Transcription factors RFX1/EF-C and ATF-1 associate with the adenovirus E1A-responsive element of the human proliferating cell nuclear antigen promoter

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ABSTRACT

The proliferating cell nuclear antigen (PCNA) is an adenovirus E1A-inducible factor that is intimately linked to the processes of DNA replication and cell cycle regulation. Previously, we defined a novel *cis*-acting element, the PCNA E1A-responsive element (PERE), that confers induction by the E1A 243R oncoprotein upon the human PCNA promoter. To better understand the regulation of PCNA expression by E1A 243R, we have identified cellular transcription factors that associate with the PERE. In electrophoretic mobility shift assays, the PERE formed three major complexes (P1, P2 and P3) with proteins in nuclear extracts from HeLa or 293 cells. Formation of complexes P2 and P3, which correlates with PCNA promoter activity *in vivo*, requires the activating transcription factor (ATF) binding site found within the PERE [Labrie et al. (1993) *Mol. Cell. Biol.*, 13, 1697–1707]. Antibody interference experiments and mobility shift assays performed with *in vitro*-synthesized protein indicated that the transcription factor ATF-1 is a major component of these complexes. Similar assays demonstrated that the hepatitis B virus enhancer-associated protein RFX1 constitutes a major component of the P1 complex. In addition, we examined the binding of proteins to the minimal E1A-responsive promoter to identify other factors important for transcription from the PCNA promoter. Mobility shift assays revealed that a fragment encompassing the region from –87 to +62 relative to the transcription initiation site forms at least five complexes, EH1-EH5, with HeLa cell nuclear extracts. The transcription factor YY1 associates with the initiator element of the PCNA promoter. The identification of these transcription factors will allow their roles in the activation of PCNA by E1A to be evaluated.

INTRODUCTION

The proliferating cell nuclear antigen (PCNA) is a critical component of the eukaryotic DNA replication and repair machinery. *In vivo*, PCNA is required for cell cycle progression and growth of both yeast and mammalian cells (1) and PCNA antisense oligonucleotides inhibit BALB/c3T3 cell proliferation (2). Human PCNA was originally identified as a protein of ~35 000 kDa through its reaction with autoantibodies (3). It is found as a trimer which appears to be the antigenic as well as the catalytically active form (4). Proliferating cell nuclear antigen probably functions as a 'sliding clamp' to increase the processivity of DNA synthesis (5). As the auxiliary factor of DNA δ (6,7), PCNA is involved in the repair of mutagen-damaged DNA (8) and is required for both leading- and lagging-strand DNA synthesis *in vitro* (9,10).

Proliferating cell nuclear antigen activity appears to be regulated through interactions with cell-cycle regulatory proteins. In normal diploid fibroblasts, but not in their SV40 virus-transformed derivatives or other transformed cell lines, PCNA exists in quaternary complexes containing a cyclin, a cyclin-dependent kinase (CDK) and the CDK-inhibitor p21 (11–14). More recently, p21 was shown to inhibit reconstituted SV40 DNA replication through a direct interaction with PCNA (15). The effect of p21 on DNA replication in this system was reversed by the addition of excess PCNA, suggesting that the level of PCNA expression in transformed cells might play a determining role in the disruption of normal cell-cycle control of DNA replication.

In this respect, the activation of PCNA expression by adenovirus is a particularly interesting model for the study of the molecular mechanisms underlying transformation. The induction of PCNA by adenovirus E1A was first described in rodent cells (16) and has since been studied in greater detail using transient expression experiments in HeLa cells (17). Proliferating cell nuclear antigen induction depends on the transforming functions of E1A common to the 243- and 289-amino acid products of the adenovirus E1A gene, hereafter referred to as E1A 243R and E1A 289R, since both are equally capable of inducing PCNA expression in the context of a viral infection or in transient expression assays (16,18). More specifically, optimal PCNA induction requires domains of E1A 243R involved in binding to the transcriptional adaptor p300 (19–21) and the retinoblastoma protein-related p107-tumor suppressor protein (20,22).

Activation of PCNA expression by E1A occurs at the transcriptional level through an increase in PCNA promoter activity (18). A PCNA E1A-responsive element, termed the PERE, resides between –59 and –45 relative to the transcription

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start site of the human PCNA promoter and contains a sequence from –52 to –45 which is homologous to an activating transcription factor (ATF) motif (23). The PERE is required for basal and E1A 243R-induced PCNA expression in transient expression assays (23) and can confer E1A-responsiveness to a heterologous promoter (24). To further elucidate the mechanism of PCNA induction by the E1A 243R oncoprotein, we have identified cellular transcription factors that associate with the E1A-responsive element of the PCNA promoter (PERE). Our studies demonstrate that ATF-1 and the hepatitis B virus enhancer-associated protein RFX1, also known as enhancer factor C (25), interact independently with the PERE. Furthermore, results from an expanded analysis of the –87 to +62 region of the PCNA promoter provide evidence that the transcription factor YY1 associates with the initiator element of the PCNA promoter. The identification of the transcription factor complexes reported here provides information essential to understanding the mechanisms of regulation of PCNA promoter activity.

MATERIALS AND METHODS

Preparation of nuclear extracts

Spinner cultures of HeLa cells (ATCC CCL2) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 100 µg of penicillin and streptomycin per ml. Human 293 cells were grown in the same medium containing 10% calf serum instead of 10% fetal calf serum. Nuclear extracts were prepared using a modification of the Dignam procedure (26). In brief, cells were harvested, rinsed with ice-cold phosphate-buffered saline and homogenized in two packed-cell volumes of Buffer A [10 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES; pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. Nuclei were pelleted by centrifugation and homogenized in 2.5 ml Buffer C per 10^9 cells [Buffer C: 20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 25% (v/v) glycerol]. The resulting supernatants were dialyzed against Buffer D [20 mM HEPES, pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM each DTT and PMSF and 20% (v/v) glycerol], quick-frozen in liquid nitrogen and stored at –70°C.

Oligonucleotides

The wild-type PERE oligonucleotide contains sequences –60 to –40 relative to the transcription start site of the human PCNA promoter while the BAM oligonucleotide harbors a 4 bp mutation (underlined) within the core of the ATF consensus binding site. Oligonucleotide 4441 harbors a 4 bp mutation between nucleotides –44 and –41:

PERE 5'-CAGCGTGGTGACGTCGCAACG-3'
BAM 5'-CAGCGTGGTG<u>GATC</u>CGCAACG-3'
4441 5'-CAGCGTGGTGACGTCG<u>ATC</u>GG-3'

The EP oligonucleotide corresponding to nucleotides 1151–1169 of the hepatitis B virus enhancer (5'-GATCCGTT-GCTCGGCAACGGCCTA-3') was supplied by Y. Shaul. The INIT oligonucleotide used for the study of the PCNA promoter-initiator binding proteins contains sequences from –9 to +12 relative to the transcription start site of the human PCNA promoter while mINIT harbors a CA to GT substitution (underlined) at positions –1 and +1:

INIT 5'-CGCGGCGGCATTAAACGGTTG-3'
mINIT 5'-CGCGGCGGG<u>GT</u>TTAAACGGTTG-3'

The oligonucleotides AAV P5 and ML TATA contain binding sites for the transcription factors YY1 and TBP corresponding to the adeno-associated virus P5 promoter –60 element (27) and the adenovirus major-late promoter TATA-box (underlined) respectively:

AAV P5 5'-AAGGGGGGC<u>AAAATGTC</u>GGGGTGG-3'
MLTATA 5'-AAGGGGGGC<u>TATAAAAG</u>GGGGTGG-3'

All of the oligonucleotides contain a 5' overhang (5'-GATC-3') for labeling. Double-stranded oligonucleotides were prepared by annealing complementary oligonucleotides in Oligo Buffer [OB: 10 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA].

