FEBS Letters 441 (1998) 200–204 FEBS 21298

Serum responsiveness of the rat PCNA promoter involves the proximal ATF and AP-1 sites

Yin-Chang Liu*, Hsueh-Wei Chang, Yi-Chyi Lai, Sheue-Ting Ding, Ji-Ling Ho

Department of Life Science, National Tsing-Hua University, Hsin-Chu 30043, Taiwan

Received 12 October 1998

Abstract We have previously shown that the rat PCNA (proliferating cell nuclear antigen) gene promoter is responsive to serum stimulation. In this study, the sequence of the promoter responsive to serum stimulation has been localized in the region between nucleotides −70 and +125 relative to the transcription initiation site. This region contains an ATF site (nucleotides −51 to −44) and an AP-1 site (nucleotides −64 to −58). Mutation at either the ATF or the AP-1 site reduced the serum responsiveness of the promoter. In gel mobility shift assays, nuclear extracts from serum stimulated cells, compared to those from quiescent cells, exhibit an increasing binding activity toward a promoter related oligonucleotide (−70 to −42) which includes the ATF site and the AP-1 site. Formation of the DNA:protein complexes requires the simultaneous involvement of ATF and AP-1 sites as either element can abrogate the complexes in the competition experiment. Both the distance and sequence are essential to complex formation. Moreover, ATF-1 but not ATF-2 (or CREB) has been identified as a major component of the complexes in the antibody supershift or interference experiment. The results of this study suggest that ATF-1 in association with other factors is involved in regulating the serum stimulation of the rat PCNA promoter activity via the proximal ATF and AP-1 sites.

Key words: Serum responsiveness; Rat proliferating cell nuclear antigen promoter; ATF site

1. Introduction

Proliferating cell nuclear antigen (PCNA) is a protein factor required for DNA synthesis and DNA repair [1–5]. In addition to its known interactions with components of the DNA replication machinery (i.e. DNA polymerase δ and replication factor C), PCNA has been found to interact directly with various proteins which are involved in cell cycle progression, DNA repair and other functions [6] including cyclin D/cyclin D dependent kinase [7], $p21^{Waf1}$ (a CDK inhibitor) [8], Gadd45 (a UV inducible protein) [9], and MyD118 (a differentiation primary response transcription factor) [10]. These interactions suggest the expression of PCNA as a potential target in the control of cell proliferation, differentiation, apoptosis and senescence. Previous studies on the regulation of PCNA gene expression have indicated that the expression of PCNA is dependent on mRNA level and is growth regulated [11–13]. Although the level of PCNA mRNA may be regulated post-transcriptionally [14], the induction of human PCNA gene expression by adenovirus E1A as well as the serum stimulation of rat PCNA gene expression are controlled at the transcriptional level [15,16]. In addition, the promoter of the human PCNA gene is solely responsible for the failure of gene expression in G1 arrested cells [17]. Promoters of the mammalian PCNA genes are TATA-less with a cluster of GC boxes in the region between nucleotides −300 and −100 relative to the transcription initiation site [15,16,18,19]. The full promoter activity is approximately within the 200 bp upstream of the +1 site. The E1A responsive region of the human PCNA promoter is localized in the 85 nucleotide region upstream of the +1 site. Furthermore, the integrity of the ATF/CRE site (nucleotides −52 to −45) of the promoter is crucial to E1A induction [20]. The same ATF/CRE site is also present in the corresponding regions of rodent PCNA promoters. Previously we have observed that the rat PCNA promoter is responsive to serum stimulation [16]. In this report, the region of the promoter responsive to serum stimulation has been identified and the importance of the proximal ATF/CRE site and its immediately upstream AP-1 site have been shown. Our data suggest that ATF-1 in association with other factors is involved in regulating the serum stimulation of the rat PCNA promoter activity via the proximal ATF site and the AP-1 site.

2. Materials and methods

2.1. Cell culture

Rat glioma (9L) cells and Chinese hamster ovary cells were provided by the American Type Culture Collection and cultured in Eagle's minimum essential medium and McCoy's 5A medium, respectively. Cells were routinely maintained in media containing 10% fetal bovine serum (FBS) at 37°C in 5% CO_2.

