Analysis of the Proliferating Cell Nuclear Antigen Promoter and Its Response to Adenovirus Early Region 1*

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The levels of the mRNA for the proliferating cell nuclear antigen (PCNA), a DNA replication factor, increase upon growth stimulation of quiescent cells. To study the transcriptional aspect of this response, we have cloned a PCNA gene fragment from size-fractionated human placental DNA. This fragment contains 1269 nucleotides upstream from the PCNA transcriptional start site and includes an Alu sequence that is transcribed in vitro. The PCNA genomic DNA promotes transcription of a linked heterologous reporter gene in HeLa and 293 cells. Transient expression assays and in vitro transcription analyses showed that 249 nucleotides of upstream sequence are sufficient for full promoter activity in HeLa cells, whereas only 172 nucleotides are needed in 293 cells. Co-transfection with a plasmid expressing the adenovirus E1 gene transactivates the PCNA promoter in HeLa cells. An E1-responsive element maps in the 85-nucleotide region immediately upstream of the site of transcription initiation.

Consistent with its role in DNA replication, expression of the proliferating cell nuclear antigen (PCNA), also known as cyclin (Mathews et al., 1984) and the DNA polymerase-α auxiliary factor (Ián et al., 1986; Bravo et al., 1987; Freligh et al., 1987a) is linked to cell growth, (Almendral et al., 1987; Mateumoto et al., 1987; Jaskulski et al., 1988; Shipman et al., 1988; Wold et al., 1988; see Mathews, 1989 for a recent review). PCNA functions in concert with DNA polymerase-δ to replicate the leading strand of an SV40 DNA template in vitro (Freligh et al., 1987b; Freligh and Stillman, 1988). The importance of its function to the replication machinery is highlighted by the high degree of evolutionary conservation of the protein. Homologs have been identified in plants (Suzuka et al., 1989), animals (Celles et al., 1987) including insects (Yamaguchi et al., 1990), yeast (Bauer and Burgers, 1990), and even a bacteriophage (Tsurimoto and Stillman, 1990).

Although PCNA protein and mRNA levels change relatively little during the cell cycle (Wold et al., 1988; Liu et al., 1989; Morris and Mathews, 1989), they increase dramatically upon growth stimulation of quiescent cells by such agents as serum, growth factors, and viral infection (Almendral et al., 1987; Bravo et al., 1987; Matsumoto et al., 1987; Zelel et al., 1987; Jaskulski et al., 1988). Like other genes involved in DNA metabolism, the induction of PCNA mRNA levels by these agents requires protein synthesis and occurs after a delay of several hours (Almendral et al., 1987; Jaskulski et al., 1988). Genes such as thymidine kinase, dihydrofolate reductase, and thymidylate synthase are transcriptionally activated during this secondary response to growth stimuli (Johnson, 1984; Jonh et al., 1985; Ito and Conrad, 1990), but it is not known whether the PCNA gene is similarly activated at the transcriptional level. However, as little as 210 nucleotides of upstream sequence from the PCNA gene are sufficient to give substantial transcriptional activity in stably transformed cells (Ottovio et al., 1990).

Adenovirus infection can cause cells to bypass normal controls of growth in culture and to produce tumors in animals (Shenk, 1989). The E1 region of the virus, which encodes both E1A and E1B, is necessary and sufficient for this process (Shenk, 1989). Viral infection activates many, if not all, of the genes activated during the secondary response to growth factors (Liu et al., 1989). The effects of E1B on transcription are probably indirect and possibly result from increasing E1A levels (Herrmann et al., 1987; Yoshida et al., 1987; Jochemsen et al., 1987; Herrmann and Mathews, 1989). E1A, on the other hand, is well characterized as a transcriptional activator of gene expression (Flint and Shenk, 1989). Most of the positive transcriptional effects of the E1 protein map to conserved region 3, which is unique to the 13 S transcript (Moran and Mathews, 1987), but a virus expressing only the 12 S E1A transcript, which lacks conserved region 3, can induce expression of the cellular genes for PCNA (Zeler et al., 1987) as well as hsp 70 (Simon et al., 1987), cdc 2 (Draetta et al., 1988), and brain creatine kinase (Kadurah-Daouk et al., 1990). The ability to induce these cellular genes might be related to the transforming properties shared by the 13 S and 12 S E1A transcripts (Moran and Mathews, 1987).