DNA probes

Oligonucleotides used as probes in electrophoretic mobility shift assays (EMSA) were labeled with [α-^{32}P]dATP (3000 Ci/mmol) and three cold NTPs using DNA polymerase I (Klenow fragment). Proliferating cell nuclear antigen promoter fragments (~150 bp) used as EMSA probes were released from PCNA-87 CAT plasmids (23) by *Eco*RI and *Hin*dIII digestion and labeled. All probes were purified on native 6% polyacrylamide gels, eluted from gel slices in OB containing 0.5% (w/v) SDS and resuspended in OB following phenol extraction and precipitation.

EMSA

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. Oligonucleotide probes (20 000 c.p.m.) were added to 20 µl binding reactions containing 5 µg of nuclear extract protein and 2 µg poly(dI-dC)-poly(dI-dC). Experiments conducted with Schneider cell extracts used 100 ng of poly(dC-dG) competitor DNA in the binding reactions (Fig. 8). Unless indicated otherwise, incubations were conducted at 25–37°C for 15 min following a 15 min preincubation at the same temperature in the absence of probe. Binding to EH87 probes was performed under similar conditions except that 1.5 µg of competitor DNA was used and incubation was at 16°C. Where indicated, unlabeled competitor oligonucleotides were added to the binding reactions at the same time as the labeled probes, in which case an equivalent volume of OB was added to control assays. Antibodies were added to the binding reactions at least 1 h prior to the addition of probe and incubation was at 4°C. Oligonucleotide-associated complexes were resolved on 0.4–1 mm-thick 5% polyacrylamide (29:1 Bis)-0.5 × TBE gels. Optimal resolution of EH87-associated complexes was obtained on 1.5 mm-thick 4.5% polyacrylamide (39:1 Bis)-0.25 × TBE gels. Gels were run in the cold room at 10 V/cm without buffer recirculation, fixed in 10% methanol/10% acetic acid, dried and exposed at –70°C with intensifying screens. Gel shift experiments with INIT and mINIT probes were run at room temperature instead of in the cold room.

Antibodies

Rabbit antibodies raised against a full-length GST fusion protein of ATF-1 (amino acids 57–271) and the sheep antiserum raised against a GST-fusion protein containing amino acids 1–30 of ATF-1 (28) were provided by S. Wagner and M. R. Green. Transcruz® supershift antibodies against ATF-1 (C41–5.1),

ATF-2 (F2BR-1), ATF-3 (C-19), CREB-1 (24HB4), CREB-2 (C-20) and YY1 (C-20) were purchased from Santa Cruz Biotechnology. Anti-RFX1/EF-C serum was provided by P. Hearing. The anti-YY1 Ig3-1 antibody (29) was provided by S. Natesan. Antibody against c-abl was provided by Y. Shaul.

In vitro transcription and translation

Plasmids for *in vitro* transcription-translation were provided by S. Wagner (ATF-1) and W. Reith and P. Hearing (RFX1). RNA encoding ATF-1 was synthesized from *Ssp*I-linearized pGEM3-ATF-1 (28) as described (30) and translated in a reticulo-cyte lysate. RFX1 RNA was transcribed from *Hind*III-linearized pHRFX1 (25) and translated in a reticulocyte lysate. Binding assays with unlabeled proteins were performed immediately following translation of ATF-1 and RFX1 synthesis reactions. ^{35}S-methio-nine-labeled proteins were synthesized in parallel reactions and the translation products analyzed in 12% polyacrylamide-SDS gels.

RESULTS

Transcription factor binding sites in the minimal E1A-responsive PCNA promoter

To understand how E1A 243R induces PCNA expression, we sought to identify the cellular transcription factors that associate with the minimal E1A-responsive PCNA promoter which is comprised of sequences between −87 and +62 relative to the transcription initiation site (18). Within this region lies the PCNA E1A-responsive element (PERE; nucleotides −59 to −45), which harbors a putative ATF binding site, as well as a putative initiator element and a degenerate TATA box (5′-TGAGA-3′). To identify proteins that associate with the minimal E1A-responsive PCNA promoter, HeLa cell nuclear proteins were incubated with probe EH87 which comprises nucleotides from −87 to +62 of the human PCNA promoter (Fig. 1A, lane 1).

HeLa nuclear extracts formed five major complexes with EH87, identified EH1 to EH5 in order of increasing mobility. Similar complexes were also observed in adenovirus-trans-formed human 293 cells (data not shown). We then performed competition experiments using unlabeled double-stranded oligo-nucleotides to determine which of these complexes contained transcription factors that we suspected might be involved in the regulation of PCNA promoter activity. Note that the double-stranded PERE oligonucleotide used in the binding assays described throughout the text also contains the sequence 5′-CAACG-3′ (nucleotides −44 to −40) in addition to the PCNA E1A-responsive element proper (nucleotides −59 to −45). Com-plexes EH1 and EH4 were efficiently suppressed by the PERE oligonucleotide, indicating that the proteins present in these complexes interacted with sequences in the PCNA promoter situated between −60 and −40. The experiment was repeated using the BAM oligonucleotide which harbors a 4 bp mutation at the core of the ATF consensus sequence that abrogates transac-tivation of the human PCNA promoter by E1A 243R in transient expression assays *in vivo* (18). The BAM oligonucleotide efficiently competed for complex EH1 but did not affect EH4 (lane 3), strongly suggesting that the PERE-binding activity required for PCNA promoter activation by E1A resides in complex EH4. Complex EH3, which was mildly affected by both the PERE and BAM oligonucleotides, will not be discussed further since we have yet to identify EH3-associated proteins.
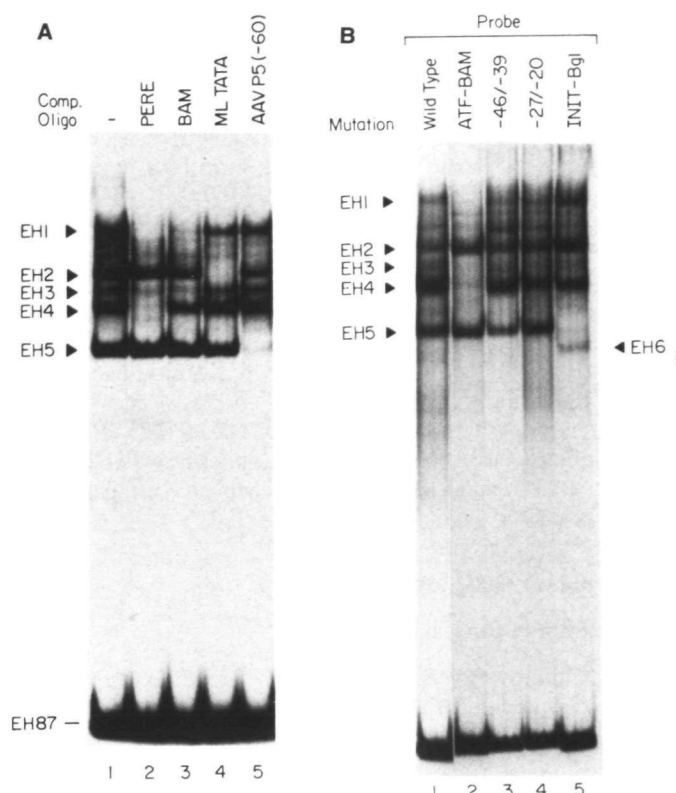

