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# Promoter Activity of the Proliferating-Cell Nuclear Antigen Gene Is Associated with Inducible CRE-Binding Proteins in Interleukin 2-Stimulated T Lymphocytes

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**The proliferating-cell nuclear antigen (PCNA) gene encodes an auxiliary factor of DNA polymerase delta and functions in DNA replication during S phase. It is expressed at much higher levels in proliferating cells than in quiescent cells. We have studied the regulatory role of the 5′-flanking sequence of the murine PCNA gene in interleukin 2 (IL-2)-responsive cloned T cells (L2). Analysis of a set of deletion constructs in transient transfection assays measuring heterologous reporter gene (luciferase) activity demonstrated that the 182-bp 5′-flanking region provides full promoter activity in IL-2-stimulated L2 cells. While many elements contribute to PCNA promoter strength in IL-2-stimulated cells, the largest decrease in activity occurred with deletion of the tandem CRE (cyclic AMP response element) binding sites located at nucleotides −37 to −52. With a gel mobility shift assay, several IL-2-inducible DNA-protein complexes were detected, including CREB (CRE-binding) and ATF1 (activating transcription factor) proteins that are specific for the PCNA-CRE sequence. Methylation interference analysis confirmed specific binding of these proteins to the CRE sites. Mutation at the PCNA-CRE motif abolishes IL-2-inducible binding and reduces substantially PCNA promoter activity. These results indicate that IL-2-stimulated PCNA transcription may be partially mediated by these CRE-binding proteins.**

The activation of antigen-specific T lymphocytes from $G_0$ to S phase usually requires several signals. Initially, antigen interacts with the T-cell antigen receptor, which leads to the transition from $G_0$ to $G_1$ and the expression of interleukin 2 (IL-2) receptor and lymphokines including IL-2. Further $G_1$ progression requires additional signals provided by the interaction of IL-2 with its high-affinity receptor, which results in the expression of genes related to $G_1$ activation, and binding of prolactin to its receptor, which results in entry into S phase and DNA replication (11, 14, 35, 38, 67).

One of the genes expressed during IL-2-driven $G_1$ progression is proliferating-cell nuclear antigen (PCNA) (49, 69). PCNA was originally described as a proliferation-associated nuclear antigen that was thought to be expressed in a cell-cycle-specific manner (8, 47). While PCNA is an auxiliary protein for DNA polymerase delta (leading-strand polymerase) (9, 59) and PCNA-associated immunofluorescence closely parallels [$^3$H]thymidine incorporation during cell cycle progression (42), synthesis and expression of PCNA can be detected in all phases of the cell cycle ($G_1$/S/$G_2$/M) in proliferating cells, suggesting that the protein is easily extractable when it is not associated with the polymerase (10, 49). Beach and coworkers showed recently that D-type cyclins can directly interact with PCNA and suggested this interaction as one possible mechanism by which the cell cycle machinery could regulate PCNA function and $G_1$/S transition (77). The ability of PCNA-specific antibodies to inhibit inducible DNA synthesis in isolated nuclei (76), and of PCNA antisense oligonucleotides to inhibit proliferation of BALB/c3T3 cells (29), underscores the important role of this protein in DNA replication.

Normal cellular proliferation is regulated by a variety of specific growth factors. The mechanisms regulating growth factor-induced increases in PCNA RNA accumulation are beginning to be defined (2, 30, 69). During growth factor stimulation of lymphocytes or 3T3 cells, there is an increase in PCNA RNA stability (12, 70). Increased PCNA transcription has been demonstrated in IL-2-stimulated T cells (70), but it could not be found in serum-stimulated 3T3 cells (12). Alternatively, a transcriptional pause or splicing block has been proposed as a mechanism for regulating PCNA RNA accumulation in 3T3 cells. This suggests that lymphocytes and fibroblasts use different mechanisms for regulating levels of PCNA RNA (1, 12, 57).

cDNA and genomic DNA of several species of PCNA have been isolated (2, 5, 44, 69, 73, 78), and the structural gene for human PCNA has been localized to chromosome 20 (36). The PCNA promoters of at least four species have been analyzed (51, 54, 56, 71, 78). Most of these studies utilized continuously proliferating cells which have little relevance to the regulatory mechanisms encountered during initial growth factor stimulation of normal quiescent cells. From these studies, it appears that humans, rats, and mice require approximately 200 bp of 5′-flanking sequence for full promoter activity and that there is differential promoter utilization in various cell lines (51, 54). Ottavio et al. have shown that the human PCNA promoter is fully active in serum-deprived murine 3T3 cells, providing further evidence that PCNA expression is not regulated at the level of transcription initiation in fibroblasts (56).

