A Complex Promoter Element Mediates Transactivation of the Human Proliferating Cell Nuclear Antigen Promoter by the 243-Residue Adenovirus E1A Oncoprotein

CLAUDE LABRIE, GILBERT F. MORRIS, AND MICHAEL B. MATHEWS*

Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724-2208

Received 13 October 1992/Returned for modification 11 November 1992/Accepted 1 December 1992

The adenovirus E1A oncoproteins interfere with the normal regulation of cellular proliferation through interactions with cell cycle regulatory proteins. In view of the essential role of proliferating-cell nuclear antigen (PCNA) in DNA replication, we performed a mutational analysis of the minimal human PCNA promoter (nucleotides -87 to +62) to define sequence elements which mediate transactivation by the 243-residue E1A protein (E1A 243R). Linker-scanning and site-directed mutants were examined for basal and E1A-induced expression of chloramphenicol acetyltransferase (CAT) from PCNA promoter-CAT reporter constructs transiently expressed in HeLa cells. The results define the *cis*-acting element required for induction of PCNA by E1A 243R as a region between -59 and -45 relative to the transcription initiation site. This PCNA E1A-responsive element (PERE), which is protected from DNase I digestion by nuclear extracts from 293 cells, includes the sequence AGCGTGG immediately upstream of the ATF binding site previously shown to be important for activation of PCNA by E1A 243R (G. F. Morris and M. B. Mathews, *J. Virol.* 65:6397-6406, 1991). Mutation of either the upstream component or the ATF site within the PERE diminishes basal promoter activity and abrogates transactivation by E1A 243R. This novel *cis*-acting element is also essential for both basal and E1A-induced expression in the context of the full-length PCNA promoter.

The processes of cell cycle control and DNA replication are intimately intertwined and strictly regulated. Completion of DNA replication is a prerequisite to cellular division (18). Hence, the DNA replication machinery constitutes a necessary target for regulation by oncogenes. The human proliferating cell nuclear antigen (PCNA) is the auxiliary protein of DNA polymerase- δ (9, 56) and is a critical component of the DNA synthesis machinery. PCNA is required for cell cycle progression and growth of both yeast (6) and mammalian (34, 43) cells. In addition to its function in leading-strand DNA synthesis during replication (55), PCNA has recently been shown to be involved in nucleotide excision repair of mutagen-damaged DNA (66). In agreement with the known association between PCNA and cellular proliferation, PCNA is induced by serum (1) as well as by growth factors (10, 35, 46), and elevated levels of PCNA protein have been observed in human tumors (for a review, see reference 45).

The induction of PCNA observed during the transformation of quiescent rodent cells by adenovirus (85) provides a unique opportunity to study the mechanisms underlying cellular transformation and transactivation by the adenovirus E1A oncoprotein products. The E1A 289- and 243-residue proteins (referred to as E1A 289R and E1A 243R) are encoded by alternatively spliced 13S and 12S transcripts, respectively, and are identical in sequence with the exception of an internal 46-amino-acid region unique to the 289R protein (47). The E1A proteins are multifunctional, possessing both transforming and transactivating properties (7, 50, 64). The E1A gene is sufficient to immortalize primary rodent cells (33), but complete transformation requires cooperation between E1A and the adenovirus E1B gene (64) or oncogenes such as *Ha-ras* (60). The transforming functions of E1A map to domains common to both the 289R and 243R

proteins (80), while the domain unique to the 289R protein endows it with potent transactivating capability (7, 50, 64). In addition to the well-characterized adenovirus early gene promoters, E1A proteins, E1A 289R in particular, have been shown to transactivate a number of cellular promoters (for a review, see reference 64).

Adenovirus E1A proteins do not directly bind DNA (20) but activate transcription through interactions with cellular transcription factors and/or the general transcription machinery. The latter mechanism has been ascribed to the 289R E1A protein, which binds specifically to the general transcription factor TFIID (32, 40). This interaction may account for TATA box-dependent transactivation of the *c-fos* (69) and *hsp70* promoters (67), as well as other promiscuous transcriptional effects of E1A 289R (7, 64, 73, 83). E1A 289R has also been shown to relieve transcriptional repression by YY1 more efficiently than E1A 243R (65). On the other hand, E1A 243R is equally capable of inducing transcription in a promoter-specific manner (28, 29, 74) by releasing the E2F/DRTF1 transcription factor from transcriptionally inactive complexes (3, 5).

We have shown that expression of the human adenovirus E1A gene in HeLa cells activates a cotransfected human PCNA promoter (48) and that both the 289R and 243R oncoprotein products are equally effective in this regard (49). Thus, the ability of the 243R oncoprotein to activate the PCNA gene may represent a link between the processes of transcription and transformation. A putative activating transcription factor (ATF) binding site (5'-TGACGTCG-3') located approximately 50 nucleotides upstream from the transcription initiation site of the PCNA promoter is necessary for transactivation of the PCNA promoter by the 243R E1A protein in HeLa cells (49). The absence of recognizable E2F sites in the PCNA promoter would suggest that novel transactivation functions reside within domains common to both the 243R and 289R proteins. These observations, cou-

* Corresponding author.

FIG. 1. Structure of the wild-type and mutant human PCNA promoter constructs. (Top) Schematic representation of the PCNA-87 CAT plasmid and location of putative regulatory elements in the PCNA promoter. PCNA promoter sequences from -87 to +62 were cloned into the *SacI*-*HindIII* sites upstream of the CAT gene in the pBACAT plasmid (16, 48). Potential transcription factor binding sites, as determined by sequence similarity to the ATF consensus sequence (22), the EivF binding site (14), the YY1 binding site (63), and the TdT initiator motif (70), are indicated by boxes whose orientation is inferred from their positions on the top or bottom strands. The 5' and 3' boundaries are indicated. (Bottom) Promoter sequences from -87 to +10 of the wild-type PCNA-87 promoter are illustrated. The ATF site is underlined and highlighted. The substitution mutants are identified by the 5' and 3' positions of the *BglII* linker (CAGATCTG). Base changes resulting from the linker-scanning mutagenesis are shown, while conserved bases are indicated by hyphens. The x in linker-scanner -82/-75 represents a deleted base. The ATF-Bam and INTR-Bgl mutants created by site-directed mutagenesis are also shown.

pled with studies of other promoters showing that the presence of an ATF site alone is not sufficient to ensure induction by E1A (67, 73, 83), prompted us to perform a detailed examination of the human PCNA promoter. To define the DNA element(s) responsible for its activity, we mutated sequences within the minimal E1A-responsive human PCNA promoter (PCNA-87 CAT) by linker-scanning and site-directed mutagenesis. The results of transient expression assays in HeLa cells demonstrate that the *cis*-acting element required for basal and E1A 243R-induced PCNA expression is a region between -59 and -45 relative to the transcription initiation site. This region is composed of the sequence AGCGTGG together with the adjacent ATF site consensus. The entire element is protected by human nuclear proteins and is also required for basal and E1A 243R-induced activity of the full-length PCNA-1265 promoter. A promoter element that specifies the site of transcriptional initiation in the TATA-less PCNA promoter was also located.

