reached the lowest level 6 h later when most cells had completed their mitotic division and entered a new cell cycle (Fig. 1C). The low level of hPCNA promoter activity did not reflect a general toxic effect of the nocodazole treatment, because SV40 promoter activity was not significantly decreased at 0 h or 3 h compared with the level observed 9 h after block release (data not shown). Due to the half-life of the luciferase (3 h), all the fluorescence curves were delayed to a certain extent compared with the corresponding FACS curves.

Together these results indicate that the promoter region contributes to the cell cycle-dependent transcriptional regulation of the hPCNA gene at least in certain cell lines. In L-O2 cultures, the hPCNA promoter is activated on entry into S

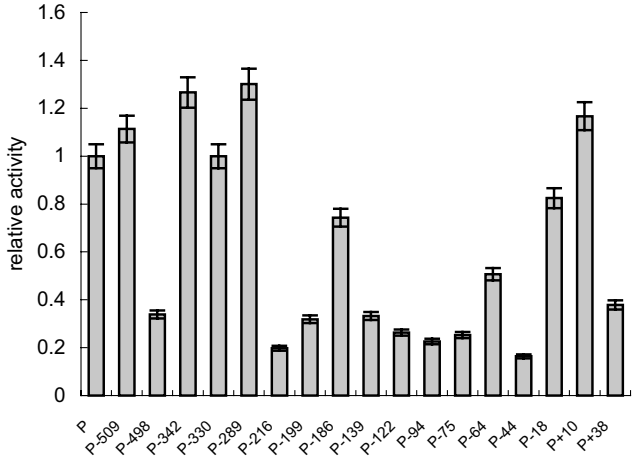

Fig. 2. Relative activity of wild-type and mutant hPCNA promoter constructs in asynchronous L-O2 cells. Every PCNA promoter activity (firefly luciferase) was normalized to the SV40 promoter activity (*Renilla* luciferase) from the same sample, and the relative activity of wild hPCNA promoter was taken as 1.

phase, reaching its maximal transcription in S phase, and is down-regulated in mitosis.

3.2. Identification of elements required for optimal activity of the hPCNA promoter in asynchronously cycling L-O2 cells

To identify the upstream cell cycle regulatory elements, we first examined the contribution of each putative element summarized in Table 1 to the optimal activity of the hPCNA promoter in L-O2 cells using transient expression assays. Reporter constructs bearing the wild-type hPCNA promoter or the 8 bp substitution-mutagenized hPCNA promoters and control construct were transfected into asynchronous L-O2 cells using LipofectAMINE. Thirty-six hours after the transfection, cell lysates were prepared to determine the luciferase expression level. Every wild or mutant promoter activity (firefly luciferase) was normalized to the SV40 promoter activity (*Renilla* luciferase) from the same sample, and the relative activity of wild hPCNA promoter was taken as 1. Results in Fig. 2 show that transcription activities of P−498, P−216, P−199, P−139, P−122, P−94, P−75, P−64, P−44, and P+38 promoters were drastically reduced compared with the wild-type promoter. In contrast, transcription activities of the P−509, P−342, P−330, P−289, P−186, P−18, and P+10 constructs were comparable to, or slightly more efficient than that of the wild-type promoter. Thus, efficient hPCNA promoter activity in cycling L-O2 cells requires the integrity of the −498, −216, −199, −139, −122, −94, −75, −64, −44, and +38 sites. The −44 element represents a perfect consensus site for the transcription factor ATF, which has been well established [9,10]. The −216 site was previously demonstrated to be a DNA damage-responsive element [20]. The −199, −122, −94, and −64 sites were also shown to be putative elements in Tommasi's in vivo footprinting data [14]. That is to say, the majority of the essential elements suggested in Fig. 2 have been described previously, except for the −498, −139, −75, and +38 sites.