2.2. Plasmids

The rat PCNA promoter plasmid, −693CAT (nucleotides −693 to +125, previously referred to as D6), was prepared as described in [16]. Two PCNA promoters of shorter length at the 5′ end (−240 to +125, and −70 to +125, respectively) were obtained by the PCR method using −693CAT as the template. They are referred to herein as −240CAT and −70CAT. The site-specific mutations of the rat PCNA promoter −240CAT at ATF site (−51 to −44) and AP-1 (−64 to −58) sites were obtained by megaprimer PCR [21] using mutated primers 5′-AGGTTGTGCATATGCAGCGCTGACC-3′ and 5′-GGTGGGTCATATGGCGCTGTGACGC-3′, respectively. To facilitate the identification, the original ATF (TGACGCCA) and AP-1 (TGGGTCA) sites were replaced with the restriction enzyme *Nde*I recognition site (CATATG). Mutations of the promoter were verified by DNA sequencing analyses. All the promoters were subcloned in the vector of pBasic-CAT (Promega Co.) in such a way that the reporter gene chloramphenicol acetyltransferase (CAT) was driven by the promoters.

2.3. DNA transfection

Transient transfection of PCNA promoters into 9L cells was performed using the DEAE-dextran method and the CAT assay was used to measure the promoter activity as described previously [16]. To avoid dish-to-dish variation of transfection efficiency, the strategy

*Corresponding author. Fax: (886) (3) 571-5934.
E-mail: lslyc@life.nthu.edu.tw

PII: S0014-5793(98)01549-X

described in [23] was used, i.e. to transfect large cells and then to trypsinize the cells after 24 h of incubation and distribute them among smaller dishes. Furthermore, in the transient expression experiments, we used the protein amount to normalize the CAT activity, for conventional promoters such as pCMV-β-gal have been shown to be serum responsive and promoters non-responsive to serum stimulation were not available to us. Nonetheless, we made efforts to minimize the weight of transfection efficiency variation in our data, by repeating the same experiment for 5–6 times. For obtaining stable transfectants of the promoters, cells were co-transfected with the promoters and pCMV-Neo, a plasmid conferring neomycin (G418) resistance on cells. Cells that survived in the presence of antibiotic were pooled together as mixed clones for experiments.

2.4. Preparation of nuclear extracts

CHO.K1 cells at 70–80% confluence were serum-starved for 48 h to reach quiescence. The quiescent cells were divided into two parts: one part of cells was continuously grown in serum-free medium and the other part of cells was cultured in medium with 10% FBS. The cells were harvested at the indicated time for nuclear preparation according to the procedure described in [22], except that an additional centrifugation step (30 000 rpm for 10 min) was adopted to clarify the nuclear extracts.

2.5. Electrophoretic mobility shift assay (EMSA)

2.5.1. Probe preparation and labeling. The oligonucleotides were prepared by synthesizer. The sense strand and the antisense strand were annealed in annealing buffer (10 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA) by the following procedure with a thermal cycler: 2 min at 88°C, 10 min at 65°C, 10 min at 37°C, and then 5 min at 25°C. The oligonucleotides were labeled by Klenow reaction or kinase reaction as described in [23]. The labeled probes were gel-purified prior to use.

2.5.2. EMSA. The DNA binding assays were performed in 1×EMSA buffer consisting of 12 mM HEPES (pH 7.6), 50 mM NaCl, 1 mM DTT and 5% (v/v) glycerol. DNA probes (~20 000 cpm) were added to 20 μl binding reactions containing 5–10 μg of nuclear extracts and 1.5 μg poly(dI-dC)-poly(dI-dC). The incubations were conducted at 4°C for 15 min following a 20 min pre-incubation at the same temperature in the absence of probe. For supershift or antibody interference assay, antibodies were added to the binding reaction 1 h prior to the addition of the probe. All antibodies were obtained from Santa Cruz Biotechnology, Inc. They were antibodies to ATF-1 (sc-243x), to ATF-2 (sc-187x), to c-jun/AP-1 (sc-44x) and to c-fos (sc-413x). DNA:protein complexes were resolved on 0.4 mm thick 5% polyacrylamide (29:1 Bis)-0.5×TBE gels. Gel electrophoresis was preformed in the cold room at 10 V/cm without buffer recirculation. The gels after separation were dried and exposed to X-ray films at −70°C with intensifying screens.