MATERIALS AND METHODS

Construction of PCNA promoter mutants. PCNA-87 CAT (Fig. 1) (48) consists of PCNA promoter and 5' RNA leader sequences from -87 to +62, relative to the transcription initiation site, cloned into the *SacI*-*HindIII* sites immediately upstream of the bacterial chloramphenicol acetyltransferase (CAT) reporter gene in pBACAT (16). Linker-scanning mutagenesis (61) of PCNA promoter sequences was done with the PCNA-87 CAT plasmid as a template. Deletions from the 5' and 3' ends of the PCNA sequences were created by digesting *SacI*- or *HindIII*-linearized PCNA-87 CAT with nuclease BAL 31. Eight-base-pair *BglII* linkers (5'-CAGATCTG-3') were ligated to digested DNA, and matching pairs of 5' and 3' deletion mutants bearing *BglII* linkers

were joined to create exact substitution mutants, with the exception of PCNA-82/-75 CAT, which is 1 bp short of a perfect substitution. The numbers which identify the linker-scanning mutants correspond to the 5' and 3' positions of the *BglII* linker relative to nucleotide +1. ATF site linker-scanning mutants -59/-52, -56/-49, and -46/-39, as well as PCNA-87 ATF-Bam (49), were cloned into the full-length PCNA-1265 CAT plasmid by using *DsaI* sites located at nucleotides -68 in the PCNA promoter (48) and +759 in CAT.

Deletion of the transcription initiation site and adjoining sequences was achieved by joining imperfectly matched linker-scanning mutants. The resulting constructs, PCNAΔ-6/+12 CAT and PCNAΔ-2/+9 CAT, contain a *BglII* linker in place of the deleted sequences. In PCNA-87/+60 INTR-Bgl (49), nucleotides +2 to +6 (nucleotide +1 is underlined), ΔTTAAA, were changed to ΔGATCT by site-directed mutagenesis.

Constructs PCNA-59/-56, -59/-54, -59/-53, -59AC, -56GA, -52TG, -43/-42, -43, and -42 CAT contain single or clustered point mutations. They were created by using an oligonucleotide-directed site-specific mutagenesis kit (Amersham UK).

All the expression and reporter plasmids used in the experiments described in this report were purified by two cesium chloride gradient ultracentrifugations followed by a polyethylene glycol precipitation to remove low-molecular-weight RNA.

Cytomegalovirus expression plasmids. Plasmids expressing the E1B 19-kDa protein (pCMV19K), the 243R polypeptide encoded by the E1A 12S RNA (pCMV12S), a truncated E1A 12S product (pCMV12S.FS), or the β-galactosidase (β-gal) gene (pON260) under the control of the cytomegalovirus promoter (8) have been described previously (49, 72, 78).

Cell culture. Monolayer cultures of HeLa cells (ATCC CCL 2) were grown under 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 100 µg each 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.

Transfections. Transient expression assays were performed in triplicate in passage 6 to 13 HeLa cells. Cells at 40 to 50% confluence were transfected by the calcium phosphate coprecipitation technique as described previously (27, 48). Each 6-cm plate received 20 µg of DNA, comprising salmon sperm carrier DNA, 10 µg of PCNA-87 CAT (or 5 µg of PCNA-1265 CAT), 0.5 µg of either pCMV12S.FS or pCMV12S, and 1 µg of pCMVβGAL as a control for transfection efficiency. Transfection of HeLa cells with the PCNA-87 CAT plasmid produces readily measurable CAT activity, but PCNA-87 CAT plasmids bearing ATF site mutations yield very low levels of CAT activity. For this reason, a plasmid expressing the 19-kDa product of the adenovirus early region 1B gene (pCMV19K, 0.5 µg per plate), which stabilizes transfected DNAs (27, 49), was included in transfections with PCNA-87 CAT-derived plasmids to augment the level of transfected reporter plasmid but was excluded from transfections with the more active PCNA-1265 CAT. The cells were washed twice with phosphate-buffered saline 16 h after transfection, and fresh medium was added to the plates. Cells were harvested 48 h after transfection.

CAT and β-gal assays. Cell extracts were prepared by freezing and thawing the cells in 0.25 M Tris-HCl (pH 8.0), and assays for CAT activity were performed with up to 50 µl of extract to yield values within the linear range of the assay as described previously (21, 48). Thin-layer chromatograms were quantified with a Betascan System (Ambis, San Diego, Calif.), and CAT activity was expressed as the percentage of chloramphenicol acetylated by 50 µl of extract incubated at 37°C for 1 h. The β-gal assay was performed as described previously (26), and β-gal activity was expressed as the optical density at 420 nm obtained with 50 µl of extract incubated at 37°C for 1 h. CAT activity, corrected for β-gal activity, is expressed relative to the level of CAT activity obtained by cotransfection of wild-type PCNA-CAT with pCMV12S.FS. The results, expressed as means ± standard errors of the means, are representative of two or more independent transfections performed in triplicate.

RNA extraction. For RNA analysis, triplicate 6-cm dishes of human 293 cells were transfected by calcium phosphate coprecipitation with 20 µg of DNA, comprising 19 µg of PCNA-CAT reporter plasmid and 1 µg of pCMVβGAL. The cells were harvested 48 h after transfection. Groups of three plates were pooled, and the cell pellets were promptly lysed in guanidinium isothiocyanate or rapidly frozen in liquid nitrogen and stored at -70°C until a later time. RNA was extracted as described previously (2) with the addition of a phenol-chloroform extraction following the cesium chloride gradient ultracentrifugation.

Primer extension. Primer extension analysis was done as described previously (2). A synthetic oligonucleotide (CAT 230, 5'-GCTCCTGAAATCTCGCC-3'), complementary to CAT nucleotides 230 to 215 inclusively and the last two nucleotides in the *Hind*III linker of pBACAT (16), was end labelled with [γ -³²P]ATP (4,500 Ci/mmol). Total cellular RNA (20 µg) purified from transfected 293 cells was annealed to 5 × 10⁴ Cerenkov counts of probe at 30°C for 12 h. Primer extension was done at 42°C for 1.5 h with 40 U of avian myeloblastosis virus reverse transcriptase (Life Sci-

ences, St. Petersburg, Fla.), 4 mM deoxyribonucleotides, and 50 U of RNasin. The reaction mixes were then treated with RNase A for 30 min at 37°C, and the proteins were removed by extraction with 25 phenol-24 chloroform-1 isoamyl alcohol. To identify the sites of transcription initiation from each PCNA-CAT reporter gene construct, the wild-type PCNA-87 CAT plasmid was denatured and subjected to DNA sequencing with the CAT 230 primer and α -³⁵S-dATP. The primer extension products were resolved on a denaturing 6% polyacrylamide gel, fixed, dried, and autoradiographed. After autoradiography, the amounts of the nonspecific 108-nucleotide extension product and PCNA-CAT message in each lane were quantitated with a Fuji BAS 2000 Bio-imaging analyzer to provide a measure of RNA recovery as well as a semiquantitative determination of PCNA-CAT mRNA levels.

DNase I footprinting. Probes for DNase I footprinting were prepared by end labelling the 3' termini of *Xho*I-*Hind*III promoter inserts from PCNA-397 CAT and PCNA-397 ATF-Bam plasmids. Proteins were preincubated on ice for 15 min in a 50-µl reaction mixture containing 50 mM Tris (pH 7.9), 12.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 0.1 M KCl, 0.1% Nonidet P-40, 2% polyvinyl alcohol, and 4.25 µg of salmon sperm DNA. Incubations in the presence of 40,000-cpm probe were at 4°C for 20 min, after which time 50 µl of a solution containing 5 mM MgCl₂ and 5 mM CaCl₂ was added. DNase I digestions were performed at room temperature for 1 min. The reactions were stopped by the addition of 90 µl of stop solution containing 20 mM EDTA (pH 8.0), 1% sodium dodecyl sulfate, 0.2 M NaCl, and 100 µg of yeast tRNA per ml. The reaction mixtures were treated with 10 µg of proteinase K for 15 min at 37°C and extracted with phenol, phenol-chloroform, and chloroform. The DNase I-treated probes were precipitated and analyzed on an 8% polyacrylamide-7 M urea gel.