To define the interactions of E1A with the PCNA promoter, we have isolated human PCNA promoter sequences. General features of the PCNA promoter were characterized by sequence comparison and in vitro transcription, and the effects of deletions on promoter activity were assayed by transfection of PCNA-CAT constructs into HeLa cells and 293 cells. Transactivation of the PCNA-CAT fusion constructs by the adenovirus E1 gene was investigated by co-transfection into HeLa cells, and an E1 responsive region in the PCNA promoter was identified.

MATERIALS AND METHODS

Plasmids—A PCNA cDNA clone, pS14 (Almendral et al., 1987), was obtained from R. Bravo (KMBL, Heidelberg, Federal Republic...
of Germany). pSV2CAT (Gorman et al., 1982) was obtained from C. Gorman (Genentech, Inc., San Francisco, CA). pEMCAT (Weeks and Jones, 1983) was obtained from N. Jones (Cold Spring Harbor, NY).

Transfection and CAT Assays—Transfection experiments were performed by the calcium phosphate precipitation technique (Herrmann et al., 1987) except that the medium was not changed prior to addition of the precipitate. 5 μg of plasmid was employed for each transfection, and salmon sperm DNA was added to bring the total to 20 μg of DNA per 6-cm plate. CAT activity was assayed as previously described (Herrmann et al., 1987) and quantified using an AMBUS Beta Scanner.

Cell Culture—Monolayer cultures of HeLa cells (ATCC CCL 2) were grown in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and 100 μg/ml penicillin and streptomycin. 293 cell monolayers were grown in the same medium with 10% calf serum instead of 10% fetal calf serum.

RESULTS

Cloning of the PCNA Promoter—As a first step toward cloning the PCNA promoter, we analyzed total genomic DNA from human placenta by digestion with the restriction endonuclease EcoRI. The resulting fragments were resolved and probed by hybridization to the entire PCNA cDNA clone or to a fragment containing the 5′ end of the cDNA (probe 1, Fig. 1B). Three genomic fragments of about 4.14, 2.75, and 1.56 kb hybridized to the complete cDNA (Fig. 1A, lane 1), while only the 2.75-kb band hybridized to the 5′ end of the cDNA (Fig. 1A, lane 2). Consistent with the cDNA sequence, the 2.75-kb fragment was cleaved by PstI, producing two smaller fragments of about 1.5 kb and 1.0 kb that hybridized to a fragment representing the 5′-half of the cDNA (probe 2, Fig. 1B; data not shown). To clone these fragments, genomic DNA was digested with EcoRI and PstI on a preparative scale and was fractionated by agarose gel electrophoresis (Fig. 1C). The fractions containing the desired PCNA gene fragments were identified by hybridization to probe 2 (Fig. 1D). Enriched plasmid libraries were prepared from these two fractions and colonies corresponding to the 1.0-kb fragment and the 1.5-kb fragment were selected by hybridization with probe 2.

Sequence of the PCNA Promoter—DNA sequence analysis of the 1.5-kb fragment revealed that it contains the 5′ end of the cDNA and upstream sequences, and that it is contiguous with the 1.0-kb clone which contains adjacent downstream cDNA sequences as depicted in Fig. 1B. The upstream 1.5-kb fragment is uninterrupted, whereas the downstream 1.0-kb fragment is interrupted twice by introns of approximately 0.7 kb and 0.1 kb. The sequence of the larger fragment is shown in Fig. 2. While this work was in progress, Travali et al. (1989) published the sequence of a human genomic clone comprising the entire PCNA coding sequence. Relative to their sequence, our sequence contains 6 insertions, 3 deletions, and 16 substitutions. Four of the changes have been confirmed by restriction analysis of our clone (marked in Fig. 2). Several of the changes are clustered and presumably represent polymorphisms in the PCNA gene, but others are isolated differences. The upstream region of the PCNA gene displays numerous homologies with consensus sequences for RNA polymerase II transcription factor binding sites. Some of these are summarized in Table I to facilitate consideration of their potential role in the expression of the PCNA gene in light of the data presented below.