3. Results and discussion

3.1. Serum responsiveness of the rat PCNA promoter is localized in the region between nucleotides −70 and +125

Previously we showed that the rat PCNA promoter (nucleotides −693 to +125) is responsive to serum stimulation [16]. To localize the region responsive to serum stimulation, we used promoters with variable deletions at the 5′ region for the study. Deletion of the 5′ sequence did not significantly affect the basal activity of the promoter unless it went over nucleotide −240 (see Fig. 1A). The shortest promoter −70CAT has only about 40% of the full activity. Nonetheless, serum responsiveness was observed with the promoters even for −70CAT. The activity of the promoter increases during the course of serum stimulation (Fig. 1B). The promoter −70CAT contains AP-1 (nucleotides −64 to −58) and ATF/CRE (nucleotides −51 to −44) consensus sites. The ATF/CRE site and its immediately upstream sequence are fairly conserved among the mammalian PCNA promoters [20]. The respective region in human PCNA promoter is re-

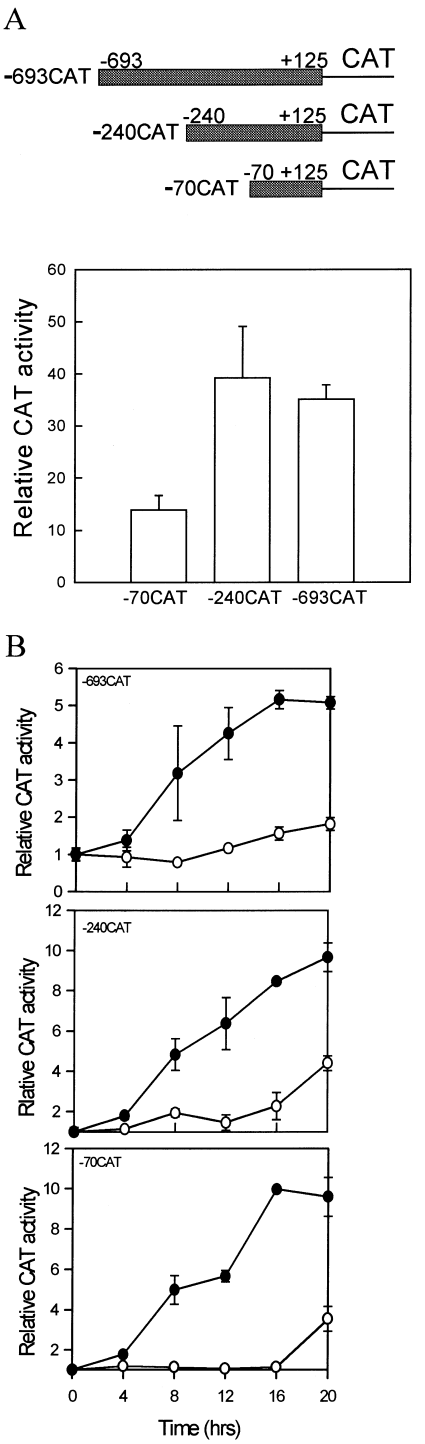

Fig. 1. A: Relative activities of the rat PCNA promoters with variable 5′ ends. Rat glioma (9L) cells were transiently transfected with a PCNA promoter as described previously [16]. Cell extracts were prepared and assayed for CAT enzyme activity at 46 h post transfection. B: Serum responsiveness of the rat PCNA promoters with variable 5′ ends. Cells (9L) were transiently transfected with a PCNA promoter as mentioned in A and recovered for 2 days in serum-free medium to become quiescent. Cells were then grown in serum-containing (●) or serum-free (○) medium and harvested at the indicated period for CAT activity assay. The change of the promoter activity during the time course is shown by the relative level to that at time zero. The activity at time zero was set at 1. The variation of transfection efficiency was controlled by the strategy as described in Section 2. Data are presented from at least three independent experiments, and the standard error bars are indicated.