RESULTS

Mutational analysis of minimal E1A-responsive PCNA promoter. The promoter elements of the PCNA gene which confer E1A inducibility reside within the 87 bp immediately upstream from the site of transcription initiation (48). This E1A-responsive region of the PCNA promoter also contains, in addition to the aforementioned ATF site homology (39, 49), other potential E1A-regulatory elements (Fig. 1), namely, a putative E1F binding site overlapping the ATF site (14) and two inversely oriented binding sites for the YY1 transcription factor (12). To identify the promoter elements that specify basal PCNA expression and those which, in addition to the ATF site, may be involved in the E1A response, we subjected the minimal E1A-responsive PCNA promoter (hereafter referred to as PCNA-87 CAT) to linker-scanning mutagenesis between nucleotides -83 and -7 (Fig. 1). Each of the resulting linker-scanning mutants changed an average of 6 of 8 bps.

Mutation of bases between -27 and -20 produces transcription initiation site heterogeneity. We performed primer extension analysis to determine whether mutations in the promoter sequences of PCNA-87 CAT alter transcription initiation site selection and to ascertain that the CAT activity measured in our assays accurately reflected PCNA promoter-driven transcription. RNA was isolated from transfected adenovirus-transformed human 293 cells rather than HeLa cells because the former are more amenable to transfection and, since they express endogenous E1A and E1B genes,

FIG. 2. Primer extension analysis of mRNA expressed by mutant PCNA promoter constructs in 293 cells. (A) Schematic representation of the PCNA-87 CAT plasmid illustrating the position of the synthetic oligonucleotide, CAT 230, complementary to CAT mRNA nucleotides 230 to 215. The stippled region of the PCNA-CAT plasmid between PCNA nucleotide +60 and CAT nucleotide 215 represents *Hind*III linker sequences. The direction of extension by avian myeloblastosis virus reverse transcriptase is indicated by an arrow. (B) Primer extension analysis. Lanes G, A, T, and C refer to dideoxynucleotide sequencing reactions on PCNA-87 CAT plasmid with CAT 230 primer. The ATF and initiation sites are marked. Lane 5, untransfected 293 cells; lanes 6 through 20, 293 cells transfected with PCNA-87 CAT or each of the indicated substitution mutants, in the usual order. Samples 8 (–76/–69), 14 (–45/–38), and 20 (–14/–7) are underrepresented, while lane 19 (–21/–14) is overrepresented. Autoradiography was performed at –70°C for 3 days with an intensifying screen. WT, wild type.

allow higher levels of expression from transfected PCNA-CAT reporter plasmids. Furthermore, the sites of transcription initiation from the full-length PCNA-CAT are identical in both cell types (48).

A correctly initiated PCNA-CAT transcript should yield a primer extension product 85 nucleotides in length (48, 75) (Fig. 2A). Adventitiously, RNA from untransfected 293 cells yielded an abundant 108-nucleotide extension product (Fig. 2B, lane 5) which is also observed in HeLa cells (data not shown) and provides a useful internal control for RNA recovery and loading. PCNA-87 CAT mRNA transcription initiates at four sites within the ATTAAG element of the PCNA promoter in 293 cells, predominantly from nucleotides A at +1 and T at +2 (Fig. 2B, lane 6). Transcription also initiates at the adenine bases located at +4 and +5, but these more downstream cap sites account for less than 15% of all transcription. Multiple start sites were not observed in previous determinations of the transcription initiation site of PCNA which identified a single cap site at +1 (48, 75). This discrepancy may result from a greater resolution achieved with the present assay, which employed a primer in close proximity to the transcription initiation site, but we cannot exclude the possibility that the multiple start sites are the result of false termination in the primer extension reactions.

With one exception, all the substitution mutants produced transcripts initiating at the same nucleotides as observed with PCNA-87 CAT, and the predominance of initiation from A⁺/T⁺ over A⁺/A⁺ was also conserved. Exceptionally, in the case of the –27/–20 construct (Fig. 2B, lane 18), the frequency of initiation at the more downstream sites equalled that at the major A⁺/T⁺ sites. In addition, initiation was detected at the A⁺ position in the ATTAAG hexanucleotide. Thus, mutation of the sequence 5'–²⁷AGAGCGCG^{–20}–3' to CAGATCTG (Fig. 1) appears to alter the ratio of initiation site in favor of more downstream bases. Since constructs –31/–24 (Fig. 2B, lane 17) and –21/–14 (lane 19) show a wild-type pattern of transcription initiation site selection, a discrete region involving bases between –24 and –21 could be involved in specifying the site of transcriptional initiation.

Site of transcription initiation is dictated by an upstream promoter element. In the absence of an identifiable TATA box in the PCNA promoter, we examined the sequences adjoining the cap site for initiator function. The initiator element has been defined as a DNA sequence located between –3 and +5 which directs transcription initiation (71). In the context of the adeno-associated virus P5 promoter, the initiator-binding protein YY1 is a target for transactivation by E1A (63). The four bases between –1 and +3 in the PCNA promoter (Fig. 3A) are identical to the corresponding nucleotides found within the initiator motifs of the terminal deoxynucleotidyltransferase (TdT) gene (70) and the adeno-associated virus P5 +1 element (63). We previously observed that mutation of nucleotides +1 to +6 (ATTAAG) in the PCNA promoter to AGATCT by the introduction of a *Bgl*II site (INTR-*Bgl* construct) did not adversely affect basal or E1A-induced PCNA-CAT expression as determined by RNase protection analysis (49).

To investigate further the role of a putative initiator element in the PCNA promoter, we examined the effect of more extensive base deletions (Δ –2/+9 and Δ –6/+12) on CAT activity and transcription initiation site selection in HeLa and 293 cells, respectively (Fig. 3B). Despite extensive mutations around the cap sites, transcription from all three constructs was efficient as evidenced by the levels of CAT activity, which approximated those observed with PCNA-87 CAT in the presence or absence of E1A 243R (Fig. 3B). These results indicate that in the presence of intact promoter elements upstream of –7, mutation of the initiation site does not influence PCNA-CAT expression.

Introduction of a *Bgl*II site at nucleotides +1 to +6 (INTR-*Bgl*), however, caused transcription to initiate at the G one nucleotide downstream from the normal start site despite the existence of an A at position +1 (Fig. 3B and C). More interestingly, larger deletions around the cap site caused a progressive downstream shift in the sites of transcription initiation which corresponded to the extent of the deletion. In fact, replacement of an 11-bp deletion from –2 to +9 with a 7-bp linker (Δ –2/+9) caused initiation to move 4 bases downstream while a partially compensatory 6-bp downstream shift occurred with mutant Δ –6/+12 in which 18 bases were replaced with an 8-bp linker. Taken with the start site heterogeneity observed with mutant –27/–20 (Fig. 2B), these results suggest that the site of transcription initiation in the PCNA gene is dictated by sequences between –29 and –25. This sequence, 5'-TGAGA-3', could conceivably bind the TATA box-binding component of TFIID (TBP). In fact, TFIID is required for transcription from both TATA-less and TATA-containing promoters (11, 57, 71), and recent observations have shown that TBP binds

FIG. 3. Mutational analysis of the transcription initiation site. (A) Comparison of the transcription initiation sequences from the PCNA (48, 75), TdT (63), adeno-associated virus P5 (AAVP5) (12), and leukocyte interferon gene cluster (LeIF-J) promoters (77). The sequences are aligned at the cap site (+1). Underlined letters indicate sequences conserved with the PCNA initiator. (B) Activity and sequence of PCNA initiation site mutants. The mutant sequences were aligned with the wild-type sequence by anchoring the sequences at +13. The numbers above each sequence identify bases retained from PCNA-87 CAT. Underlined bases indicate the sites of transcription initiation in 293 cells for each construct. Sequence similarities with the LeIF-J initiation site are shaded. CAT activity measurements were made in HeLa cells. (C) Primer extension in 293 cells. Lanes 5, 6, and 7, primer extension products from 293 cells transfected with Δ-6/+12, Δ-2/+9, and INTR-Bgl plasmids, respectively. Lanes G, A, T, and C refer to dideoxynucleotide sequencing reactions on PCNA-87 CAT plasmid with the same oligonucleotide primer (CAT 230) used in the primer extension reactions. Autoradiography was performed at -70°C for 3 days with an intensifying screen.

to sequences approximately 30 nucleotides upstream from the transcription initiation site of TATA-less promoters (82).