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EcoRI HindIII PCNA

| | | |
|---|---|---|
| -39 | 5'-CCGAATTCAAGCTTTCGCTGACAGGGAAAAGCGC-3 | -20 |
| -80 | 5'-CCGAATTCAAGCTTTTGAGGAGAGGTGGGTGGAT-3' | -61 |
| -110 | 5'-CCGAATTCAAGCTTCCTTGCTCAAACCACGGGTA-3' | -91 |
| -144 | 5'-CCGAATTCAAGCTTGTCAGCCCCGCCTTTGCATA-3' | -125 |
| -182 | 5'-CCGAATTCAAGCTTAACCCCGTGATGCCCCTCGC-3' | -163 |
| -503 | 5'-ATGAATTCAAGCTTGGAGAAGCGTTCACGTTAAGAGG-3' | -481 |
| +143 | 5'-ATGAATTCAAGCTTGGTGGCGGAGTTGTGGCGACTA-3' | +122 |

FIG. 1. Oligonucleotides used for PCR amplification of the 5'-flanking region of the PCNA gene. They are listed in 5' to 3' direction with the PCNA sequence underlined and with the numbers indicating the position within the promoter to which the sequence corresponds.

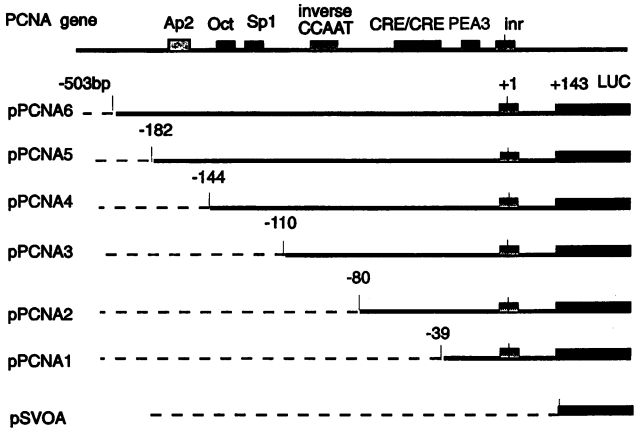



FIG. 2. Schematic diagram of PCNA 5'-flanking region and pPCNA-LUC deletion constructs. The 5'-flanking sequence and 143 bp of nontranslated sequence of the PCNA gene are represented by a black line. The luciferase gene coding sequence is shown by the solid box. The pSVOA vector sequence is shown by a dashed line. The locations of some putative protein binding sites are illustrated by shaded boxes. The 5' ends of all the deletion constructs are as indicated.

In the present study, deletion mutants of the PCNA 5'-flanking sequence were analyzed to define the *cis*-acting elements in the murine PCNA promoter which are required for its promoter activity and IL-2 responsiveness. The 5'-flanking sequence was found to confer an IL-2 response upon a heterologous luciferase reporter. Full promoter activity in IL-2-stimulated T cells requires several sequence elements; tandem CRE binding sites at nucleotides (nt) −37 to −52 and a region downstream of nt −40 contributed substantially to its transcriptional regulation. In addition, results from gel mobility shift assay, methylation interference, and antibody supershift assay show that IL-2 induces several binding activities including CREB and ATF1 proteins that bind specifically to the PCNA-CRE site. Promoter mutations at the CRE motif which abolish CRE binding substantially reduced PCNA promoter activity in cloned T cells, which suggests a possible role for CREB and ATF1 proteins in mediating IL-2-induced PCNA transcription.

## MATERIALS AND METHODS

**Plasmids.** A 1.8-kb PCNA genomic sequence was isolated previously from a BALB/c liver genomic library and subcloned into the pBluescript vector (71). The pPCNA-LUC plasmids were constructed by inserting PCR-amplified DNA containing the PCNA promoter (−1600 to +143) into a *Hin*dIII site of the promoterless pSVOA/LΔ5' vector (the numbering is relative to the transcription start site at +1). The pSVOA/LΔ5' vector has been described previously (16) and will be referred to as pSVOA-LUC in this report. 5'-deletion constructs were made by PCR (see Fig. 2) (60). Oligonucleotides used for PCR amplification of the 5'-flanking region of the PCNA gene contained a *Hin*dIII site and an *Eco*RI site for subsequent cloning. They are listed in Fig. 1.