An Upstream Alu Sequence—Hybridization of the 1.5-kb genomic clone to total genomic DNA revealed the presence of a repetitive DNA element (data not shown). In vitro assays at high template concentrations, which favor transcription by
RNA polymerase III, gave evidence of RNA polymerase III transcription of the upstream sequence. Transcription of a PCNA construct containing 1265 nucleotides of upstream sequence produced several RNA species in the 300-500 nucleotide range (Fig. 3, lane 2). Their synthesis was resistant to a low concentration of α-amanitin (Fig. 3, lane 1), consistent with RNA polymerase III transcription, and they were not transcribed from truncated templates containing 560 nucleotides or less of upstream sequence (Fig. 3, lane 3). These data place a RNA polymerase III transcription unit between -1269 and -560. A homology search of the entire 1.5-kb clone with a member of the human Alu family, located upstream of the c-globin gene (DiSegni et al., 1981), revealed an Alu family member between -1164 and -844 in the PCNA promoter (underlined in Fig. 2). The region of homology includes consensus sequences for RNA polymerase III promoter elements (DiSegni et al., 1981), and, by analogy to its homolog in the e-globin promoter, the Alu sequence in the PCNA promoter should be transcribed in the same direction as the PCNA gene itself. The e-globin Alu sequence is transcribed in vivo (Allan and Paul, 1984) as well as in vitro (DiSegni et al., 1981) and is co-regulated with the e-globin promoter (Wu et al., 1990).
TABLE I

Sequence similarities in the PCNA promoter

Factor binding motifs were compared to sequences upstream of +1 in the PCNA gene. Boldface represents homology between the consensus sequence and a similar sequence in the PCNA gene. Location is given in terms of nucleotides in the PCNA sequence: decreasing numbers indicate that the motif is in the same orientation as the PCNA gene; increasing numbers indicate that the sequence is inverted relative to the direction of transcription of the gene. * marks the site of transcription initiation. Steroid response elements were compared as half-palindromes, because perfect palindromes for steroid response elements are rarely found (Beato, 1989).

| Site           | Consensus sequence | PCNA sequence | Location | Reference |
|----------------|--------------------|---------------|----------|-----------|
| CAT            | CCAAT              | CCAAT         | −95 −100 | McKnight et al., 1989 |
|                |                    | CCAAT         | −145 −141 |           |
| SP1            | GGGGGG             | GGGCGGGGCC    | −129 −120 | Jones et al., 1988 |
|                | T AAT              | AAGCGGGGCC    | −159 −168 |           |
|                |                    | GAGCGGGGGA    | −193 −184 |           |
|                |                    | CTTGGGGGGG    | −523 −514 |           |
| AP-3           | AAA TGTGG G TTT    | TGGGAGAT      | −233 −242 | Jones et al., 1988 |
| AP-4           | CATCTGTGG          | CATATGTGG     | −230 −238 | Jones et al., 1988 |
|                |                    | CAGCTCTGG     | −395 −403 |           |
| PRD I Octamer  | GAACTGAAAGT GCATTGCAT | ATTGGCAT     | −349 −408 | Jones et al., 1988 |
|                |                     | ATTTCAGCAG    | −405 −401 |           |
| SPH            | AAG ATGCA          | AAAATATGCA    | −405 −413 | Jones et al., 1988 |
|                | T                   |              |          |           |
| Terminal deoxynucleotidyltransferase initiator | GGCCTCACTCTGGAGAC | GGGCGGATTAACGGTT | −6 +11 | Smale and Baltimore, 1989 |
| Initiator consensus | ATTT CN1 GCCA | ATTAACCGGTGCA | +1 +14 | Means and Farnham, 1990 |