ATF site is required for both basal and E1A-induced expression of PCNA-87 CAT. The effects of PCNA promoter mutations on basal PCNA promoter activity were assessed by cotransfection of each mutant construct with a plasmid expressing a truncated E1A protein devoid of transcriptional activity (pCMV12S.FS). Compared with basal PCNA-87 CAT activity, cotransfection of the wild-type

PCNA-87 promoter with a plasmid expressing the 243R oncoprotein of adenovirus E1A (pCMV12S) caused a 17-fold increase in CAT activity (Fig. 4). On the other hand, linker scanners -59/-52 and -56/-49, which contained mutations within the putative ATF site (5'-TGACGTCG-3') as well as sequences upstream of the ATF, caused 90 and 100% reductions in basal CAT activity, respectively, and completely abolished transactivation by pCMV12S, indicating that an intact ATF site is required for both basal and E1A

FIG. 4. Effect of linker substitutions within the PCNA-87 promoter on basal CAT activity and transactivation by E1A 243R in HeLa cells. Each lane is designated with the PCNA-CAT construct assayed (Fig. 1). PCNA-CAT reporter plasmids were cotransfected with pCMV12S.FS (Control) or pCMV12S (E1A 243R). In this and subsequent figures, CAT activity was corrected for β -gal activity generated from a cotransfected reporter plasmid and expressed as the means $\pm$ SEM relative to the level of CAT activity obtained by cotransfection of wild-type PCNA-CAT with pCMV12S.FS.

243R-induced expression of PCNA-87 CAT. Indeed, the -56/-49 construct is analogous to the previously described ATF-Bam mutation (49) (Fig. 1) in that it mutates bases at the core of the ATF consensus sequence and would be expected to be extremely deleterious. In fact, both the ATF-Bam mutant and -56/-49 greatly diminish basal CAT activity and abrogate the response to pCMV12S. The -59/-52 construct, on the other hand, contains a single T-to-G change at position -52 within the ATF site consensus and an additional 6 of 7 bp change between nucleotides -59 and -53, suggesting that the T is critical for ATF function and/or that the E1A-responsive element in the PCNA promoter extends upstream from the ATF site toward -59. These possibilities are addressed below.

Mutation of sequences in the PCNA promoter between -45 and -36, and between -27 and -14, also failed to achieve the levels of CAT activity observed with the wild-type promoter in response to E1A 243R, suggesting that these mutations compromise some as yet undefined aspect of PCNA promoter function. Examination of ATF site sequences from several mammalian and viral promoters (41, 59) revealed that the promoter of the E1-responsive human DNA polymerase- β gene (81) contains, as does PCNA, a CAAC tetranucleotide immediately downstream (nucleotides -44 to -41 in the PCNA promoter) of an ATF consensus sequence. Mutation of the A nucleotides to C at positions -43 and -42, singly or in combination, had little effect on basal or E1A 243R-induced levels of CAT activity (data not shown), indicating that the sequences immediately downstream of the ATF site are not critical. Likewise, mutation of either of the putative YY1 binding sites in the PCNA promoter did not enhance basal PCNA expression as might have been expected if they were involved.

Effect of ATF site mutations in full-length PCNA promoter. The sequences upstream of -87 in the PCNA promoter contain several potential binding sites for potent transactivators such as C/EBP and Sp1 which may account for the 10-fold-greater basal activity of PCNA-1265 CAT compared with PCNA-87 CAT (48). Moreover, octamer elements found within the full-length promoter may also mediate transactivation by E1A (13). To determine the functional significance of ATF site mutations in the context of the full-length promoter, we cloned three deleterious ATF site mutants, namely, -59/-52, -56/-49, and ATF-Bam, and a mutant displaying wild-type activity, -46/-39, into the PCNA-1265 CAT plasmid. These constructs were assayed for basal CAT activity and E1A responsiveness in HeLa cells.

In agreement with previous observations (48), cotransfection of PCNA-1265 CAT with pCMV12S produced a two-fold increase in CAT activity over the level observed with pCMV12S.FS (Fig. 5A). In keeping with the results observed with the -87 promoter (Fig. 4), the -46/-39 linker-scanning mutant evinced an essentially wild-type response to pCMV12S in the context of the full-length promoter. Furthermore, mutations of the ATF site which proved deleterious in the -87 promoter also compromised -1265 function, causing 80 to 90% decreases in basal promoter activity and abolishing E1A 243R responsiveness. Likewise, a PCNA-397 CAT promoter construct (48) harboring the ATF-Bam mutation produced residual PCNA-CAT activity and was insensitive to transactivation by E1A 243R (Fig. 5B). RNA analysis of the ATF-Bam mutation in the -397 (Fig. 5B) and -87 (49) PCNA promoters revealed results similar to the CAT analysis. These results indicate that the putative ATF site performs an essential function in the

FIG. 5. Effect of ATF site mutations on full-length PCNA promoter function in HeLa cells. (A) Transactivation of the PCNA-1265 CAT promoter by the E1A 243R protein. (Top) Each lane is designated with the PCNA-CAT construct assayed. PCNA-CAT reporter plasmids were cotransfected with pCMV12S.FS (dark bars) or pCMV12S (hatched bars). (Bottom) Sequences of PCNA-1265 CAT ATF site mutants. The putative ATF site is underlined. (B) Wild-type PCNA-397 CAT plasmid or the PCNA-397 CAT construct harboring the ATF-Bam mutation (ATF-Bam) was transfected into HeLa cells in the presence (+) or absence (-) of E1A 243R-expressing plasmid. RNase protection analysis was performed as described previously (49).

PCNA promoter for which upstream DNA elements cannot substitute.

ATF site is necessary but not sufficient for induction of PCNA by E1A 243R. To determine the upstream boundary of the E1A 243R-responsive element of the PCNA promoter and to establish whether the putative ATF site is sufficient for basal and E1A 243R-induced PCNA expression or, alternatively, that sequences upstream of -52 are also required, we created mutants which reproduce single or clustered mutations found in the -59/-52 construct. These mutants, together with -59/-52, -56/-49, ATF-Bam, and -46/-39, were assayed by cotransfection in HeLa cells (Fig. 6).