3.3. A putative E2F site (−84 to −75) contributes to cell cycle-regulated hPCNA transcription

We set out to identify the promoter elements that might be required for serum responsiveness among all the putative binding sites summarized in Table 1. Reporter constructs bearing the wild-type hPCNA promoter or the mutant hPCNA promoters and pGL_2-SV40 control construct were transfected in L-O2 cells that were then synchronized by serum starvation. G_0 and S phase cultures were harvested before serum addition and 15 h after release respectively. Luciferase assays showed that the activity of the wild-type hPCNA promoter was low in G_0 phase and increased in S phase, and the 8 bp substitutional mutations did not affect the serum responsiveness of any of the mutant constructs except P−75, which bears a mutation within the E2F-like site from −84 to −75. As shown in Fig. 3A, the mutation of the −75 E2F motif abolished the response of the hPCNA promoter to growth stimulation, and this feature of the site is emphasized in Fig. 3B. The remaining increasing activity detected could be due to incomplete cell synchronization. We further examined the activity of the −75 E2F site-mutated hPCNA promoter during the cell cycle in L-O2 cells following the protocols used in Fig. 1A,B. P−75 and control constructs were transfected in L-O2 cultures that were then synchronized by serum starvation and hydroxyurea block respectively. Cell cycle block and

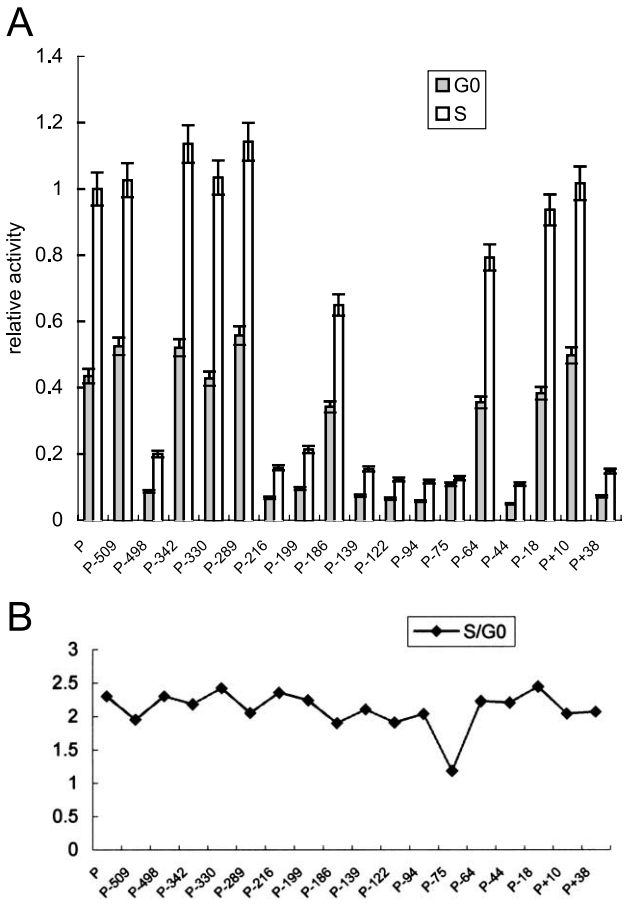

Fig. 3. Identification of elements required for serum responsiveness. A: Relative activity of wild-type and mutant hPCNA promoter constructs in growth-arrested (shaded histograms) and S phase-restimulated (open histograms) L-O2 cells. The mean value obtained from the wild-type promoter in S phase cells was taken as 1. B: Comparison of the ratios between the promoter activities in S phase cells and in growth-arrested cells.

re-entry were monitored by FACS analysis. It was shown that the ratios between the −75 mutated promoter activity (firefly luciferase) and the SV40 early promoter activity (*Renilla* luciferase) were slightly elevated following serum stimulation (Fig. 4A), and were hardly raised after hydroxyurea block release (Fig. 4B), substantiating the role of the −75 E2F motif in cell cycle-related regulation. Together these data indicate that the E2F motif may contribute to both G_1/S and maximal S phase induction of the hPCNA promoter in synchronized L-O2 cells. In view of the low degree of cell synchrony (see Fig. 1), it is likely that the contribution of the −75 E2F element has been underestimated.