Figure 1. Association of cellular transcription factors with the minimal E1A-responsive promoter and identification of PERE-associated complexes. (A) An end-labeled PCNA promoter fragment from −87 to +62 relative to the transcription initiation site (EH87) was incubated with 5 μg HeLa nuclear extract and the protein–DNA complexes resolved on a 4.5% native polyacryl-amide gel. The major probe-associated complexes observed in lane 1 are labeled EH1–EH5 in order of increasing mobility. Oligonucleotide competi-tions (lanes 2–5) were performed with 5 pmol unlabeled double-stranded oligonucleotides corresponding to the PERE, a mutated PERE (BAM), a TBP-binding site (MLTATA) or a YY1-binding site (AAV P5). (B) Probes derived from the wild-type PCNA-87 CAT construct (EH87) or from promoter constructs harboring mutations in the PERE (ATF-BAM, −46/−39), putative TATA element (−27/−20) or the putative initiator element (Init-Bgl), were incubated with HeLa nuclear extract and the protein-DNA complexes resolved on a native gel. Complex EH6 corresponds to a novel Init-Bgl-associated complex.

We obtained preliminary evidence for the association of other transcription factors with the PCNA promoter. The MLTATA and AAV P5 oligonucleotides harbor sites derived from the adenovi-rus major late (MLTATA) and the adeno-associated virus P5 promoters (AAV P5) which bind the general transcription factor TBP and the initiator binding protein YY1, respectively. Com-plexes EH2 and EH5 were specifically abrogated by MLTATA and AAV P5 respectively (lanes 4 and 5). These results imply the association of a TATA box-binding complex (EH2) and a complex containing the transcription factor YY1 (EH5) with the PCNA promoter. It is noteworthy that complexes EH1, EH3 and EH4 were not significantly affected by the MLTATA and AAV P5 competitor oligos. Thus, the formation of each of the five major EH complexes was affected by at least one competitor oligo-nucleotide.

To localize the regulatory elements corresponding to com-plexes EH1–EH5, we performed gel retardation assays employ-

ing EH87 probes with mutations derived from PCNA-CAT linker-scanning constructs (23). In agreement with the competition assays, the ATF-BAM probe derived from this construct failed to form complex EH4 (Fig. 1B, lane 2). However, despite its abrogation by a competing BAM oligonucleotide (Fig. 1A), complex EH1 did not associate with the BAM probe. A possible explanation would be that the BAM mutation is deleterious for binding of proteins to the ATF site and to a possible adjoining site but that proteins may still bind to this second element at the high concentrations of oligonucleotide used in the competition assays. Mutation of nucleotides –46 to –39 (EH4639) reduced EH1 formation without affecting complex EH4. Since this mutation permits wild-type activity of the PCNA promoter in transfection experiments (23), these data also point to EH4 as the relevant complex as far as induction by E1A 243R is concerned (lane 3).

A mutation in the region of the putative initiator element (EHINIT-BgI) caused a decrease in the intensity of complex EH5 and the appearance of a new complex, EH6 (lane 5), suggesting that this is a site for the binding of transcription factor YY1. In support of these results, the INIT-BgI mutation disrupts a consensus YY1 binding site (5′-CATT-3′) located from –1 to +3 of the PCNA promoter. On the other hand, although the competition experiments suggested the association of a TATA-box binding complex on the PCNA promoter (Fig. 1A, lane 4), a probe containing a *Bgl*II linker substitution from –27 to –20 (EH2720) which disrupts the putative TATA element did not adversely affect EH2 formation (lane 4). Mobility shift assays employing mutant probes derived from other PCNA-CAT linker-scanning constructs also failed to localize complex EH2 (data not shown). The characterization and mapping of complex EH2 remains to be determined.

Defining PERE-associated complexes

To examine PERE-binding complexes more specifically, HeLa cell nuclear extracts were incubated with the PERE oligonucleotide (Fig. 2A). We detected three major PERE-binding activities in HeLa cells labeled P1, P2 and P3. Two of these, P2 and P3, appear to be specific for ATF-binding since they were unaffected by competition with the BAM oligonucleotide (lane 5) but disappeared upon addition of PERE (lane 4). The formation of complexes P2 and P3 was relatively temperature-independent whereas complex P1 was sensitive to elevated temperatures (Fig. 2B). In fact, appreciable amounts of P2 and P3 were observed when binding reactions were incubated at 75°C prior to the addition of probe, while P1, on the other hand, was sensitive to temperatures as low as 55°C. Similar experiments conducted with EH87 probe and with fractionated HeLa nuclear extracts (data not shown) lead us to believe that P1 corresponds to at least part of the binding activity in EH1, while P2 and P3 correspond to EH4.