ElA-responsive elements

| Site           | Consensus sequence | PCNA sequence | Location | Reference |
|----------------|--------------------|---------------|----------|-----------|
| ATF/CRE        | A CGTCA            | ACGTCG       | −50 −45  | Buckbinder et al., 1989 |
|                | T                  | TCGTCA       | −260 −265 |           |
| EivF           | G GTACGT           | GGTACGT      | −53 −47  | Cortes et al., 1988 |
| AAV P5         | TTTGGCCAG          | CTTGGCCAG    | −42 −47  | Chang et al., 1989 |
|                | T                  | GCTTGGCCAG   | −18 −8   |           |
| Polymerase β palindrome | GTGACGTCAAC | GTGACGTCCGAC | −53 −41 | Widen et al., 1988 |
| AP-1           | TGACTCA            | TGACTCA      | −819 −813| Jones et al., 1988 |

Steroid response elements

| Site           | Consensus sequence | PCNA sequence | Location | Reference |
|----------------|--------------------|---------------|----------|-----------|
| GRE            | AGAACA N1 TGGTCT   | AGAAACA      | −216 −221| Beato, 1989 |
|                |                    | TGGTCT       | −473 −479|           |
| ERE            | AGGTCA N2 TGACCT   | TGACTC       | −1092 −1097| Beato, 1989 |
| TRE            | AGGTCACTGACCT      | AGGTGATCCACCT| −1098 −1111| Beato, 1989 |

It is not yet known if the Alu sequence functions in PCNA gene expression.

The Transcription Initiation Site—Preliminary attempts to map the site of transcription initiation by the S1 nuclease technique suggested a location about 40 nucleotides upstream from the 5′ end of the nearly complete human cDNA clone of Almendral et al. (1987). In the experiment shown in Fig. 4B, a broad DNA band of approximately 217 nucleotides was protected efficiently by poly(A)+ or total cellular RNA (lanes 2 and 3), but not by poly(A)−RNA (lane 1). The same end-labeled DNA probe was digested with a second enzyme to produce a 51-nucleotide fragment for 5′ end mapping by the primer extension method (Fig. 4A). Dideoxy chain termination reactions (lanes A, C, G, T) were carried out in parallel with the unblocked primer extension reaction (lane P) and gave a partial sequence that matched the cDNA sequence of Almendral et al. (1987). This verified that the primer was specific for PCNA mRNA. The unblocked primer extension product was about 4 nucleotides longer than the fragment protected against nuclease S1, probably because of overdigestion by the nuclease in this AT-rich region. The position deduced for the 5′ end of the PCNA mRNA, indicated in Fig. 2 by conversion to lower case, lies 160 nucleotides upstream of the translation initiation codon. Similar results have been obtained by Traval et al. (1989), who located the site of transcription initiation by primer extension to the same position as the 5′ terminus of a full length PCNA cDNA clone (Jaskulski et al., 1988). Initiation at the A residue designated here as +1 is consistent with the transcriptional start site being fixed by a surrounding initiator element (see Table I and below).

Transient Expression in HeLa Cells—To discover whether the 1.5-kb clone contains a functional promoter, the region from −1265 to +60 was fused to a reporter gene (chloramphenicol acetyltransferase, CAT) and transfected into HeLa cells. The upstream sequence from the PCNA gene promoted the synthesis of CAT (Fig. 6), albeit 20–40 times less effectively than the SV40 early promoter (data not shown). To identify regions of the PCNA promoter important for its function, we produced a series of upstream deletion mutants of the PCNA promoter, all fused at +60 to the CAT reporter.
gene (Fig. 5). The CAT activities obtained in a transient expression assay with these constructs are shown in Fig. 6A. Roughly equivalent levels of CAT activity were observed in HeLa cells whether the construct contained -1265, -560, -397, or as little as -249 nucleotides of upstream PCNA genomic sequences. Deletion of the region between -249 and -172 reduced the activity in HeLa cells by approximately 40%. Within this region is a potential Spl site at -190 and -172 to -87 reduced the activity in HeLa cells by approximately 40%. Within this region is a potential Sp1 site at -190 and partial homology to overlapping Ap-3 and Ap-4 sites at -235 (Table I). Removal of sequences from -172 to -87 reduced the activity of the promoter further, to levels only slightly higher than the basal levels observed with the parent clone lacking inserted PCNA promoter sequences, pBACAT. The region from -172 to -87 contains two CCAAT boxes and two Sp1 sites (Table I). The largest upstream deletion tested, a -147 to +60 construct, removed a site homologous to the ATF consensus sequence (Table I). CAT activity generated by the -147 to +60 construct did not routinely exceed that from the pBACAT control, except in the most sensitive experiments conducted with more extract and longer assay incubation times (Fig. 6B). A summary of the relative CAT activities for each of the constructs, averaged over several experiments, is shown in Fig. 5.