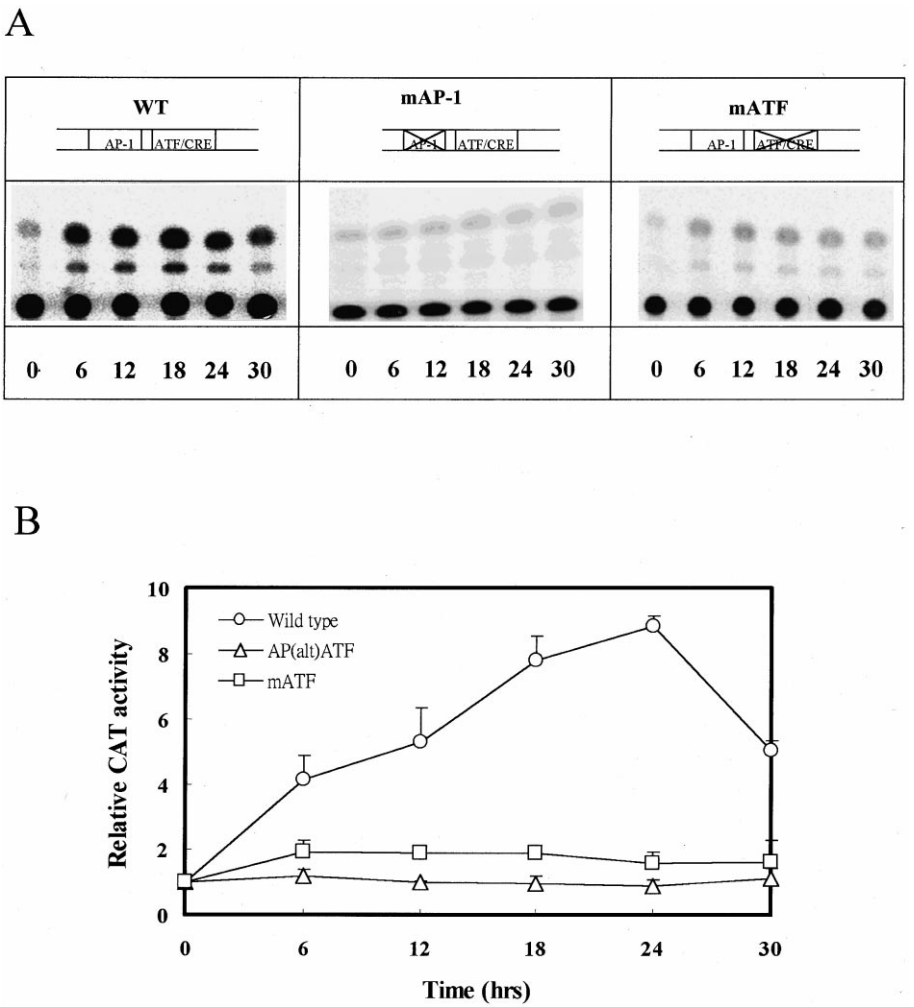

Fig. 2. Effects of mutations of the ATF and AP-1 sites on serum responsiveness of the rat PCNA promoters. CHO.K1 stable transfectants of wild type or mutant rat PCNA promoters were serum-starved for 2 days and then serum-stimulated again for the indicated interval before harvest for the CAT assay. A: Typical results of a CAT analysis. Each CAT activity assay contained a constant amount of crude extract. The intervals (6, 12, 18 etc.) are in hours. B: Serum responsiveness of the wild type and mutant PCNA promoters. The change of activity for each promoter during the time course is shown by the relative level to that at time zero. The activity at time zero was set at 1. Data are presented from at least three independent experiments, and the standard error bars are indicated.

sponsible for E1A activation [19]. In order to investigate if the region of the rat PCNA promoter is important to serum stimulation, we constructed promoters with site-directed mutations at either the ATF/CRE (−51 to −44) or the AP-1 (−64 to −58) site. For convenience, these mutant promoters are referred to as mATF and mAP-1, respectively.

3.2. *Serum responsiveness of the rat PCNA promoter involves the proximal ATF/CRE and AP-1 sites*

Typical results of the promoter activity assays are shown in Fig. 2A, indicating that both mutant promoters have lower basal activity than the wild type, and particularly the mAP-1 is notably weak. Hence, the ATF/CRE and the AP-1 sites are important to the basal activity of the promoter. Furthermore, serum responsiveness is significantly reduced in both mATF and mAP-1 as compared to the wild type (see Fig. 2B), indicating that the integrity of the ATF/CRE and AP-1 sites is crucial to the serum responsiveness of the promoter.