ATF-1 associates with the PERE

The presence of an ATF site in the PERE suggested that one or several members of the ATF transcription factor superfamily might associate with the PCNA promoter. In addition, the heat-stability of complexes P2 and P3 was consistent with the possibility that ATF-1 is a component of these complexes (31). To test this hypothesis, PERE-binding assays with HeLa nuclear extracts were conducted in the presence of three different antibodies raised against ATF-1. As shown in Figure 3, both complexes P2 and P3,
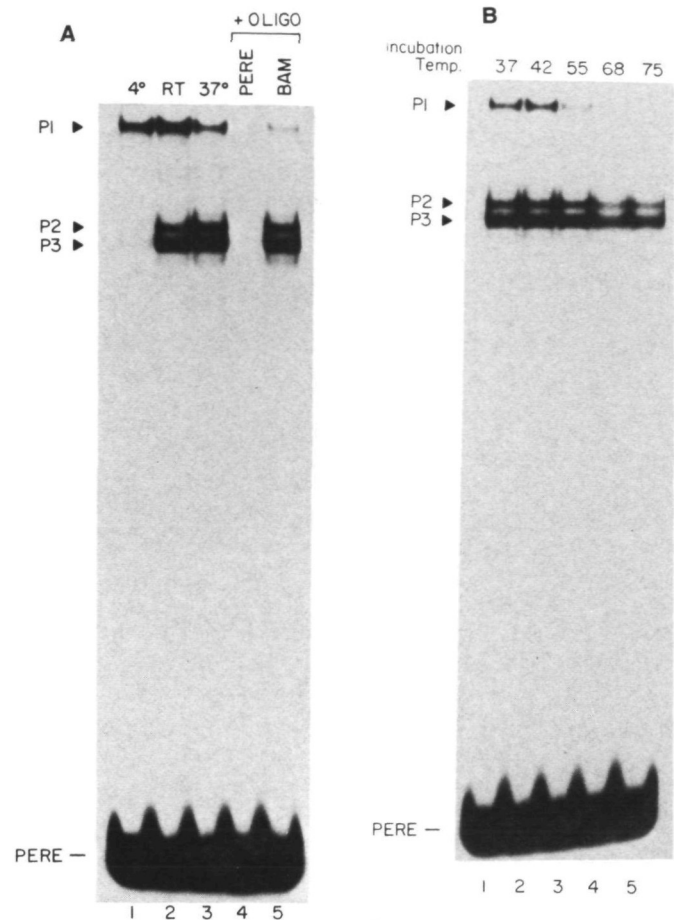

Figure 2. PERE-associated complexes. (A) Radiolabeled PERE oligonucleotide corresponding to nucleotides –60 to –40 relative to the transcription start site of the human PCNA promoter was incubated with 5 µg HeLa cell nuclear extract at 4°C, room temperature (RT) or 37°C and the protein–DNA complexes resolved on a native 5% polyacrylamide gel. Three major complexes, denoted P1, P2 and P3 are identified. Incubations at 37°C were performed in the absence (lane 4) or presence of 5 pmol unlabeled PERE or BAM oligonucleotides (lanes 4 and 5) (Note that P1 migrates ~1 cm into the gel). (B) To determine the heat-sensitivity of PERE–protein interactions, binding reactions were incubated at temperatures ranging from 37 to 75°C preceding the addition of labeled PERE probe.

but not complex P1, were supershifted by a mouse monoclonal antibody raised against ATF-1 (lane 2). Similar results were obtained with a rabbit polyclonal antibody raised against amino acids 57–271 of ATF-1 (lane 3). Since ATF-1 shares 64% amino acid identity with CREB-1, we also tested a sheep polyclonal antibody raised against the first 30 amino acids of ATF-1 expressed as a glutathione-S–transferase-ATF-1 fusion protein (28). This antibody does not react with CREB-1, but it also supershifted complexes P2 and P3 (lane 4) whereas a preimmune serum did not (lane 5). Together these results indicate that ATF-1 is a major component of PERE-associated complexes P2 and P3. As shown below, CREB-1 does not appear to be contained in these complexes.

As an additional proof of ATF-1 binding to the PERE, we examined the PERE-binding ability of ATF-1 synthesized *in vitro* in a rabbit reticulocyte lysate (Fig. 4). Several PERE-associated
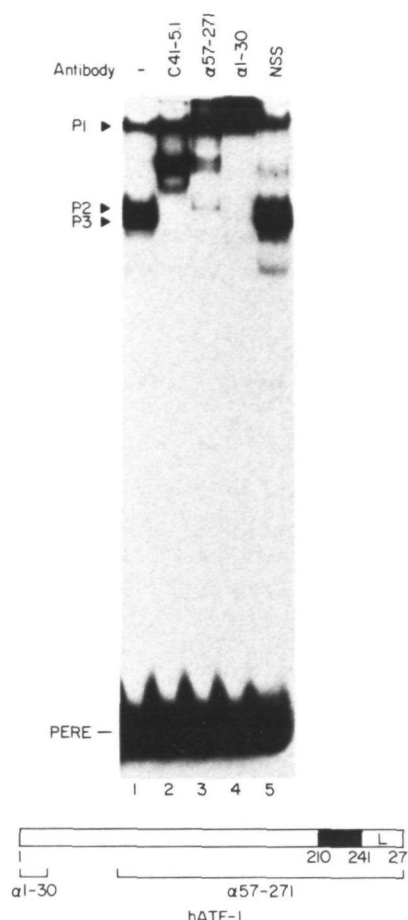
Figure 3. ATF-1 is a major component of complexes P2 and P3. Labeled PERE probe was incubated with HeLa cell extracts and the protein–DNA complexes analyzed by gel retardation assay as in Figure 2. Extracts were incubated in the absence (lane 1) or presence (lanes 2–4) of antibodies to the transcription factor ATF-1: C41–5.1 (100 ng), α-57–271 (1 µl of a 1/5 dilution) and α-1–30 (1 µl) C41–5.1 is a mouse monoclonal antibody while α-57–271 and α-1–30 are polyclonal rabbit and sheep antibodies respectively, raised against the corresponding amino acids of ATF-1 expressed as GST-fusion proteins. As a control, nuclear extract was incubated with normal sheep serum (lane 5) A schematic diagram of ATF-1 is shown below for reference. The basic region (amino acids 210–240) and the leucine zipper (amino acids 241–271) of ATF-1 are indicated.

Figure 4. *In vitro*-translated ATF-1 binds to PERE. Full-length ATF-1 was synthesized *in vitro* (IVT-ATF-1) and incubated with α^{32}P-labeled PERE probe. IVT-ATF-1 formed a specific complex, ◁ (lane 5), which migrated in the vicinity of complexes P2 and P3 observed in HeLa nuclear extract (lane 1). Lane 2, HeLa nuclear extract and reticulocyte lysate; lane 3, BSA alone (5 µg of Boehringer Manheim BSA was present in all binding reactions containing reticulocyte lysate); lane 4, BSA and reticulocyte lysate; lanes 5–8, programmed reticulocyte lysate alone or in the presence of unlabeled competitor PERE (lane 6), BAM oligonucleotide (lane 7) or anti-ATF-1 antibody (lane 8).