In agreement with the data presented in Fig. 4, mutant -46/-39 exhibited wild-type levels of basal CAT activity and attained levels of CAT activity comparable to those observed with PCNA-87 CAT in response to pCMV12S. The results presented in Fig. 6 demonstrate that sequences located between nucleotides -59 and -53, upstream of the putative ATF site, are required for full transactivation of the PCNA promoter by the E1A 243R protein. All the mutations upstream of and including the ATF site diminished basal PCNA promoter activity from 62% of wild type observed

FIG. 6. Detailed analysis of PERE mutations in the PCNA-87 promoter. (Top) Transactivation by the E1A 243R protein. Each lane is designated with the PCNA-CAT construct assayed. HeLa cells were transfected with PCNA-CAT reporter plasmids and pCMV12S.FS (Control) or pCMV12S (E1A 243R). The increases in CAT activity observed with the wild-type PCNA-87 promoter and mutants -59AC, -59/-56, -56GA, -59/-54, and -46/-39 in the presence of E1A 243R were statistically significant ($P < 0.01$). On the other hand, the slight differences in the levels of CAT activity observed when E1A 243R was cotransfected with mutants -59/-53, -59/-52, -52TG, -56/-49, and ATF-Bam were not statistically significant. (Bottom) Sequences of PCNA-87 CAT ATF site mutants.

with -59AC to 10% of wild type with ATF-Bam. These mutants also failed to achieve wild-type levels of CAT activity in response to E1A 243R. Cotransfection with pCMV12S effectively divided the mutants into two groups: those that were essentially nonresponsive to E1A 243R and those that evinced a partial response to E1A 243R. The former group contains mutations between nucleotides -53 and -47, while the latter contains mutations between nucleotides -59 and -54. In fact, mutant -59AC achieved approximately 50% of wild-type levels of CAT activity when cotransfected with pCMV12S. A single G-to-A mutation at position -56 (-56GA) reduced the level of PCNA induction by E1A 243R to 28% of the wild-type response. Similar effects were observed with mutants -59/-56 and -59/-54, which harbor additional base changes between -59 and -54. On the other hand, mutation of bases -53 and -52, either in the context of mutants -59/-53 or -59/-52 or singly, as in mutant -52TG, severely impaired induction of PCNA-CAT activity by pCMV12S. These mutants, like -56/-49 and ATF-Bam which mutate the core of the ATF consensus site, show little or no response to E1A 243R in repeated experiments.

Although a low level of PCNA-CAT activity derived from correctly initiated PCNA-CAT mRNA (49) persists after mutation of the ATF site (Fig. 5 and 6), sequences between -53 and -45 are required for optimal basal PCNA promoter activity and for activation by E1A 243R. Since sequences

FIG. 7. DNase I footprints on PCNA promoter. The wild-type (lanes 1 to 3) or ATF-Bam (lanes 4 to 6) PCNA-397 CAT promoter constructs were digested with DNase I in the presence of nuclear proteins from 293 cells. Lanes 1 and 4, A+G sequencing ladder; lanes 2 and 5, no protein added (-); lanes 3 and 6, 35 μ g of crude nuclear extract (+) from human 293 cells. The positions of the Sp1, ATF, and initiation sites are marked.

between -59 and -54 are also necessary, we conclude that the PCNA E1A-responsive element, PERE, includes the sequence AGCGTGG together with the adjoining putative ATF binding site.

Binding of nuclear proteins to PERE. To establish a biochemical link between its critical function in transactivation of the PCNA promoter by E1A 243R in vivo and the underlying mechanism of transactivation, we examined the PERE for binding to nuclear proteins. The wild-type PCNA-CAT promoter composed of nucleotides -397 to +62 was incubated with crude nuclear extract prepared from human 293 cells and subjected to DNase I digestion (Fig. 7, lane 3). Consistent with methylation interference experiments showing that nuclear proteins from hamster cells contact multiple bases in the PCNA promoter between -62 and -41 (53), we observed protection of a region of the PCNA promoter corresponding to the PERE as determined by mutational analysis. This suggests that one or more proteins involved in PCNA expression interact with the PERE. Protection was also observed over the Sp1 site located between -129 and -120. Mutation of the ATF site abolished protection over

	-60	-50	-40
Rat PCNA	gTCAGCGCTGTG	AcGcC	acaaC
Mouse PCNA	aTCAGCGCTGTG	CGTC	atgaC
Human PCNA	gaCAGCGTgGTG	AcGTC	gcaacg
Consensus	gtcAGCGctGTG	AcGTC	-caacc
RERE	CCGTGACGNT GG		
ATF Consensus	TGACGTCA		
AP-1 Consensus	TGAC-TCA		

FIG. 8. Alignment of PCNA promoter sequences. Promoter sequences from the human (48, 75), mouse (84), and rat (51) PCNA genes were aligned by using the GENALIGN multiple sequence alignment program (Intelligenetics, release 5.4). In the consensus sequence, nucleotides conserved in all three species are indicated by capital letters, while lowercase letters indicate dominant bases. Nucleotides corresponding to the PERE are underlined. The RERE (37), ATF, and AP-1 consensus sequences are shown below the derived PCNA promoter consensus sequence.

the entirety of the PERE but did not affect the Sp1 footprint (Fig. 7, lane 6). We conclude that PERE function, whether measured as the induction of PCNA-CAT expression by E1A 243R or as protein-binding capability, requires an intact ATF site.

DISCUSSION

The adenovirus E1A oncoproteins override normal regulation of cellular proliferation through interactions with cell cycle regulatory proteins (19, 25, 54, 76, 79). In view of its essential role in DNA replication, activation of PCNA by E1A may constitute an integral part of the transformation process. Indeed, induction of DNA synthesis in quiescent cells requires PCNA (34). Consequently, an understanding of the mechanism used by the 243R E1A oncogene to activate expression of PCNA will provide valuable information on the molecular mechanisms underlying cellular transformation. We previously showed that a putative ATF site is required for the transactivation of PCNA by the 243R E1A protein (49). The present data identify sequences flanking the putative ATF site which are required for optimal basal and E1A 243R-induced PCNA expression, indicating that the ATF site in itself is not sufficient to ensure basal expression and mediate transactivation by the 243R E1A protein. These findings define a novel E1A-responsive element in the PCNA promoter, PERE, composed of the sequence AGCGTGG and the contiguous putative ATF site (TGACGTGCG).

The high degree of conservation of PERE sequences in the human (48, 75), rat (51), and mouse (84) PCNA promoters (Fig. 8) supports the importance of this element to PCNA promoter function. Furthermore, the observation that promoter elements upstream of the PERE in the full-length -1265 promoter cannot substitute or compensate for PERE mutations indicates that this element is critical for both basal and E1A 243R-induced PCNA expression. Accordingly, the ATF site, and by extension the PERE, may function both to direct the assembly of a preinitiation complex and to provide a target for transactivation by E1A 243R. In this regard, studies with the E4 promoter have shown that ATF interacts with the general transcription factor TFIID to assemble a functional preinitiation complex in TATA element-containing promoters (23, 31).

The binding of nuclear proteins to the PERE in an ATF site-dependent manner suggests two possible modes of pro-

tein-DNA interaction at the PERE. Both scenarios can accommodate the recruitment of multiprotein complexes to the PERE. In the first case, the PERE would constitute a single site, binding a factor that remains to be identified. Alternatively, the upstream heptanucleotide (AGCGTGG) and the putative ATF site could function as two distinct but cooperating sites binding an unknown factor together with a member of the ATF family. In view of the footprinting data and the quantitatively distinct effects of PERE mutations on either side of nucleotide -54, binding to the ATF component of PERE would constitute a critical stabilizing interaction at the PCNA promoter.

The particular context of the PCNA promoter ATF site, located as it is within a larger E1A-responsive element, is a distinguishing feature of the PCNA promoter. Transactivation of other E1A 243R-responsive promoters such as the human hsp70 (38, 68, 73, 83) and adenovirus E2 (28, 44, 52) promoters does not require ATF sites present in these promoters, suggesting that PERE may recruit to the ATF site a specific member of the ATF superfamily, or a factor with similar DNA binding properties. The ATF superfamily comprises the members of the ATF/CREB and fos/jun families (24) which classically bind to ATF (TGACGTCA) or AP-1 (TGACTCA) recognition sites, respectively. The ability of these related leucine zipper proteins to form selective intra- and interfamily heterodimers generates a variety of ATF proteins with altered DNA binding specificities (22, 24). Moreover, cross-talk between the ATF/CREB and AP-1 signal transduction pathways at the transcriptional level provides an additional dimension of complexity (17, 30, 62). More recently, the CAAT box/enhancer binding protein has been shown to bind to ATF sites and stimulate transcription (4). The resulting multiplicity of interactions allows for intricate transcriptional regulatory mechanisms at an ATF site. To date, however, it has not been possible to identify a member of the ATF superfamily which mediates transactivation by the 243R E1A protein (42).