3.4. The putative E2F site (−84 to −75) in the hPCNA promoter shows cell cycle-regulated interaction with DNA binding factors

Since a group of cell cycle-related genes are regulated by the E2F family in a coordinate manner [21], the −75 E2F-like site from −84 to −75 seemed particularly worth investigating. We then examined the interactions of the oligonucleotide encompassing the −75 E2F site with DNA binding factors. In electrophoretic mobility shift assays, two high molecular weight complexes were formed with the nuclear extracts from asynchronous L-O2 (Fig. 5A, lane 2). The complexes were com-

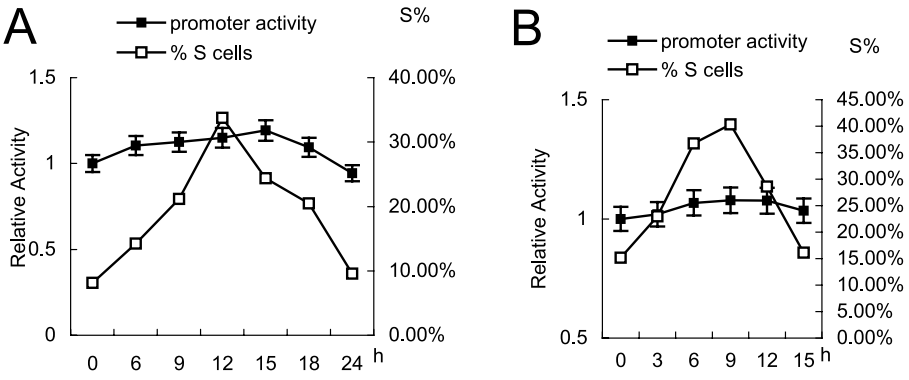

Fig. 4. Transient expression assays of −75 E2F site-mutated hPCNA promoter activity during the cell cycle. The proportions of the cells in corresponding phases in transfected cultures monitored by FACS are also shown. The promoter strengths were calculated as firefly/*Renilla* activity in each transfected sample. The relative activity at 0 h was taken as 1. Mean and S.D. values were calculated from at least three independent assays. A: The relative activities of the −75 mutated promoter in G_0-arrested and restimulated L-O2 cells. B: The relative activities of −75 mutated promoter in G_1/S-arrested and restimulated L-O2 cells.

petitively disrupted by excess of the cold −75 E2F oligonucleotide (Fig. 5A, lanes 4 and 5), as well as by the cold E2F consensus sequence (Fig. 5A, lanes 6 and 7), but not by the cold mutant −75 E2F oligonucleotide bearing an 8 bp substitutional mutation which abolishes the E2F binding site (Fig. 5A, lanes 8 and 9). Thus the −75 E2F site is actually an E2F binding site, and both of the bands represent E2F-containing complexes. Interactions established during cell cycle progression were analyzed using the nuclear extracts from growth-arrested and S phase L-O2 cells respectively (Fig. 5B). It is exciting that the electrophoretic mobility shift pattern of the −75 E2F sequence showed a change between G_0 and S phase cultures. The −75 E2F site formed two complexes with S phase nuclear extracts, whereas the minor band (both in content and in molecular weight) disappeared when the nuclear extracts were from growth-arrested cells (Fig. 5B, lanes 1 and 2), indicating that the minor band represented an S phase-specific complex. These results did not reflect a lower content of transcription factors in extracts from G_0, compared with S phase cells, because the TATA box binding protein had a comparable abundance in extracts from both sources (Fig. 5B, lanes 3 and 4). The coincidence of S phase-specific E2F site occupancy and transactivation of the hPCNA promoter suggests that this complex functions largely as a transcriptional activator. Formation of the S phase-specific complex was interfered with by addition of anti-E2F-1 antibody KH95 (Fig. 5A, lane 3), a monoclonal antibody that recognizes an epitope between amino acids 342 and 386 of human E2F-1 and does not show any cross-reactive properties with other members of the E2F family [22]. Hence E2F-1, rather than other members of the E2F family, is the component of the S phase-specific complex, while the major E2F-containing