complexes were formed with unprogrammed reticulocyte lysate (lane 4). Although these complexes were specifically competed by PERE oligonucleotide (lane 6), they migrated faster than complexes P2 and P3 and were unaffected by an antibody against ATF-1 (lane 8). On the other hand, reticulocyte lysate programmed with transcript of the full-length ATF-1 cDNA formed a single protein–DNA complex migrating in the vicinity of complex P3 (lane 5). The specificity of the PERE-ATF-1 interaction was confirmed by oligonucleotide competition with the BAM oligo (lane 7) and coincubation with an anti-ATF-1 antibody (lane 8). It is tempting to speculate that the homodimer observed in reticulocyte lysates corresponds to HeLa P3 complex, but more definitive experiments will be required to determine if other proteins are present in complexes P2 and P3 or if these complexes reflect post-translational modifications of ATF-1 such as phosphorylation.
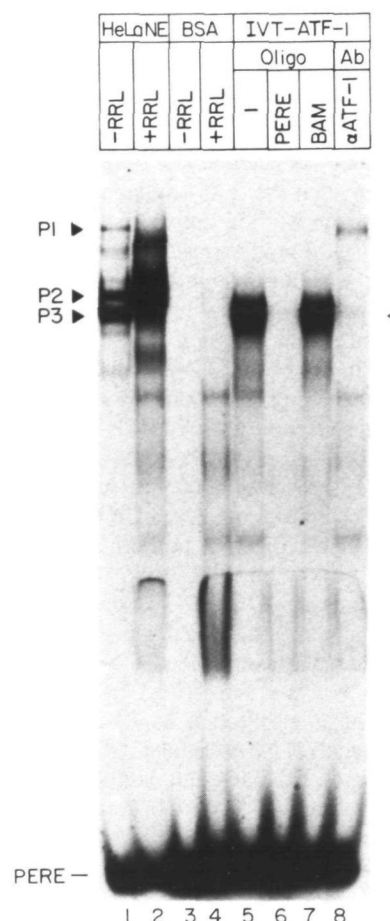
ATF-1 binding proteins

ATF transcription factors bind DNA as heterodimers or homodimers (30). To date, ATF-1 has been shown to dimerize with itself or with CREB-1 (28,32). However, while complexes P2 and P3 could be supershifted by as little as 25 ng of monoclonal antibody against ATF-1 (Fig. 3), up to 1 µg of monoclonal antibody raised against CREB-1 failed to disrupt either complex (Fig. 5A, lane 2). Similar results were obtained with antibodies raised against several other members of the ATF family, including ATF-2 and CREB-2 (lanes 1 and 3), as well as ATF-3, Jun and Fos (data not shown). To confirm our findings in another cell line we performed gel shift assays with nuclear extract from adenovirus-transformed 293 cells. The similar gel shift pattern obtained with 293 cell extract indicated that the adenovirus E1 proteins, present at high levels in 293 cells, do not greatly influence the complexes detected. In agreement with results obtained in HeLa cells, only
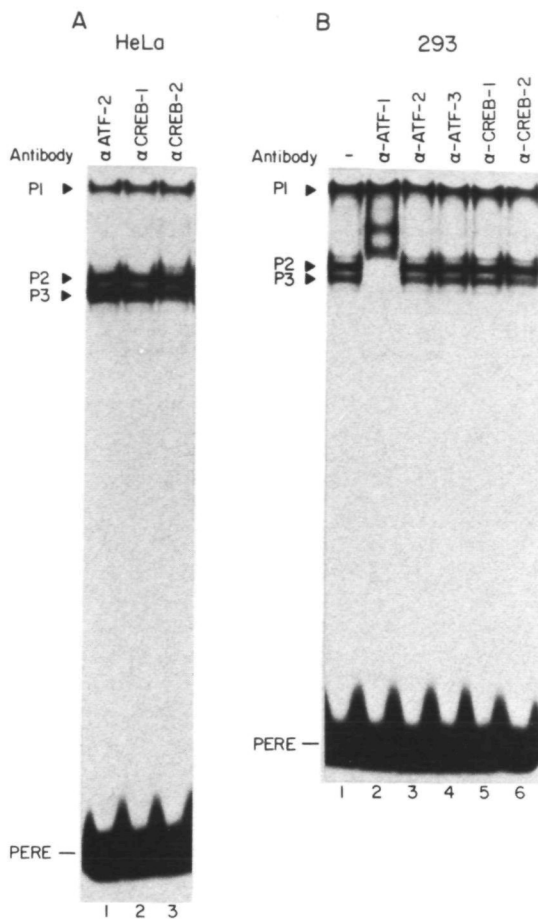
Figure 5. PERE-binding proteins in HeLa and 293 cells. (**A**) Radiolabeled PERE probe was incubated with 5 μg HeLa cell nuclear extract and the resulting protein–DNA complexes analyzed by gel retardation assay. Extracts were incubated at 4°C with 1 μg of antibodies to the ATF-2, CREB-1 and CREB-2 transcription factors prior to addition of the probe (lanes 1–3, respectively) (**B**) 5 μg of 293 nuclear extract was incubated with PERE probe and the resulting complexes resolved on a native 5% polyacrylamide gel. Complexes P1–P3 are identified. 293 extract was incubated in the absence (lane 1) or presence (lanes 2–6) of monoclonal or purified polyclonal antibodies to the transcription factors ATF-1, ATF-2, ATF-3, CREB-1 and CREB-2. 25 ng of anti-ATF-1 antibody was used in lane 2 while 1 μg of the indicated antibodies were used in lanes 3–6.

ATF-1 could be detected in PERE-associated complexes in 293 cells (Fig. 5B). None of the antibodies affected P1. While it is possible that the antibodies used do not recognize their cognate proteins under the assay conditions used, the experiments described above clearly identify ATF-1 as the major component of PERE-associated complexes.

Enhancer factor C (RFX1) binds to the PERE

Since the PERE oligonucleotide-associated P1 complex was sensitive to competition by both the PERE and BAM oligo-nucleotides (Fig. 2), we considered the possibility that this oligonucleotide, which encompasses the region from –60 to –40 of the PCNA promoter contains a second element distinct from the ATF site. The sequence 5′-GCAACG-3′ located between nucleotides –45 and –40 corresponds to one of two repeats found in the hepatitis B virus enhancer EP element (33). Addition of

unlabeled EP oligonucleotide to PERE-binding assays effectively eliminated complex P1 (Fig. 6, lane 4) but P1 was unaffected by coincubation with a mutated EP oligonucleotide (data not shown) and was reduced but not abolished with a PERE oligonucleotide harboring a 4 bp mutation between –44 and –41 (oligonucleotide 4441, lane 3). These results raised the possibility that the c-abl tyrosine kinase, which has been shown to bind to the HBV EP element and whose activity is regulated by the retinoblastoma protein (34,35), might associate with the human PCNA promoter. However, three different antibodies raised against c-abl failed to specifically affect complex P1 (data not shown). On the other hand, an antiserum against enhancer factor-C (EF-C), which also interacts with the HBV EP element (36), specifically supershifted complex P1 (lane 5), strongly suggesting that EF-C might associate with the PERE probe. The specificity of the supershift by anti-EF-C antibody was confirmed by the resistance of the P1 complex to normal rabbit serum and an anti-YY1 antibody (C-20) (lanes 6 and 7). To substantiate the importance of the 5′-GCAACG-3′ repeat between nucleotides –45 and –40 for complex P1 formation we incubated HeLa cell nuclear extract with radiolabeled 4441 probe in place of wild type PERE probe. Significantly, the 4441 probe formed complexes P2 and P3, but not P1 (lane 8).