3.3. *Serum stimulation increases the binding activity in nuclear extract toward the rat PCNA promoter related oligonucleotide*

To study the nature of the regulatory proteins which modulate the promoter activity, we performed EMSAs. The DNA probe in the EMSA experiments was an oligonucleotide containing the promoter sequence between nucleotides −70 and −42, which includes both the AP-1 and the ATF sites. Results of a typical EMSA experiment are shown in Fig. 3, indicating that formation of specific DNA:protein complexes is increased due to serum stimulation (compare lanes 2 and 4 or lanes 3 and 5). Furthermore, complex formation was abrogated in the presence of either ATF or AP-1 oligomers (lanes 6 and 7), suggesting that complex formation involves simultaneously ATF and AP-1 sites, i.e. the regulatory proteins form co-complexes with these two sites. As the two sites are relatively close to each other (6 bases apart), it is of interest to know if the distance or the sequence between the two sites is essential to complex formation. For this purpose, we per-

formed EMSA experiments with two mutant oligonucleotides as DNA probes. One of these mutant probes is referred to as del-space in which the sequence between AP-1 and ATF was shortened; the other is referred to as alt-space in which the sequence was altered while the distance remained the same (see Fig. 4A). Results of a typical experiment are shown in Fig. 4B (lanes 2–4), indicating that each mutant oligonucleotide gives similar but distinct patterns as compared to that of the wild type oligonucleotide. Seven complexes (1–7) were identified with the wild type probe, while only four complexes (2–5) were seen with the alt-space probe. In addition, with the alt-space probe, the formation of the DNA:protein complexes was apparently less effective (compare lanes 3 and 2 in Fig. 4B). On the other hand, with the del-space probe, two distinct complexes (* and **) were detected. Furthermore, some of the common complexes may be slightly enhanced with the del-space probe. Nevertheless, both alt-space and del-space lost the ability to form complex 1. Taken together, the data suggest that both the distance and the sequence of the space are essential to complex formation.

For identifying the protein components in the EMSA complexes, several antibodies of choice were included in the binding reaction mixture for antibody interference or supershift assays. In the presence of ATF-1 antibody (lane 6 of Fig. 4B), the complexes 3 and 4 were clearly supershifted, indicating that ATF-1 is a major component of the complexes. In contrast to ATF-1, c-jun (or c-fos) is apparently not a major component in the complexes as the antibody to c-jun (or c-fos) only interfered with the formation of complex 1. While the ATF site may be recognized by dimers of various members of the ATF family or the CREB family, other members of the families such as ATF-2 or CREB were not detected in the complexes (data not shown). Furthermore, the complexes were totally abrogated when the nuclear extracts were pretreated with phosphatase (data not shown), suggesting that phosphorylation of the transcription factors is critical to the serum stimulation of the rat PCNA promoter. Consistent with this finding is the observation that the activity of the promoter was induced in the presence of okadaic acid, a phosphatase inhibitor (unpublished results). At the moment, our experiences suggest that serum stimulation of the promoter involves the signal transduction pathways which lead to the phosphorylation of ATF-1 (and other associated factors). However, ATF-1 or related transcription factors may be phosphorylated

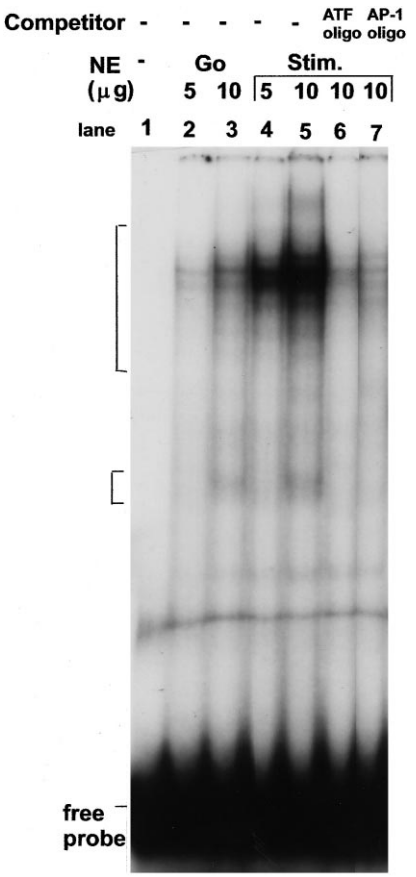

Fig. 3. EMSA of nuclear protein:rat PCNA promoter related oligonucleotide complexes. The nuclear extracts (NE) used in lanes 2 and 3 were obtained from quiescent (G_0) cells and those used in lanes 4–7 were obtained from serum-stimulated (10 h) cells. Nuclear extract used in lanes 2 and 4 was 5 μg, while in lanes 3 and 5–7 it was 10 μg. No nuclear extract was added in lane 1. The rat PCNA promoter related oligonucleotide (−72 to −42, see Fig. 4A) was ^{32}P-labeled. Lanes 6 and 7 contained excess cold ATF oligomer (5′-CAGCGCTGTGACGCCACAACC-3′) and AP-1 oligomer (5′-CGCTTGATGACTCAGCCGAA-3′), respectively. The specific DNA:protein complexes are indicated by brackets.