**Cells.** The cloned murine T-helper-cell line L2 was maintained as previously described (61, 62, 66). L2 cells were cultured at 37°C under 5% $CO_2$ in Dulbecco's modified Eagle's medium supplemented with 1.5 mM glutamine, 0.55 mM arginine, 1 mM pyruvate, 10 mM MOPS (morpholinepropanesulfonic acid [pH 7.2]), 50 μM 2-mercaptoethanol, 0.24 mM asparagine, 0.136 μM folic acid, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal bovine serum (DMEM-CM). Eight days following exposure to irradiated allogenic spleen cells and 5 to 10 U of IL-2 per ml, L2 cells were purified by Ficoll-Hypaque density gradient centrifugation. At this time, the cells are in a $G_1$ resting state (67). To ensure that the cells were in a resting state, L2 cells were cultured for 24 h in fresh medium without IL-2. After 24 h, $1.0 \times 10^7$ L2 cells in 10 ml of medium were stimulated with 100 to 150 U of IL-2 per ml. Human recombinant IL-2 was a gift of Cetus (Emeryville, Calif.) (65). This substance was 98% pure and contained 0.01 ng of endotoxin per $3.6 \times 10^6$ U of IL-2; specific activity was evaluated by the manufacturer. The mouse thymoma cell line EL4 was grown in DMEM-CM. Freshly prepared splenocytes were from CBA/J mice, separated from erythrocytes by density gradient centrifugation through Ficoll-Hypaque (1.090 g/ml). Splenocytes at a concentration of $6 \times 10^6$/ml in DMEM-CM containing 500 ng of concanavalin A (ConA) per ml were incubated for 72 h prior to extraction of proteins.

**DNA transfection and luciferase assay.** PCNA-LUC constructs were transfected transiently into EL4 cells by the DEAE-dextran method as previously described (4, 20), except that the cells were harvested for assay 18 h after transfection when luciferase activity was expressed maximally. L2 cells in a $G_1$ resting state grown in IL-2-free DMEM-CM were transfected by the DEAE-dextran method. Briefly, resting cells were washed with Tris-buffered saline twice, pelleted, and resuspended in 600 μl of a solution containing equal parts DNA and DEAE-dextran (1 mg/ml) and incubated at 37°C for 30 min. The cells were washed twice with Tris-buffered saline and incubated at 37°C with or without 150 U of IL-2 per ml until harvesting at 53 h posttransfection, which was optimal for IL-2-induced luciferase expression in L2 cells. Cells were harvested, washed with $Ca^{2+}$- and $Mg^{2+}$-free phosphate-buffered saline, and lysed by freeze-thawing. Luciferase activity was assayed as previously described with a Berthold Lumat LB 9501 luminometer and expressed in relative light units (7). Quantitation of transfected luciferase DNA by slot blotting was used to correct for variation in transfection efficiency as described previously (43).

**Gel mobility shift assay.** Cellular extracts were prepared from induced and noninduced cells according to the protocol of Dignam et al. (17). Protein concentration was quantitated with the Bradford reagent (Bio-Rad Laboratories, Richmond, Calif.). Gel mobility shift assay was performed as previously described (21). Briefly, 5,000 to 15,000 cpm of $\gamma$-$^{32}$P-labeled duplex oligonucleotide probe and 3 to 5 μg of cell extract were

TABLE 1. Transient expression of pPCNA-LUC constructs in L2 and EL4 cells[a]

| pPCNA-LUC construct | L2 | | | | | EL4 | |
|---|---|---|---|---|---|---|---|
| | −IL-2 | | +IL-2 | | Fold induction | Luciferase activity (light units) | Relative promoter activity (%) |
| | Luciferase activity (light units) | Relative promoter activity | Luciferase activity (light units) | Relative promoter activity | | | |
| pPCNA6-LUC | 3,250 | 100 | 129,000 | 100 | 40 | 147,000 | 100 |
| pPCNA5-LUC | 2,240 | 69 | 178,000 | 138 | 79 | 52,000 | 36 |
| pPCNA4-LUC | 1,860 | 57 | 157,000 | 122 | 84 | 44,000 | 30 |
| pPCNA3-LUC | 1,600 | 49 | 135,000 | 105 | 85 | 16,000 | 11 |
| pPCNA2-LUC | 1,200 | 37 | 103,000 | 79 | 85 | 8,000 | 5 |
| pPCNA1-LUC | 490 | 15 | 20,600 | 16 | 42 | 2,300 | 1.5 |
| pSVOA-LUC | 130 | 4 | 480 | 0.4 | 4 | 300 | 0.2 |
| pRSV-LUC | 1,140 | | 159,000 | | 140 | 186,500 | |

[a] pPCNA-LUC constructs were transiently transfected into L2 cells by the DEAE-dextran method. L2 cells were stimulated by 150 U of recombinant IL-2 per ml. Luciferase activities of each 5′-deletion construct in the presence and absence of IL-2 were measured 53 h after transfection. EL4 cells were transiently transfected with 3 μg of pPCNA-LUC constructs by the DEAE-dextran method. The luciferase activities of the full-length (pPCNA-LUC) and the 5′-deletion constructs were measured 18 h after transfection. All results were normalized to luciferase DNA content by DNA slot blotting. The experiments were performed as duplicates and repeated at least three times, and a typical result (with mean value of duplicates) is shown here. Luciferase activities of all deletion constructs were compared with full-length (−520 promoter) promoter activity (designated as 100%) and listed as relative promoter activity (percent). Mock-transfected cells show background luciferase activity (i.e., no cell extract added to luciferase assay) of about 150 light units.