The growth- and E1A 289R-inducible *c-jun* promoter (15) contains a structural homolog of the PERE. In a situation analogous to the ATF site contained within the PERE, the most proximal of five direct repeats of a *cis*-acting element present in the retinoic acid and E1A response element (RERE) of the *c-jun* promoter contains an AP-1 binding site (37). Moreover, 9 of 11 PERE nucleotides between -56 and -46 are found in this RERE repeat (Fig. 8). As with the PCNA promoter ATF site (49), mutation of the *c-jun* AP-1 site severely impaired transactivation by E1A. Although the role of the sequences upstream of the AP-1 site and the effect of E1A 243R have not been specifically addressed (37), the remarkable similarity between the E1A-responsive elements of the PCNA and *c-jun* promoters suggests that the two genes may interact with similar transcriptional regulators.

In contrast to the promiscuous and potent transactivating properties displayed by E1A 289R (64), a distinguishing feature of the E1A 243R protein appears to be promoter-specific transactivation. Induction of human hsp70 by E1A 243R (68), for example, requires the TATAA sequence (38), while E2F-dependent transactivation by E1A 243R has been clearly established (3, 28). The unique sequence characteristics of the PERE, the equipotency of the E1A 243R and E1A 289R proteins as transactivators of PCNA (49), and the dependence of both basal and E1A 243R-induced PCNA expression on PERE imply that transactivation of PCNA by E1A may utilize an as yet undefined transactivating function of the 243R E1A protein.

Insight into the mechanism underlying transactivation of

the PCNA promoter by E1A 243R may be drawn from mutational studies of the protein itself. The 243R E1A protein contains several distinct functional domains (47) that are involved in promoter-specific transactivation. Conserved regions 1 and 2 of E1A 243R are required for E2F-dependent transactivation (58), while the N-terminal domain of E1A 243R is required for TATAA-dependent transactivation of the human hsp70 promoter (38). Activation of PCNA, on the other hand, is dependent on regions of E1A 243R that collectively bind to p300, p107, and cyclin A (36), suggesting that the activation of PCNA expression by E1A 243R involves multiple known E1A-binding proteins. Since these regions include the N-terminal domain of the protein, it is not excluded that transactivation of PCNA by E1A 243R may proceed, at least in part, through a mechanism similar to that observed with the hsp70 promoter (38). Alternatively, transactivation of PCNA by E1A 243R may employ an undefined cellular protein-binding function of E1A 243R.

While the mechanism of activation of PCNA expression by E1A 243R remains unknown, the requirement for PERE for maximal expression from the PCNA promoter indicates that this element constitutes a binding site for one or more cellular transcription factors which specifically target E1A 243R to the PCNA promoter. The identification of the E1A 243R-responsive element of the human PCNA promoter will facilitate the characterization of the protein(s) involved in the transactivation of PCNA by E1A 243R and contribute to our understanding of the mechanisms by which E1A activates DNA replication.

ACKNOWLEDGMENTS

We thank M. Gilman and W. Herr for helpful comments. We are indebted to summer students M.-D. Galibert and B. Lee for their valued assistance, to R. Packer for cell culture, and to A. Stenlund for plasmid pON260.

This work was supported by grant CA 13106 from the National Cancer Institute and by a postdoctoral fellowship to C.L. from the Canadian Medical Research Council.

REFERENCES

1. Almendral, J. M., D. Huebsch, P. A. Blundell, H. Macdonald-Bravo, and R. Bravo. 1987. Cloning and sequence of the human nuclear protein cyclin: homology with DNA-binding proteins. *Proc. Natl. Acad. Sci. USA* **84**:1575-1579.
2. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1991. *Current protocols in molecular biology*. John Wiley & Sons, Inc., New York.
3. Bagchi, S., P. Raychaudhuri, and J. R. Nevins. 1990. Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A trans-activation. *Cell* **62**:659-669.
4. Bakker, O., and M. G. Parker. 1991. CAAT/enhancer binding protein is able to bind to ATF/CRE elements. *Nucleic Acids Res.* **19**:1213-1217.
5. Bandara, L. R., and N. B. La Thangue. 1991. Adenovirus E1A prevents the retinoblastoma gene product from complexing with a cellular transcription factor. *Nature (London)* **351**:494-497.
6. Bauer, G. A., and P. M. J. Burgers. 1990. Molecular cloning, structure, and expression of the yeast proliferating cell nuclear antigen gene. *Nucleic Acids Res.* **18**:261-265.
7. Berk, A. J. 1986. Adenovirus promoters and E1A transactivation. *Annu. Rev. Genet.* **20**:45-79.
8. Boshart, M., F. Weber, G. Jahn, K. Dorsch-Häsler, B. Fleckenstein, and W. Schaffner. 1985. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* **41**:521-530.
9. Bravo, R., R. Frank, P. A. Blundell, and H. Macdonald-Bravo. 1987. Cyclin/PCNA is the auxiliary protein of DNA polymerase- δ . *Nature (London)* **326**:515-517.
10. Bravo, R., and H. Macdonald-Bravo. 1984. Induction of the nuclear protein 'cyclin' in quiescent mouse 3T3 cells stimulated by serum and growth factors. Correlation with DNA synthesis. *EMBO J.* **3**:3177-3181.
11. Carcamo, J., L. Buckbinder, and D. Reinberg. 1991. The initiator directs the assembly of a transcription factor IID-dependent transcription complex. *Proc. Natl. Acad. Sci. USA* **88**:8052-8056.
12. Chang, L.-S., Y. Shi, and T. Shenk. 1989. Adeno-associated virus P5 promoter contains an adenovirus E1A-inducible element and a binding site for the major late transcription factor. *J. Virol.* **63**:3479-3488.
13. Chellappan, S. P., and J. R. Nevins. 1990. DNA octamer element can confer E1A trans-activation, and adenovirus infection results in a stimulation of the DNA-binding activity of OTF-1/NFIII factor. *Proc. Natl. Acad. Sci. USA* **87**:5878-5882.
14. Cortes, P., L. Buckbinder, M. A. Leza, N. Rak, P. Hearing, A. Merino, and D. Reinberg. 1988. EivF, a factor required for transcription of the adenovirus EIV promoter, binds to an element involved in E1A-dependent activation and cAMP induction. *Genes Dev.* **2**:975-990.
15. De Groot, R., N. Foulkes, M. Mulder, W. Kruijer, and P. Sassone-Corsi. 1991. Positive regulation of *jun/AP-1* by E1A. *Mol. Cell. Biol.* **11**:192-201.
16. Déry, C. V., C. H. Herrmann, and M. B. Mathews. 1987. Response of individual adenovirus promoters to the products of the E1A gene. *Oncogene* **2**:15-23.
17. Engel, D. A., U. Muller, R. W. Gedrich, J. S. Eubanks, and T. Shenk. 1991. Induction of *c-fos* mRNA and AP-1 DNA-binding activity by cAMP in cooperation with either the adenovirus 243- or the adenovirus 289-amino acid E1A protein. *Proc. Natl. Acad. Sci. USA* **88**:3957-3961.
18. Enoch, T., and P. Nurse. 1991. Coupling M phase and S phase: controls maintaining the dependence of mitosis on chromosome replication. *Cell* **65**:921-923.
19. Ewen, M. E., Y. Xing, J. B. Lawrence, and D. M. Livingston. 1991. Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. *Cell* **66**:1155-1164.
20. Ferguson, B., B. Krippel, O. Andrisani, N. Jones, H. Westphal, and M. Rosenberg. 1985. E1A 13S and 12S mRNA products made in *Escherichia coli* both function as nucleus-localized transcription activators but do not directly bind DNA. *Mol. Cell. Biol.* **5**:2653-2661.
21. Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2**:1044-1051.
22. Hai, T., and T. Curran. 1991. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc. Natl. Acad. Sci. USA* **88**:3720-3724.
23. Hai, T., M. Horikoshi, R. G. Roeder, and M. R. Green. 1988. Analysis of the role of the transcription factor ATF in the assembly of a functional preinitiation complex. *Cell* **54**:1043-1051.
24. Hai, T., F. Liu, W. J. Coukos, and M. R. Green. 1989. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Genes Dev.* **3**:2083-2090.
25. Harlow, E., P. Whyte, B. R. Franza, Jr., and C. Schley. 1986. Association of adenovirus early-region 1A proteins with cellular polypeptides. *Mol. Cell. Biol.* **6**:1579-1589.
26. Herbolme, P., B. Bourachot, and M. Yaniv. 1984. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. *Cell* **39**:653-662.
27. Herrmann, C. H., and M. B. Mathews. 1989. The adenovirus E1B 19-kilodalton protein stimulates gene expression by increasing DNA levels. *Mol. Cell. Biol.* **9**:5412-5423.
28. Hiebert, S. W., S. P. Chellappan, J. M. Horowitz, and J. R. Nevins. 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. *Genes Dev.* **6**:177-185.
29. Hiebert, S. W., M. Lipp, and J. R. Nevins. 1989. E1A-dependent trans-activation of the human *MYC* promoter is mediated by the