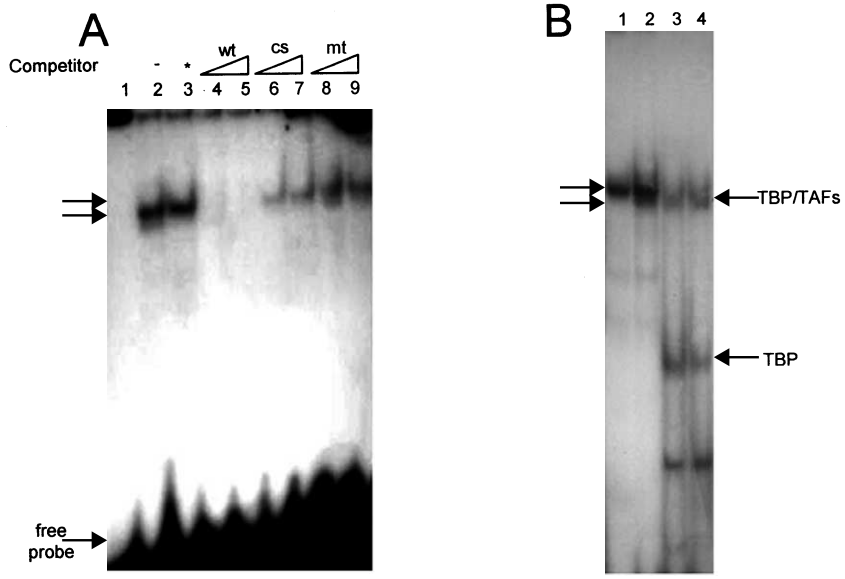

Fig. 5. Protein binding features of the ^{32}P-labeled −75 E2F sequence. A: Electrophoretic mobility shift assays of −75 E2F oligonucleotide alone (lane 1) or incubated with nuclear extracts from asynchronously growing L-O2 cultures (lanes 2–9), which contained no competitor DNA (lane 2), increasing (50-fold and 100-fold excess) amounts of cold −75 E2F oligonucleotide (wt) (lanes 4 and 5, cold consensus E2F sequence (cs) (lanes 6 and 7) or cold mutant −75 E2F oligonucleotide (mt) (lanes 8 and 9), as indicated by the triangles at the top of the gel. The asterisk (lane 3) represents the interference experiment with anti-E2F-1 antibody. B: Electrophoretic mobility shift assays using extracts from growth-arrested (lanes 1 and 3) and S phase (lanes 2 and 4) L-O2 cells, with ^{32}P-labeled −75 E2F (lanes 1 and 2) and ^{32}P-labeled TATA (lanes 3 and 4) oligonucleotides. The shifted products are marked by the arrow.

complex (both in content and in molecular weight) was not interfered with by the anti-E2F-1 antibody (Fig. 5A, lane 3), suggesting that the E2F member contained in the major complex is not E2F-1.

4. Discussion

PCNA is regulated by a combination of mechanisms that act at both transcriptional and post-transcriptional levels [8]. It has been reported that introns, rather than the 5′ promoter, may be responsible for cell cycle-regulated hPCNA transcription [11,13,14]. However, the data in this work demonstrate that the activity of the hPCNA promoter fluctuates during the cell cycle in L-O2 cells. The construct carrying a substitutional mutation in the −75 E2F site showed only 25% as much transient hPCNA luciferase expression in asynchronous L-O2 cells as compared with the original pGL_2-hPCNA, and furthermore lost the response to serum stimulation, suggesting that the putative E2F site 5′-TTCCCCGCAA-3′ located at positions −84 to −75 is required both for optimal promoter activity and for serum-induced hPCNA transactivation at the onset of S phase. In electrophoretic mobility shift assays the ^{32}P-labeled −75 E2F sequence formed two high molecular weight complexes with nuclear extracts from asynchronous or S phase L-O2 cultures, whereas only the major complex was seen with G_0 extract. Thus the minor complex was the S phase-specific one. Interference with complex formation by anti-E2F-1 antibody showed that this complex indeed contains E2F-1. This is the first demonstration of the E2F site from −84 to −75 as both a positive regulatory element and a serum-responsive element in the hPCNA 5′ promoter region.

As for the other three elements required for the optimal activity of hPCNA promoter suggested in Fig. 2, the −498 site is shown to be a cell type-specific element in our work (data not shown). Functional analysis of the −139 site has not yet been done. The cell type-specific role of the +38 E2F-like site will be elucidated in another paper (in preparation).