We also performed RNase protection assays to examine the PCNA-CAT transcripts generated in transfected HeLa cells. RNA from the transient expression of each upstream deletion construct gave rise to a protection product of the correct size and partial homology to overlapping Ap-3 and Ap-4 sites at -235 (Table I). Control assays, with tRNA or RNA from untransfected cells or cells transfected with pBACAT, did not produce a protection product that mapped to this site. For these constructs, the levels of correctly initiated transcripts correlated well with the amount of CAT activity detected (compare Fig. 6, A and C), and a simultaneous RNase protection assay for a globin clone co-transfected with each deletion produced similar signals in each case. Longer PCNA-CAT protection products were observed in each lane (Fig. 6C, bands I–4) and probably correspond to transcripts from cryptic promoters upstream of the inserted PCNA genomic fragment. These read-through transcripts were more prominent than the PCNA-CAT mRNA protection product with the -87 and -147 deletions. Read-through transcription might lower the amount of correctly initiated PCNA-CAT mRNA by promoter occlusion (Adhya and Gottesman, 1982; Vales and Darnell, 1989; Wu et al., 1990); perhaps the inverted CCAAT motif at -147 in the PCNA promoter serves to reduce the amount of read-through transcription from upstream promoter sequences, as is the case in the adenovirus major late promoter (Connelly and Manley, 1989).

Possible Initiator Element—Some promoters that lack TATA elements possess a sequence encompassing the transcription initiation site, termed the initiator sequence (Smale and Baltimore, 1989; Means and Farnham, 1990), that serves to designate the site of transcription initiation. The PCNA promoter lacks a suitably placed TATA box and displays some homology near its cap site to the published initiator sequences (Table I). To assess the role of sequences near the cap site in the function of the PCNA promoter, sequences from -560 to -2 nucleotides upstream of the cap site were fused to the CAT reporter gene (Fig. 5, bottom line). This cap site deletion construct gave rise to 30% less CAT activity in HeLa cells than the -560 to +60 construct (Figs. 5 and 6A), suggesting that sequences between -1 and +60 play some role in gene expression. On the other hand, no decrease in CAT
mRNA was evident when the major RNase protection product (band 5) arising from the cap site deletion construct was compared with the product derived from the longer clone (Fig. 6C). However, the RNase protection analysis is likely to overestimate the production of mRNA by the -560 to -2 clone; since the sequence of this construct diverges from that of the probe at the 5' end of the CAT gene, any transcripts initiating upstream of this point, including the read-through transcripts referred to above, would be scored in band 5. It is probable that some of the upstream transcription initiations scored in this assay do not give rise to a functional CAT mRNA, thereby accounting for the apparent discrepancy between the CAT assay and the RNase protection experiment.

To obtain more direct transcriptional information, each of the CAT constructs described above was tested for its template activity in a HeLa nuclear extract. The predicted α-amanitin-sensitive run-off product was observed with all of the templates carrying upstream deletions (Fig. 6D). The yields of run-off product observed approximated the activity of the promoters in the transient expression assays described above. The -560 to -2 clone did not produce a prominent amanitin-sensitive run-off product, but a greater amount of amanitin-sensitive transcription was observed within the region 50 to 100 nucleotides longer than the predicted run-off transcript (bracketed in Fig. 6D). Presumably, these arise from heterogeneous upstream starts on the -560 to -2 template, reflecting deletion of an element (such as an initiator element) involved in determining the site of transcription initiation. Alternatively, the deletion of sequences including the cap site might simply produce a more deleterious effect on transcription in vitro than in vivo.