The results of the antibody supershift experiment were confirmed by examining the PERE-binding ability of *in vitro*-synthesized (IVT) EF-C (Fig. 7). EF-C is identical to the major histocompatibility complex class II regulatory factor RFX1 (25). Reticulocyte lysate programmed with the full-length RFX1 cDNA formed a PERE-associated complex that migrated in the vicinity of complex P1 (lane 3). A faster migrating PERE-asso-ciated complex was observed in the unprogrammed reticulocyte lysate (lane 2). Although formation of this complex was reduced by the PERE and 4441 oligonucleotides (lanes 4 and 5), it was not affected by the EP oligonucleotide or any of the antibodies tested. In contrast, the IVT-RFX1 complex disappeared upon addition of PERE or EP oligonucleotide but was not eliminated by coincuba-tion with oligonucleotide 4441 (lanes 4–6). Moreover, the PERE-associated complex formed in the presence of IVT-RFX1 was specifically supershifted by the addition of anti-RFX1 antiserum but was unaffected by preimmune rabbit serum or an unrelated antibody (anti-ATF-1) (lanes 7–9). In accordance with these results, IVT-RFX1 did not associate with labeled 4441 oligonucleotide (lane 10) but did form a complex with labeled EP probe (lane 11).

YY1 protein associates with the initiator element of the PCNA promoter

The transcription factor YY1 has been shown to bind to the initiator element and mediate transactivation by E1A (27,29). The observation that an oligonucleotide corresponding to the YY1-binding site situated at –60 in the AAV P5 promoter can compete for one of the PCNA promoter-associated complexes (Fig. 1), raised the possibility that YY1 interacts with PCNA promoter elements. Furthermore, our results with the EH INIT-Bgl probe (Fig. 1B, lane 5) suggested that the binding site for YY1 on the PCNA promoter resides between nucleotides –1 to +3 with respect to the transcription initiation site. To determine whether YY1 binds to the initiator element of the PCNA promoter, we performed binding assays using a double-stranded oligonucleotide probe comprised of nucleotides –9 to +12 relative
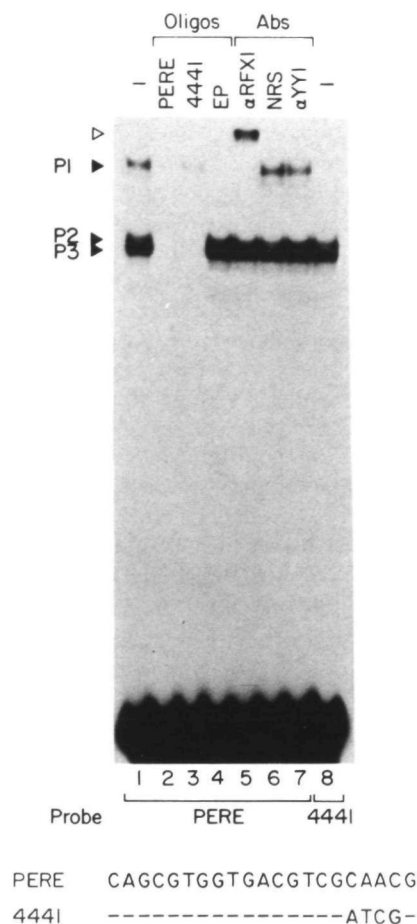
Figure 6. RFX1 is a major component of the P1 complex. HeLa cell nuclear extract (5 µg) was incubated with radiolabeled PERE probe (lanes 1–8) and the resulting protein–DNA complexes analyzed by gel retardation assay. Complexes P1, P2, and P3 are denoted. Extract was incubated in the absence (lane 1) or presence of excess unlabeled competitor oligonucleotides PERE (lane 2), 4441 (lane 3) or EP (lane 4) As shown below, oligonucleotide 4441 contains a 4 bp mutation in nucleotides –44 to –41 of the PERE ·oligonucleotide (5′-ATCG-3′ instead of 5′-CAAC-3′) which disrupts the putative RFX1-binding site contained within the PERE. EP corresponds to an RFX1 binding site present in the hepatitis B virus enhancer. In lanes 5–7, nuclear extract was preincubated with antiserum raised against RFX1 (lane 5), normal rabbit serum (NRS, lane 6) or an antibody against YY1 (lane 7) A supershifted RFX1 complex observed in the presence of anti-RFX1 antiserum is denoted by a ▷ (lane 5). As a control, HeLa extract was incubated with labeled 4441 oligonucleotide (lane 8).

Figure 7. *In vitro*-synthesized RFX1 binds to the PERE. RFX1 was synthesized *in vitro* (IVT) using a reticulocyte lysate programmed with mRNA generated from linearized pHRFX-1 plasmid. Incubation of IVT-RFX1 with radiolabeled PERE probe (lane 3) resulted in the formation of a specific complex that co-migrated with complex P1 observed in HeLa cell nuclear extract (lane 1). The unlabeled competitor oligonucleotides PERE, 4441 and EP were added to binding reactions containing IVT-RFX1 (lanes 4–6), while antiserum to RFX1, normal rabbit serum (NRS) or monoclonal antibody to ATF-1 were added in lanes 7–9. A supershifted complex, is denoted in lane 4. As controls, radiolabeled 4441 (lane 10) and EP (lane 11) oligonucleotides were incubated with IVT-RFX1. As in Figure 5, 5 µg of BSA was present in the binding reactions shown in lanes 2–11. The unmarked bands in lane 1 were observed due to the longer exposure time required for detection of the IVT-RFX1–PERE complex.

to the site of transcription initiation (INIT). As illustrated in Figure 8, incubation of INIT with HeLa nuclear extracts gave rise to two retarded complexes referred to as YS and YF (YY1 slow and YY1 fast). Oligonucleotide competition experiments with the AAV P5 (–60) oligo abrogated YF formation and reduced YS formation (lane 4). Furthermore, complexes YS and YF disappeared upon addition of the INIT oligonucleotide (lane 5). In addition, neither complex was detected when nuclear extracts were incubated with a probe (mINIT) harboring a CA to GT mutation at positions –1 and +1 (lane 6), nor did the mutated oligonucleotide compete for wild-type INIT associated YS and YF complexes (data not shown). Together these results establish
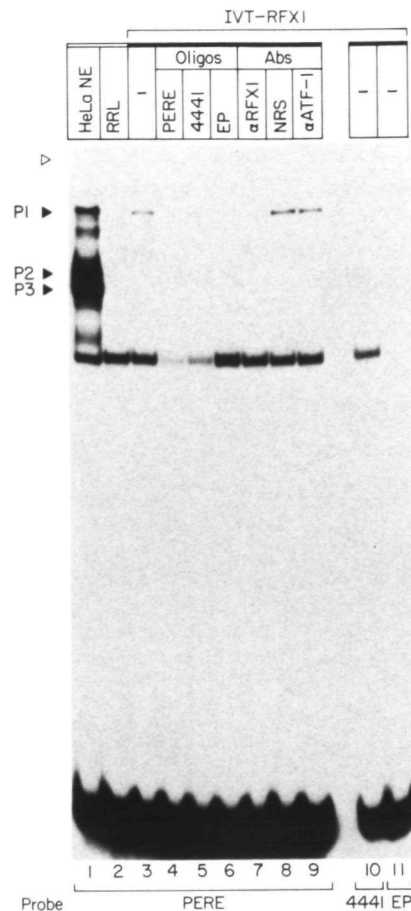
that YS and YF result from specific interactions with the PCNA promoter initiator element.