The −75 E2F-like site contains an 8-of-10 match to the central 10 bp of an inverted repeat E2F element (TTTC/GCCGCC/GAAA) [23]. It should be noted that some reported E2F sites, such as site 1 of the E1A enhancer [24] and the dihydrofolate reductase E2F site [25], possess a dyad symmetry similar to that of the −75 E2F site [23]. An increasing number of reports document the central role of the transcription factor E2F in coordinating transcription during the cell cycle, particularly in the induction of specific genes at the G_1/S transition [21,26]. Although comparison of the hPCNA promoter with murine, *Drosophila*, tobacco, and rice PCNA promoters showed little conservation upstream of the transcription start site, E2F elements have been characterized in these PCNA genes [14,27–30], indicating that these E2F elements may be general features of eukaryotic PCNA genes which fall into the group of G_1/S-induced genes mentioned above. Interestingly, an inverted repeat E2F sequence identified in the tobacco PCNA promoter was located just from −83 to −74 [28], similar to the −75 E2F site in this paper, which implies the significance of the sites in the regulation of the PCNA promoter.

The E2F family comprises six factors related in their DNA binding specificity and expressed at specific phases of the cycle, and each member can heterodimerize with DP1 or DP2, giving in all 12 different DNA binding transcriptional regulators which function as either transcriptional activators or repressors, depending on their target genes and the phase of the cell cycle [21]. In electrophoretic mobility shift assays two complexes formed between the −75 E2F oligonucleotide and L-O2 nuclear extracts, and the S phase-specific minor complex contain E2F-1. It has been well established that E2F-1 is rapidly transactivated after growth stimulation and functions as the major activator in late G_1/S phase [31]. Therefore, it is likely that this E2F-1-containing complex is a transcriptional activator in L-O2 cells on entry into S phase, while the E2F member contained in the major complex is not E2F-1. The in vitro data showed that the major band did not vary significantly between G_0 and S phase. Due to the excess of ^{32}P-labeled oligonucleotide, it seemed that the major complex existed constantly in L-O2 nuclei. Given these findings, we propose that the major complex may assume the opposite responsibility to the minor, and in vivo, where the protein complexes are dominant, the S phase-specific complex could competitively bind to the −75 E2F site at the onset of S phase. The occupancy of E2F sites was initially observed to be cell cycle-dependent, and functional E2F sites were thought either to be occupied by free E2F activator only when the gene was triggered at the G_1/S transition [26], or to be bound by the E2F retinoblastoma repressor only when the gene was inactive in G_0 and G_1 phase cells [32]. However, it was later found that E2F sites can be occupied continuously during the cell cycle, and thus there may be a switch between a repressing and an activating complex [14,33]. The data presented here are consistent with the latter situation, in that the major complex supposed to be a repressor constantly exists in the nuclei, while the S phase E2F-1-containing complex is observed in G_1/S induction. The identification of the E2F member contained in the major complex and the other components of the two complexes, as well as the detailed mechanism involved in the switch between complexes of different functions, needs further investigations. In fact, we could not rule out the possibility that the major band may include several DNA–protein complexes because of the complexity of the E2F family and the associated factors, such as DP and pocket protein family.

It has been proposed by several groups that E2F is involved in hPCNA regulation during cell cycle progression through an intron-associated binding site [11–14]. Tommasi et al. showed that the E2F site located in the first intron at position +583 plays a role in activation of the hPCNA gene at the onset of S phase, and their in vivo footprinting data even did not show the footprint of the −75 E2F site during the cell cycle in normal human foreskin fibroblasts (HF39) [14]. In our own work, the 5′ promoter of hPCNA did not respond to serum stimulation in HeLa, MCF-7, or HepG2 cell lines (data not shown). It is quite possible that the abundance of E2F-1, or other factors contained in the S phase-specific complex, is too low to function in these cell lines, which may be one of the reasons why other researchers deduced that 5′ promoter did not contribute to S phase induction. In fact, expression of E2F-regulated genes mostly results from the concerted action of two E2F elements through the cell cycle [22,25,34]. Thus, the −75 E2F site, the +583 E2F element, and even other unknown E2F sites in the hPCNA gene most likely cooperate to switch the transcription from negative to positive regulation at the onset of S phase, which is consistent with the observation that the fluctuation range of exogenous hPCNA promoter activity during the cell cycle was less than that of

endogenous PCNA synthesis in the parallel experiments (data not shown), and the coordination model depends on the cell line and the physiological condition.