**Transient Expression in 293 Cells**—Zerler et al. (1987) demonstrated that PCNA gene expression is induced by the adenovirus E1A gene. To characterize the effects of E1A on the PCNA promoter, we first assayed the function of the PCNA promoter in 293 cells, which express both the adenovirus E1A and E1B gene products. Fig. 7 recapitulates in 293 cells the experiments illustrated in Fig. 6 for HeLa cells. The site of transcription initiation was identical in the two cell types (Figs. 6C and 7C), and many of the results obtained in the two cell types were similar, so only the differences will be mentioned here. The PCNA promoter was more active in 293 cells than in HeLa cells; thus, in these cells, CAT expression from the -1265 to +60 construct approached that of the SV40 promoter (data not shown). In HeLa cells, the -172 construct exhibited less promoter strength than the longer constructs, but its activity in 293 cells was undiminished both in transient expression assays and in nuclear extracts (Figs. 5 and 7, A and D). The deletion of sequences at the cap site (in the -560 to -2 construct) had a much more deleterious effect on CAT activity in 293 cells than in HeLa cells (Figs. 5, 6A, and 7A), suggesting that the region from -1 to +60 is responsive to transactivation by E1. As with HeLa cells, the cap site deletion clone produced disparate RNase protection and CAT assay results (Fig. 7, A and C). As noted above, the discrepancy is probably due to the detection of transcripts that do not produce a CAT protein in the RNase protection assay. In 293 cell nuclear extracts, the -560 to -2 template also did not produce a prominent run-off product consistent with transcription initiation immediately following the promoter sequences (Fig. 7D). These observations support the idea that the region from -1 to +60 determines the site of transcription initiation.

**Transactivation by the Adenovirus E1 Region**—The elevated expression of PCNA-CAT constructs in 293 cells compared to HeLa cells suggested that the PCNA promoter is transactivated by the E1 region of adenovirus. In an attempt to demonstrate this directly, we co-transfected HeLa cells with PCNA-CAT constructs and an E1A expression plasmid, but did not consistently observe the expected transactivating effect. However, the simultaneous expression of both transforming genes of adenovirus (E1A and E1B, from plasmid pE1) reproducibly elicited a substantial transactivation of the PCNA-CAT construct; about 6-fold in the experiment of Fig. 8. To define the region of the PCNA promoter that is responsive to E1, each of the deletion mutants illustrated in Fig. 5 was tested by co-transfection with pE1 into HeLa cells. pE1 increased the expression of all the promoter fusion constructs except the -46 to +60 construct and the pBACAT control (Fig. 8). In a limited number of experiments, a slight response was seen with the -46 clone, but the effect never approached the magnitude of the response observed with the other clones.

Consistent with results obtained in 293 cells, the -560 to -2 clone was usually transactivated by E1 about half as well as the control (-560 to +60), possibly indicating a role for the initiator element in the E1 response. It is clear, however, that at least one E1-responsive element lies downstream of -87 since the -87 to +60 construct was transactivated by E1 (Fig. 8). Because the -560 to -2 clone was also E1-responsive, at least one E1-responsive element lies upstream of -2. These...
Human PCNA Promoter

FIG. 6. Transient expression of PCNA-CAT in HeLa cells. A, CAT activity in HeLa cells. Equal amounts of DNA for each PCNA-CAT construct were co-transfected into HeLa cells with a U2-promoter so that factors regulating its activity might be studied in more detail; here we have examined the response of the PCNA promoter to the transforming genes of adenovirus.