Antibody interference experiments demonstrated that YY1 is present in both complexes associated with the PCNA promoter initiator. YF was disrupted by coincubation with a commercially available polyclonal antibody (lane 2), as well as by anti-YY1 Ig3-1 (lane 4), a monoclonal antibody that has been shown to disrupt, rather than supershift, YY1-containing complexes (29,37). On the other hand, the YS complex was disrupted by anti-YY1 Ig3-1, but not by the polyclonal antibody. This result suggests that the epitope recognized by the polyclonal antibody, namely amino acids 395–414, is inaccessible in complex YS. To confirm that YY1 binds to the PCNA promoter initiator, we incubated INIT oligonucleotide with extracts prepared from
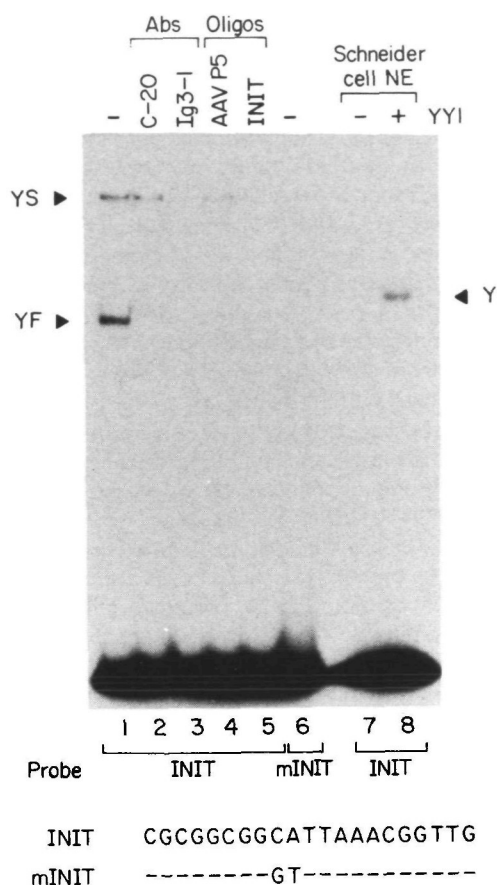
Figure 8. Transcription factor YY1 associates with the initiator element of the human PCNA promoter. Two complexes, identified as YS (YY1 slow) and YF (YY1 fast) on the basis of their relative mobilities, were observed upon incubation of HeLa cell nuclear extract (5 µg) with a radiolabeled double-stranded oligonucleotide (INIT) composed of nucleotides from −9 to +12 relative to the transcription initiation site of the human PCNA promoter (lane 1). Additional binding reactions contained monoclonal antibodies to YY1 (lanes 2 and 3) or excess competitor oligonucleotides corresponding to a known YY1 binding site (AAV P5 oligo) (lane 4) or wild type PCNA promoter initiator element (INIT) (lane 5). As a negative control, the mINIT oligonucleotide was used as probe (lane 6). The sequences of the INIT and mINIT oligonucleotides (sense strand only) are shown at the bottom of the figure. The mINIT oligonucleotide contains a CA to GT mutation at positions −1 and +1. In lanes 7 and 8, the INIT probe was incubated with extracts prepared from Schneider cells (lane 7) or from Schneider cells transfected with a YY1-expressing plasmid (lane 8). An INIT-binding complex observed in YY1-expressing Schneider cells is identified (Y) .
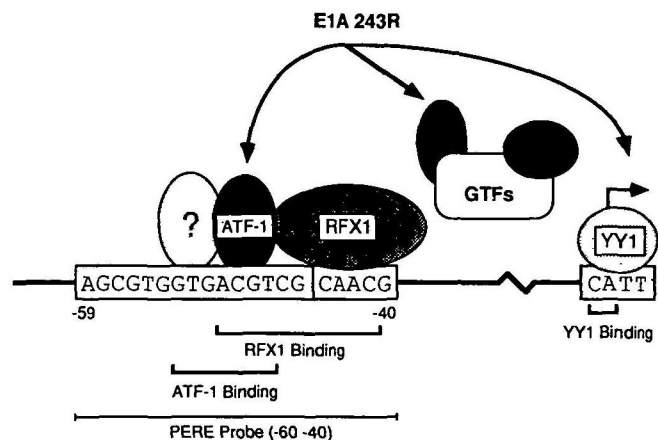
Drosophila Schneider SL2 cells, which lack detectable YY1 (29) (lane 7), as well as with extracts prepared from YY1-expressing Schneider cells (lane 8). INIT-binding activity was observed only with the YY1-expressing Schneider cell extract, thus reproducing the results observed with HeLa nuclear extracts. In contrast to HeLa cells, however, Schneider cell YY1 formed a single complex Y which migrated between HeLa cell YS and YF, implying that an additional factor (or factors) present in HeLa cells but absent from Schneider cell extract can form an additional complex with YY1.

Figure 9. Structure and activation of the human PCNA promoter. Arrows indicate possible sites for regulation of PCNA promoter-associated transcription factors by E1A 243R. The PCNA E1A 243R-responsive element (PERE, boxed) is comprised of nucleotides from −59 to −45 relative to the transcription initiation site. Nucleotides −44 to −40 which bear homology to the hepatitis B virus enhancer RFX1 binding site. The PERE oligonucleotide used in the binding assays contains both elements. ATF-1 and RFX1 bind to overlapping sites contained within the PERE. Co-occupancy of the PERE by both ATF-1 and RFX1 is shown for illustrative purposes only, since these proteins are present in distinct PERE-associated complexes. Mutations between −50 and −41 abolish RFX1 binding (BAM and 4441 mutations), while nucleotides −53 to −47 are critical for ATF-1 binding (BAM mutation and unpublished observations). In addition, the transcription factor YY1 binds to the initiator element of the human PCNA promoter. The consensus YY1 binding site is boxed and the CA dinucleotide required for YY1 binding (mINIT mutation) is bracketed. GTFs: general transcription factors.