- E2F factor. *Proc. Natl. Acad. Sci. USA* **86**:3594-3598.
30. Hoeffler, J. P., P. J. Deutsch, J. Lin, and J. F. Habener. 1989. Distinct adenosine 3',5'-monophosphate and phorbol ester responsive signal transduction pathways converge at the level of transcriptional activation by the interactions of DNA-binding proteins. *Mol. Endocrinol.* **3**:868-880.
 31. Horikoshi, M., T. Hai, Y.-S. Lin, M. R. Green, and R. G. Roeder. 1988. Transcription factor ATF interacts with the TATA factor to facilitate establishment of a preinitiation complex. *Cell* **54**:1033-1042.
 32. Horikoshi, N., K. Maguire, A. Kralli, E. Maldonado, D. Reinberg, and R. Weinmann. 1991. Direct interaction between adenovirus E1A protein and the TATA box binding transcription factor IID. *Proc. Natl. Acad. Sci. USA* **88**:5124-5128.
 33. Houweling, A., P. J. van den Elsen, and A. J. van der Eb. 1980. Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. *Virology* **105**:537-550.
 34. Jaskulski, D., J. K. deRiel, W. E. Mercer, B. Calabretta, and R. Baserga. 1988. Inhibition of cellular proliferation by antisense oligodeoxynucleotides to PCNA cyclin. *Science* **240**:1544-1546.
 35. Jaskulski, D., C. Gatti, S. Travali, B. Calabretta, and R. Baserga. 1988. Regulation of the proliferating cell nuclear antigen cyclin and thymidine kinase mRNA levels by growth factors. *J. Biol. Chem.* **263**:10175-10179.
 36. Kannabiran, C., G. F. Morris, C. Labrie, and M. B. Mathews. 1993. The adenovirus E1A 12S product displays functional redundancy in activating the human proliferating cell nuclear antigen promoter. *J. Virol.* **67**:507-515.
 37. Kitabayashi, I., Z. Kawakami, R. Chiu, K. Ozawa, T. Matsuoka, S. Toyoshima, K. Umesono, R. M. Evans, G. Gachelin, and K. Yokoyama. 1992. Transcriptional regulation of the *c-jun* gene by retinoic acid and E1A during differentiation of F9 cells. *EMBO J.* **11**:167-175.
 38. Kraus, V. B., E. Moran, and J. R. Nevins. 1992. Promoter-specific transactivation by the adenovirus E1A12S product involves separate E1A domains. *Mol. Cell. Biol.* **12**:4391-4399.
 39. Lee, K. A. W., T.-Y. Hai, L. SivaRaman, B. Thimmappaya, H. C. Hurst, N. C. Jones, and M. R. Green. 1987. A cellular protein, activating transcription factor, activates transcription of multiple E1A-inducible adenovirus early promoters. *Proc. Natl. Acad. Sci. USA* **84**:8355-8359.
 40. Lee, W. S., C. C. Kao, G. O. Bryant, X. Liu, and A. J. Berk. 1991. Adenovirus E1A activation domain binds the basic repeat in the TATA box transcription factor. *Cell* **67**:365-376.
 41. Lin, Y.-S., and M. R. Green. 1988. Interaction of a common cellular transcription factor, ATF, with regulatory elements in both E1a- and cyclic AMP-inducible promoters. *Proc. Natl. Acad. Sci. USA* **85**:3396-3400.
 42. Liu, F., and M. R. Green. 1990. A specific member of the ATF transcription factor family can mediate transcription activation by the adenovirus E1A protein. *Cell* **61**:1217-1224.
 43. Liu, Y.-C., R. L. Marraccino, P. C. Keng, R. A. Bambara, E. M. Lord, W.-G. Chou, and S. B. Zain. 1989. Requirement for proliferating cell nuclear antigen expression during stages of the Chinese hamster ovary cell cycle. *Biochemistry* **28**:2967-2974.
 44. Loeken, M. R., and J. Brady. 1989. The adenovirus E1A enhancer. Analysis of regulatory sequences and changes in binding activity of ATF and E1IF following adenovirus infection. *J. Biol. Chem.* **264**:6572-6579.
 45. Mathews, M. B. 1989. The proliferating cell nuclear antigen, PCNA, a cell-cycle regulated DNA replication factor, p. 89-120. In E. Wang and H. R. Warner (ed.), *Growth control during cell aging*. CRC Press, Inc., Boca Raton, Fla.
 46. Matsumoto, K., T. Moriuchi, T. Koji, and P. K. Nakane. 1987. Molecular cloning of cDNA coding for rat proliferating cell nuclear antigen (PCNA)/cyclin. *EMBO J.* **6**:637-642.
 47. Moran, E., and M. B. Mathews. 1987. Multiple functional domains in the adenovirus E1A gene. *Cell* **48**:177-178.
 48. Morris, G. F., and M. B. Mathews. 1990. Analysis of the proliferating cell nuclear antigen promoter and its response to adenovirus early region 1. *J. Biol. Chem.* **265**:16116-16125.
 49. Morris, G. F., and M. B. Mathews. 1991. The adenovirus E1A transforming protein activates the proliferating cell nuclear antigen promoter via an activating transcription factor site. *J. Virol.* **65**:6397-6406.
 50. Nevins, J. R. 1989. Mechanisms of viral-mediated trans-activation of transcription. *Adv. Virus Res.* **37**:35-83.
 51. Ohashi, Y., Y. Sawada, T. Moriuchi, and K. Fujinaga. 1992. Analysis of the 5' flanking region of the rat proliferating cell nuclear antigen (PCNA) gene. *Biochim. Biophys. Acta* **1130**:175-181.
 52. Phelps, W. C., S. Bagchi, J. A. Barnes, P. Raychaudhuri, V. Kraus, K. Münger, P. M. Howley, and J. R. Nevins. 1991. Analysis of trans activation by human papillomavirus type 16 E7 and adenovirus 12S E1A suggests a common mechanism. *J. Virol.* **65**:6922-6930.
 53. Pietrzakowski, Z., H. Alder, C.-D. Chang, D.-H. Ku, and R. Baserga. 1991. Characterization of an enhancer-like structure in the promoter region of the proliferating cell nuclear antigen (PCNA) gene. *Exp. Cell Res.* **193**:283-290.
 54. Pines, J., and T. Hunter. 1990. Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. *Nature (London)* **346**:760-763.
 55. Prelich, G., M. Kostura, D. R. Marshak, M. B. Mathews, and B. Stillman. 1987. The cell-cycle regulated proliferating cell nuclear antigen is required for SV40 DNA replication in vitro. *Nature (London)* **326**:471-475.
 56. Prelich, G., C.-K. Tan, M. Kostura, M. B. Mathews, A. G. So, K. M. Downey, and B. Stillman. 1987. Functional identity of proliferating cell nuclear antigen and a DNA polymerase- δ auxiliary protein. *Nature (London)* **326**:517-520.
 57. Pugh, B. F., and R. Tjian. 1991. Transcription from a TATA-less promoter requires a multisubunit TFIID complex. *Genes Dev.* **5**:1935-1945.
 58. Raychaudhuri, P., S. Bagchi, S. H. Devoto, V. B. Kraus, E. Moran, and J. R. Nevins. 1991. Domains of the adenovirus E1A protein required for oncogenic activity are also required for dissociation of E2F transcription factor complexes. *Genes Dev.* **5**:1200-1211.
 59. Rooney, R. J., P. Raychaudhuri, and J. R. Nevins. 1990. E4F and ATF, two transcription factors that recognize the same site, can be distinguished both physically and functionally: a role for E4F in E1A trans activation. *Mol. Cell. Biol.* **10**:5138-5149.
 60. Ruley, H. E. 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature (London)* **304**:602-606.
 61. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 62. Sassone-Corsi, P., L. J. Ransone, and I. M. Verma. 1990. Cross-talk in signal transduction: TPA inducible factor *jun/AP-1* activates cAMP responsive enhancer elements. *Oncogene* **5**:427-431.
 63. Seto, E., Y. Shi, and T. Shenk. 1991. YY1 is an initiator sequence-binding protein that directs and activates transcription in vitro. *Nature (London)* **354**:241-245.
 64. Shenk, T., and J. Flint. 1991. Transcriptional and transforming activities of the adenovirus E1A proteins. *Adv. Cancer Res.* **57**:47-85.
 65. Shi, Y., E. Seto, L.-S. Chang, and T. Shenk. 1991. Transcriptional repression by YY1, a human GLI-Krüppel-related protein, and relief of repression by adenovirus E1A protein. *Cell* **67**:377-388.
 66. Shivji, M. K. K., M. K. Kenny, and R. D. Wood. 1992. Proliferating cell nuclear antigen is required for DNA excision repair. *Cell* **69**:367-374.
 67. Simon, M. C., T. M. Fisch, B. J. Benecke, J. R. Nevins, and N. Heintz. 1988. Definition of multiple, functionally distinct TATA elements, one of which is a target in the hsp70 promoter for E1A regulation. *Cell* **52**:723-729.
 68. Simon, M. C., K. Kitchener, H.-T. Kao, E. Hickey, L. Weber, R. Voellmy, N. Heintz, and J. R. Nevins. 1987. Selective induction of human heat shock gene transcription by the adenovirus E1A gene products, including the 12S E1A product. *Mol. Cell. Biol.* **7**:2884-2890.
 69. Simon, M. C., R. J. Rooney, T. M. Fisch, N. Heintz, and J. R.