Transcription Factor Binding Sites for Basal Transcription—Potential transcription factor binding sites in the PCNA promoter are highlighted in Table I. Since sequences upstream of -249 had no effect on PCNA promoter activity in HeLa or 293 cells, sites further upstream would appear to be inconsequential although they may be important for PCNA expression in other cell types or during different cellular responses. For example, there is good homology to an octamer motif in the PCNA promoter at -350 (8/8) and -400 (7/8) and this motif is known to respond to the herpes viral transactivator VP-16 in HeLa cells (Stern et al., 1989) and have both positive (Scheidereit et al., 1987) and negative (Leonardo et al., 1989) effects depending on cell type. In contrast to results obtained by Travali et al. (1989) with baby hamster kidney cells transformed with the human PCNA gene, we did not observe a negative effect on PCNA promoter activity of sequences between -560 and -397. Parenthetically, a negative effect of the fourth intron of the PCNA gene on its expression in the absence of serum has been reported (Ottavio et al., 1990), but is not addressed here.

Observations show that an E1-responsive element exists between -87 and -2, but do not preclude the possibility that the PCNA promoter contains more than one such element.

DISCUSSION

Since PCNA synthesis correlates closely with cell growth, further understanding of its expression might provide a basis for a better appreciation of the cellular mechanisms involved in controlling cell growth. We have cloned the PCNA promoter so that factors regulating its activity might be studied in more detail; here we have examined the response of the PCNA promoter to the transforming genes of adenovirus.

The two homologies with the CCAAT element at -95 and -145 may be functionally relevant, since removal of the region encompassing these sites severely impairs expression from the PCNA promoter in both 293 cells and HeLa cells (Fig. 5). Promoters for other secondary response genes required for DNA metabolism possess CCAAT boxes; for example, a CCAAT motif is involved in the serum response of the human thymidine kinase promoter (Lipson et al., 1989) which possesses two functionally important CCAAT homologies (Arcot et al., 1989) and in the S phase specific transcription of the human histone H1 gene (LaBella et al., 1989) and a rat H2b globin clone. One-fifth of a freeze-thaw extract from the transfection of a 6-cm plate was incubated with [3H]chloramphenicol for 20 min. The acetylated [3H]chloramphenicol was separated by ascending thin layer chromatography. Nontransfected cell extracts were assayed in parallel (---). B, CAT activity of the weaker promoters; as in A, except that one-half of each extract was assayed for 60 min. C, RNase protection assay for CAT mRNA. Cytoplasmic RNA was isolated from two additional 6-cm plates from the transfection experiment shown in A. One-half of the RNA sample was assayed by RNase protection with PCNA-CAT and globin riboprobes. As negative controls, equal amounts of RNA from untransfected cells (---) or calf liver tRNA (tRNA) were assayed in parallel. Bands 3–4 are read-through transcripts while band 5 is of the size expected for PCNA-CAT transcripts of the -560 to -2 construct. To ensure that the probe was in excess, an equal amount of RNA from the -1265 transfection was assayed with twice as much probe (2x probe). End-labeled DNA marker sizes are shown at left.
FIG. 7. Transient expression of PCNA-CAT in 293 cells. A, CAT activity in 293 cells: as for Fig. 6A, except using one-tenth of the extract per assay. B, CAT activity of the weaker promoters in 293 cells. One-half of the extract from a different transfection experiment, performed in duplicate, was assayed as in Fig. 6B. C, RNase protection assay for CAT mRNA: as in Fig. 6C. D, transcription of the mutant constructs in 293 cell nuclear extracts: as in Fig. 6D except that only the −560 to −2 construct was incubated with α-amanitin (2 μg/ml).

FIG. 8. Stimulation of PCNA-CAT expression by El. PCNA-CAT constructs were co-transfected into HeLa cells with (+) or without (−) pE1. One-half of the extract from each plate was incubated for 60 min with [3H]-chloramphenicol to assay CAT activity. Two other constructs, E3CAT and SV2CAT, were assayed with and without E1 in parallel. The lane designated CAT is an assay with bacterial CAT enzyme.

gene (Hwang et al., 1990). The mouse DHFR gene possesses two CCAAT motifs downstream of the cap site that are important for DHFR transcription in vitro (Farnham and Means, 1990) although their role in vivo is not known. The CCAAT motif and the family of proteins that bind this element have been implicated in developmental control of gene expression, as a serum response element, in the regulation of energy metabolism, and in S phase specific transcription (McKnight et al., 1989), and it will be important to address their participation in transcription of the PCNA promoter.