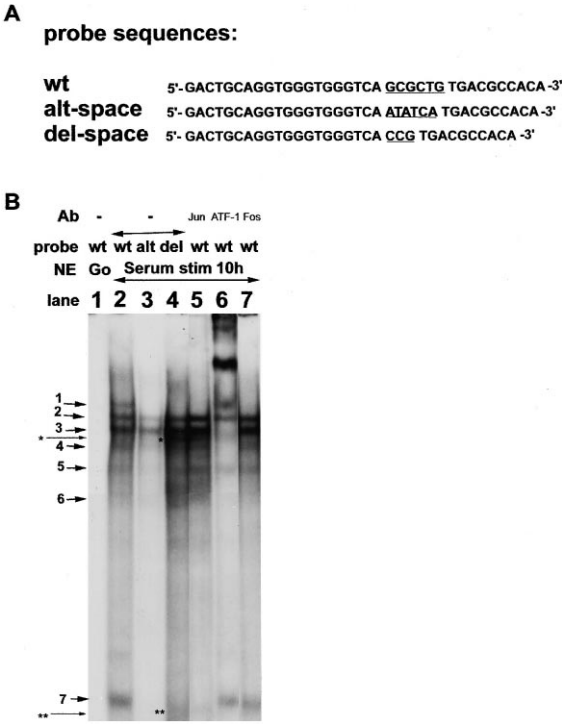

Fig. 4. Effects of alterations of space sequence between the ATF (−51 to −44) and the AP-1 (−64 to −58) sites on the formation of rat PCNA promoter related oligonucleotide:nuclear protein complexes. A: Promoter related probes: wt, oligonucleotide with the wild type space sequence (GCGCTG); alt-space, oligonucleotide with an altered space sequence (ATATCA); del-space, oligonucleotide with a deleted space sequence (CCG). Only the sequences of the sense strands are shown. B: EMSA using wild type (wt), alt-space (alt) or del-space (del) oligonucleotides as probes. The nuclear extract (NE) used in lane 1 was obtained from quiescent (G_0) cells and those used in lanes 2–7 were obtained from serum-stimulated (10 h) cells. Antibodies to c-jun (lane 5), ATF-1 (lane 6) or c-fos (lane 7) were included in the binding reactions with the wild type probe. Free probes in the gel are not shown. Two distinct complexes formed by the del-space probe are indicated by * and **.

by various protein kinases including cAMP-dependent protein kinase [24], Ca^{2+}/calmodulin-dependent protein kinase [25] and mitogen-activated protein kinases [26,27]. Each protein kinase represents a different signaling pathway. Hence, identification of the protein kinase leading to the activation of the rat PCNA promoter will be carried out in the future. As in the rat PCNA promoter, the ATF site of the human PCNA promoter is essential to E1A activation and ATF-1 is a major component of the PCNA-E1A responsive element protein complexes [28]. The resemblance suggests that E1A activation and serum stimulation may share common signaling pathways. However, a recent study has found that CREB but not ATF-1 was able to mediate transactivation of a minimal PCNA-CAT by E1A [29]. Hence, the role of ATF-1 in the regulation of the human (and rat) PCNA promoter activity should be further investigated.

In conclusion, we report herein the study on the *cis*- and *trans*-acting elements that may be involved in the serum stimulation of the rat PCNA promoter. The feature of serum responsiveness is localized in the region between bases −70 and +125, and the proximal ATF and AP-1 sites in this region are essential to both the basal activity and serum stimulation of the rat PCNA promoter. These two sites appear to crosstalk as either ATF or AP-1 oligomers was able to abrogate the whole DNA binding complex. To the best of our knowledge, this phenomenon is the first to be reported. In addition, we have not identified a case similar to the rat PCNA promoter that possesses both ATF and AP-1 sites in the vicinity. Based upon our data, we hypothesize that ATF-1 and its associated factors require the integrity of the ATF site and its immediately upstream AP-1 for transactivating the rat PCNA promoter in response to serum stimulation.

Acknowledgements: We are thankful to Mr. B.-S. Tzang for his technical assistance in the construction of the mutant promoters. We also thank Drs. Margaret D.T. Chang and A.A. Vyas for their valuable comments on the manuscript. The study was supported by Grant NSC87-2311-B007-029 from the National Science Council, Taiwan.

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