- Nevins. 1990. E1A-dependent trans-activation of the *c-fos* promoter requires the TATAA sequence. *Proc. Natl. Acad. Sci. USA* **87**:513–517.
70. Smale, S. T., and D. Baltimore. 1989. The “initiator” as a transcription control element. *Cell* **57**:103–113.
71. Smale, S. T., M. C. Schmidt, A. J. Berk, and D. Baltimore. 1990. Transcriptional activation by Sp1 as directed through TATA or initiator: specific requirement for mammalian transcription factor IID. *Proc. Natl. Acad. Sci. USA* **87**:4509–4513.
72. Spaete, R. R., and E. S. Mocarski. 1985. Regulation of cytomegalovirus gene expression: α and β promoters are *trans* activated by viral functions in permissive human fibroblasts. *J. Virol.* **56**:135–143.
73. Taylor, I. C. A., and R. E. Kingston. 1990. E1a transactivation of human HSP70 gene promoter substitution mutants is independent of the composition of upstream and TATA elements. *Mol. Cell. Biol.* **10**:176–183.
74. Thalmeier, K., H. Synovzik, R. Mertz, E.-L. Winnacker, and M. Lipp. 1989. Nuclear factor E2F mediates basic transcription and trans-activation by E1a of the human *MYC* promoter. *Genes Dev.* **3**:527–536.
75. Travali, S., D.-H. Ku, M. G. Rizzo, L. Ottavio, R. Baserga, and B. Calabretta. 1989. Structure of the human gene for the proliferating cell nuclear antigen. *J. Biol. Chem.* **264**:7466–7472.
76. Tsai, L.-H., E. Harlow, and M. Meyerson. 1991. Isolation of the human *cdk2* gene that encodes the cyclin A- and adenovirus E1A-associated p33 kinase. *Nature (London)* **353**:174–177.
77. Ullrich, A., A. Gray, D. V. Goeddel, and T. J. Dull. 1982. Nucleotide sequence of a portion of human chromosome 9 containing a leukocyte interferon gene cluster. *J. Mol. Biol.* **156**:467–486.
78. White, E., and R. Cipriani. 1990. Role of adenovirus E1B proteins in transformation: altered organization of intermediate filaments in transformed cells that express the 19-kilodalton protein. *Mol. Cell. Biol.* **10**:120–130.
79. Whyte, P., K. J. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg, and E. Harlow. 1988. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature (London)* **334**:124–129.
80. Whyte, P., N. M. Williamson, and E. Harlow. 1989. Cellular targets for transformation by the adenovirus E1A proteins. *Cell* **56**:67–75.
81. Widen, S. G., P. Kedar, and S. H. Wilson. 1988. Human β -polymerase gene. Structure of the 5'-flanking region and active promoter. *J. Biol. Chem.* **263**:16992–16998.
82. Wiley, S. R., R. J. Kraus, and J. E. Mertz. 1992. Functional binding of the “TATA” box binding component of transcription factor TFIID to the –30 region of TATA-less promoters. *Proc. Natl. Acad. Sci. USA* **89**:5814–5818.
83. Williams, G. T., T. K. McClanahan, and R. I. Morimoto. 1989. E1a transactivation of the human HSP70 promoter is mediated through the basal transcriptional complex. *Mol. Cell. Biol.* **9**:2574–2587.
84. Yamaguchi, M., Y. Hayashi, F. Hirose, S. Matsuoka, T. Moriuchi, T. Shiroishi, K. Moriwaki, and A. Matsukage. 1991. Molecular cloning and structural analysis of mouse gene and pseudogenes for proliferating cell nuclear antigen. *Nucleic Acids Res.* **19**:2403–2410.
85. Zerler, B., R. J. Roberts, M. B. Mathews, and E. Moran. 1987. Different functional domains of the adenovirus E1A gene are involved in regulation of host cell cycle products. *Mol. Cell. Biol.* **7**:821–829.