DISCUSSION

We have characterized cellular transcription factors that associate with the adenovirus-responsive human PCNA promoter to gain further insight into the molecular mechanism of transcriptional activation by the E1A 243R oncoprotein and its relation to the transformation process (Fig. 9). We previously showed that the PCNA E1A-responsive element (PERE) which is required for induction of PCNA by E1A 243R and confers E1A-responsiveness onto a heterologous promoter *in vivo* is comprised of nucleotides −59 to −45 relative to the transcription initiation site (23,24). The activating transcription factor-1 (ATF-1) was shown to interact with the ATF binding site of the E1A-responsive element of the human PCNA promoter (PERE) and appears to constitute a major component of PERE-associated complexes required for PCNA induction by E1A 243R. The identification of enhancer factor C (EF-C/RFX1) in association with an element overlapping the 3′ end of the PERE contributes a new participant to the regulation of PCNA promoter activity. Moreover, the demonstration that YY1 binds to the initiator element of the PCNA promoter implicates an additional player in the regulation of expression from TATA-less promoters. Finally, the identification of a complex competed by the MLTATA oligo on the PCNA promoter suggests that general transcription factors such as TBP play a role in regulating transcriptional expression of PCNA.

The role of EF-C/RFX1 in the regulation of PCNA promoter activity is unknown at the present time. RFX1 transactivates the HBV enhancer and is required for MHC class II gene expression

(25,38). However, RFX1 binding appears dispensable for PCNA activation by E1A 243R in HeLa cells since a promoter construct containing a mutation in the RFX1 binding site is efficiently activated by E1A 243R (23). The possibility that RFX1 plays an essential role in PCNA expression in HeLa cells cannot be ruled out, however, as mechanisms can be envisaged in which RFX1 would associate with the PCNA promoter without binding to DNA sequences, perhaps via protein–protein interactions. Furthermore, RFX1 may play a more crucial role in PCNA activity in cell types other than HeLa cells. It is of interest to note that RFX1 and ATF-1 are present in different PERE-associated complexes, P1 and P2-P3, respectively. This might imply that they do not co-occupy the PERE and thus may exert opposite effects on PCNA promoter activity. An alternative and equally attractive explanation would be that ATF-1 and RFX1 may serve to relay different intracellular signals to the human PCNA promoter. In contrast to the human PCNA promoter, the PERE-RFX1-binding element (5'-GCAACG-3') is not found in murine promoter sequences near the ATF-CRE consensus site (39), suggesting that this element is dispensable or else confers an additional regulatory mechanism upon the human PCNA promoter. As a further difference, the murine promoter contains putative binding sites for the transcription factors PEA3 and E2F (40,41).

There is no doubt, however, as to the important functional role of the ATF-CRE site present in the human and murine PCNA promoters. The ability of E1A 243R to transactivate PCNA promoter constructs in transient expression assays correlates with the presence of PERE-associated complexes P2 and P3, which contain the ATF-1 protein. Moreover, increased binding of nuclear proteins to a human PCNA promoter probe encompassing the PERE (–68 to –36) is observed upon growth stimulation of Syrian hamster fibroblasts (40). In addition, the ATF-CRE motif present in the murine promoter binds ATF-1 and contributes to the inducibility of the mouse PCNA promoter by interleukin-2 (41). The association of ATF-1 with critical regulatory elements in both the human and murine PCNA promoters indicates that this transcription factor may lie at the convergence of multiple transcriptional regulatory pathways. ATF-1 has been shown to mediate cAMP- and calcium-dependent transcriptional regulation, in some instances as efficiently as CREB (28,42). However, despite the association of ATF-1 with the adenovirus E1A-inducible human PCNA promoter and the Tax-dependent human T-cell leukemia virus 1 (HTLV-1) enhancer (43), GAL4-ATF-1 fusion proteins have been notoriously refractory to activation by viral transactivators such as E1A or Tax (44,45), suggesting that a protein that associates with ATF-1 may in fact constitute a target for E1A. We have shown that ATF-1 is present in two PERE-associated complexes, P2 and P3, therefore allowing for the formation of ATF-1 homodimers and heterodimers. Although other unknown proteins may dimerize with ATF-1, CREB is the only member of the ATF transcription factor family known to form heterodimers with ATF-1 (28,32,42). However, as a GAL4 fusion protein, CREB is only weakly activated by E1A (44) and we have not detected CREB in PERE-associated complexes. It should also be noted that while the PCNA promoter-associated complexes EH4, P2 and P3 clearly contain ATF-1, complexes EH1 and P1, which are at least partly ATF-site dependent, contain protein(s) that do not react with antibody to ATF-1. In fact, the deleterious effect of the BAM mutation on the binding of RFX-1 to the EH87 probe suggests that EH1 may in fact constitute a

multi-protein complex (Fig. 1). The nature and significance of this complex remains to be established.

We have used other immunological reagents to identify candidate PERE-binding proteins. ATF-2 has recently been implicated in the induction of the human c-*jun* gene by 243R (46). Neither ATF-2, the first ATF family member shown to mediate transcription activation by E1A 289R (45,47), nor CREB2, which is also E1A 289R-responsive (44), appeared to associate with the PERE. Moreover, no association was detected between ATF-2 and a –46/–39 PCNA promoter construct in which the ATF site was converted to a perfect palindrome (5'-TGACGTCA-3'), suggesting that discrete PERE nucleotides may dictate specific protein-DNA interactions (unpublished observations). Additional experiments will be necessary to identify which ATF family member(s) interact(s) with ATF-1. To this end, we have embarked on the purification of PERE-binding proteins using DNA affinity chromatography and have detected four proteins in the range of 37–50 kDa that associate with the PERE in UV-crosslinking experiments (unpublished observations). The identities of these proteins remain to be determined.

The existence of two PCNA promoter initiator-associated complexes containing YY1 in HeLa cells suggests that the initiator may also be the site of regulatory events involving protein–protein interactions. YY1 has been shown to interact with the transcription factors Sp1 and c-*myc* (48,49). YY1 appears to influence transcription factor function by regulating promoter structure (37). Moreover, YY1, along with TFIIB and RNA polymerase II, is sufficient to direct basal transcription from a TATA-less promoter *in vitro* (50). Interestingly, E1A 243R has also been shown to relieve transcriptional repression by YY1 (27,29). However, previous experiments using linker-scanning mutants of the human PCNA promoter harboring substitutions or deletions within the initiator element did not reveal a requirement for YY1 (23). As postulated at that time, it is likely that the mutations we studied encoded cryptic initiator elements. This impression was confirmed by the observation that complex EH5 (and EH6) that form on the PCNA-Init-Bgl probe are disrupted by coincubation with anti-YY1 antibody in a DNA-binding assay (B.L., unpublished observations). In any event, how these varied activities of YY1 relate to PCNA promoter activity will require further investigation.

Finally, it is noteworthy that none of the PCNA promoter-binding proteins identified here, namely ATF-1, RFX1 or YY1, is present in the same EH-associated complexes (Figs 1 and 9). While this may stem from an inherent instability of higher-order complexes in the gel systems used, it seems more likely to reflect their lower abundance. This mutually exclusive binding may also reflect different states of transcriptional 'alertness' of the PCNA promoter. The identification of PCNA promoter-binding transcription factors and their respective binding sites will allow the detailed examination of the dynamic interrelationships between these proteins and their respective roles in the transcriptional activation of PCNA activity.

ACKNOWLEDGEMENTS

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