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References

[1] Miyachi, K., Fritzler, M.J. and Tan, E.M. (1978) J. Immunol. 121, 2228–2234.
[2] Prelich, G. and Stillman, B. (1988) Cell 53, 117–126.
[3] Nichols, A.F. and Sancar, A. (1992) Nucleic Acids Res. 20, 2441–2446.
[4] Warbrick, E. (2000) BioEssays 22, 997–1006.
[5] Bravo, R. and Celis, J.E. (1980) J. Cell Biol. 84, 795–802.
[6] Bravo, R. and Celis, J.E. (1985) FEBS Lett. 182, 435–440.
[7] Morris, G.F. and Mathews, M.B. (1989) J. Biol. Chem. 264, 13856–13864.
[8] Chang, C.D., Ottavio, L., Travali, S., Lipson, K.E. and Baserga, R. (1990) Mol. Cell. Biol. 10, 3289–3296.
[9] Morris, G.F. and Mathews, M.B. (1991) J. Virol. 65, 6397–6406.
[10] Lee, B.H. and Mathews, M.B. (1997) Proc. Natl. Acad. Sci. USA 94, 4481–4486.
[11] Ottavio, L., Chang, C.D., Rizzo, M.G., Travali, S., Casadevall, C. and Baserga, R. (1990) Mol. Cell. Biol. 10, 303–309.
[12] Ottavio, L., Chang, C.D., Rizzo, M.G., Petralia, S., Travali, S. and Baserga, R. (1990) Biochem. Biophys. Res. Commun. 169, 509–516.
[13] Alder, H., Yoshinouchi, M., Prystowsky, M.B., Appasamy, P. and Baserga, R. (1992) Nucleic Acids Res. 20, 1769–1775.
[14] Tommasi, S. and Pfeifer, G.P. (1999) J. Biol. Chem. 274, 27829–27838.
[15] Wang, L., Li, M., Yan, P.J. and Lu, C.D. (2000) Acta Biochim. Biophys. Sin. 32, 451–458.
[16] Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nucleic Acids Res. 11, 1475–1489.
[17] Tomita, N., Horiuchi, M., Tomita, S., Gibbons, G.H., Kim, J.Y., Baran, D. and Dzau, V.J. (1998) Am. J. Physiol. 275, F278–F284.
[18] Baserga (1991) J. Cell Sci. 98, 433–436.
[19] Guarguaglini, G., Battistoni, A., Pittoggi, C., Di Matteo, G.., Di Fiore, B. and Lavia, P. (1997) Biochem. J. 325, 277–286.
[20] Morris, G.F., Bischoff, J.R. and Mathews, M.B. (1996) Proc. Natl. Acad. Sci. USA 93, 895–899.
[21] Wells, J., Graveel, C.R., Bartley, S.M., Madore, S.J. and Farnham, P.J. (2002) Proc. Natl. Acad. Sci. USA 99, 3890–3895.
[22] Di Fiore, B., Guarguaglini, G., Palena, A., Kerkhoven, R.M., Bernards, R. and Lavia, P. (1999) J. Biol. Chem. 274, 10339–10348.
[23] Wade, M., Blake, M.C., Jambou, R.C., Helin, K., Harlow, E. and Azizkhan, J.C. (1995) J. Biol. Chem. 270, 9783–9791.
[24] Kovesdi, I., Reichel, R. and Nevins, J.R. (1986) Cell 45, 219–228.
[25] Blake, M.C. and Azizkhan, J.C. (1989) Mol. Cell. Biol. 9, 4994–5002.
[26] Nevins, J.R. (1992) Science 258, 424–429.
[27] Kosugi, S., Suzuka, I. and Ohashi, Y. (1995) Plant J. 7, 877–886.
[28] Egelkrout, E.M., Robertson, D. and Hanley-Bowdoin, L. (2001) Plant Cell 13, 1437–1452.
[29] Yamaguchi, M., Hayashi, Y. and Matsukage, A. (1995) J. Biol. Chem. 270, 25159–25165.
[30] Huang, D.Y. and Prystowsky, M.B. (1996) J. Biol. Chem. 271, 1218–1225.
[31] Nevins, J.R. (1998) Cell Growth Differ. 9, 585–593.
[32] Tommasi, S. and Pfeifer, G.P. (1995) Mol. Cell. Biol. 15, 6901–6913.
[33] Tommasi, S. and Pfeifer, G.P. (1997) J. Biol. Chem. 272, 30483–30490.
[34] Chaboute, M.E., Clement, B. and Philipps, G. (2002) J. Biol. Chem. 277, 17845–17851.