There are four potential binding sites for Sp1 in the PCNA promoter. Removal of the distal Sp1 site has little effect in either HeLa or 293 cells, but removal of the three other sites at −190, −165, and −125 might be involved in the decline of PCNA-CAT expression observed with the larger deletions. Repeated Sp1 sites are a recurrent theme of late response genes including the genes for DHFR (Dynan et al., 1986), thymidine kinase (Arcot et al., 1989), and thymidylate synthase (Deng et al., 1986). The PCNA promoter of Drosophila, which paradoxically may not produce Sp1 (Courey et al., 1989), also possesses homology with Sp1 sites (Suzuka et al., 1989). Presumably the synergistic properties of Sp1 (Courey et al., 1989) and the repeated arrangement of Sp1 sites in late response genes would produce a large enhancement of transcription during periods of rapid growth. The recent observation that a negative regulator of transcription can bind Sp1-related sequences (Kageyama and Pastan, 1989) could add another layer of complexity to the PCNA promoter.

**El-responsive Elements in the PCNA Promoter**—The differences that we observe in relative CAT activity between 293 cells and HeLa cells for the various deletion constructs could be relevant to the interaction of El with the PCNA promoter. From the HeLa 293 cell comparison one might predict that an El-responsive element lies downstream of −249, since the −249 to +60 construct is fully active in both cell types. Co-
transfection experiments in HeLa cells indicate that E1 responsiveness resides in the −87 to +60 region. Removal of sequences between −1 and +60 reduces the E1 response slightly, but does not abrogate it, suggesting that the main E1 response element lies between −87 and −2.

Since E1B is not known to function at the transcriptional level, and E1A does, we will consider E1A-responsive elements in the −87 to −2 region. A likely candidate is the ATF homology at −50 (Table I). Each of the adenovirus early promoters except E1B contains at least one E1A-responsive ATF site. The ATF site can bind a large family of proteins that may include the immunologically related AP-1 family (Hai et al., 1988). Taylor and Kingston (1990) found that an AT1′ site requires specific core promoter elements (TATA motifs) for activity, but an ATF site (12/13 homology to the PCNA promoter) can bind to adjacent sites in the promoters of the human myc and hamster DHFR genes and may be involved in the response of these promoters to serum (Blake and Azizkhan, 1989; Hiebert et al. 1989). The DNA polymerase β promoter is also transactivated by co-transfection of E1 (Widen et al., 1988). Although a -acting E1A-responsive element has not been identified, mutation of the ATF palindromic reduces DNA polymerase β promoter activity in 293 cells by 70% (Widen et al., 1988).

Another potential E1A-responsive target in this region is the sequence TTGGCAGC at −45 (Table I) which is the upstream half of an imperfect inverted repeat with the second half 25 nucleotides downstream. This sequence is part of a direct repeat that confers E1 responsiveness on the adenovirus F5 promoter (Chang et al., 1989). This inverted repeat arrangement is also reminiscent of the E1A-responsive E2F sites of the adenovirus E2A gene. E2F can bind to adjacent sites in the promoters of the human myc and hamster DHFR genes and may be involved in the response of these promoters to serum (Blake and Azizkhan, 1989; Hiebert et al., 1989). No additional E1A-responsive elements can be identified by sequence comparisons of the −87 to −2 region.

This report maps sequences in the PCNA promoter required for basal activity and localizes a region that responds to co-transfection of E1 and less well to E1A. It is probable that the E1B effects are mediated through increasing E1A levels, but a cooperative interaction of E1A and E1B cannot be discounted. We are presently examining the effects of each of the transcripts of the E1 region individually and in combination on the activity of the PCNA promoter.

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