mixed at room temperature for 15 min in 20 μl of binding buffer containing 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 1 μg of poly(dI-dC), and 5% glycerol. The binding complex was separated by 4.5% polyacrylamide gel electrophoresis at 4°C in buffer containing 25 mM Tris, 190 mM glycine, and 1 mM EDTA at 25 mA. Gels were then dried and autoradiographed. Competition experiments were performed by incubating unlabeled duplex oligonucleotide with extracts for 5 min before the addition of labeled probe. Duplex oligomers were annealed from complementary strands by heating to 90°C and cooling slowly to room temperature and gel purified. For antibody supershift and antibody blocking experiments, the nuclear extract was first incubated with an antibody (diluted 1 to 25) for 10 min and then assayed for binding in the gel mobility shift assay.

The origin of each antibody is as follows: anti-Fos family antibody RR26/4 and anti-Jun family antibody 636/3, R. Bravo, Bristol-Myers Squibb Institute (34); anti-ATF1 monoclonal antibody which recognizes ATF1 but not CREB protein in Western blotting (immunoblotting) and gel mobility shift assay, S. Hinrichs (55); anti-ATF1 and anti-ATF2 antibodies, M. Green, University of Massachusetts; and anti-CREB antibody R-1090, J. F. Habener, Harvard Medical School (74).

The oligonucleotides used in the binding assay are as follows: PCNA-CRE (mouse PCNA) (71), −63 5′-GATCA GCGCTGTGGCGTCA TGACCTCG CTGACAG-3′ −30; AP1 (collagenase AP1) (3), 5′-AAAGCA TGACTCA CTCA GGG-3′; and μE3 (39), 5′-CTAGAGCAGGTCATGTGGCA AGGGTCGA-3′.

**Methylation interference.** The methylation interference assay was performed essentially as previously described (4). PCNA-CRE probes of both the coding and the noncoding strand were uniquely labeled at 5′ ends and were partially methylated with dimethyl sulfate. These probes were each incubated with L2 cell nuclear extract and poly(dI-dC) in binding reactions. After gel electrophoresis, free DNA and protein-DNA complexes were electrophoretically transferred to NA-45 DEAE membrane (Schleicher & Schuell) and eluted according to the supplier's protocol. After cleavage with piperidine, the various DNA fractions were analyzed by urea-polyacrylamide gel electrophoresis (15%) and autoradiography.

**PCR primer-mediated mutagenesis.** Site-specific mutagenesis was accomplished as described previously (60) by a two-step PCR method. The first step amplified, in two separate PCRs, the sequences upstream and downstream of the desired mutation. In the second step, equimolar amounts (0.1 μg) of the two primary PCR products were combined for secondary PCR amplification with the two outside primers. This method produced a fragment (nt −182 to +143) of the PCNA promoter with the desired mutations (see Fig. 4A). *Hin*dIII sites flanked this fragment, allowing cloning into the pSVOA-LUC vector. The DNA sequence of all mutated PCNA promoters was determined to ensure that the desired product was prepared and to confirm the correct orientation in the vector.

## RESULTS

**(i) PCNA promoter activity in IL-2-stimulated cloned T lymphocytes.** Cloned T lymphocytes (e.g., L2 cells) possess many of the characteristics of normal activated T lymphocytes in that they maintain their specific antigenic reactivity, they produce lymphokines in response to antigenic stimulation, and they proliferate in response to IL-2 (18, 63). Because they achieve a $G_1$ resting state after weekly passage, L2 cells have served as an excellent model for studying IL-2-stimulated PCNA expression and $G_1$ activation (67, 70).

There is no single established efficient transfection method to introduce DNA into normal or cloned T lymphocytes. After trying numerous transfection methods, we established a transient transfection system employing DEAE-dextran in which L2 cells are transfected at a low, but measurable, efficiency with pPCNA-LUC constructs.

To study the functional role of the 5′-flanking region of the murine PCNA gene and to evaluate the relative contribution of particular *cis* elements to promoter activity, a genomic DNA fragment carrying the PCNA 5′-flanking region (−1600 to +143) was subcloned, and deletion mutants were produced by PCR amplification. All PCNA sequences were ligated at +143 to a luciferase coding sequence in the pSVOA-LUC vector (Fig. 2) (16). These constructs were transfected transiently into L2 cells and a continuously proliferating T-cell thymoma cell line, EL4. The promoterless plasmid pSVOA-LUC containing the luciferase sequence was used as a negative control. Plasmid pRSV-LUC was transfected as a positive control which carries the Rous sarcoma virus enhancer-driven luciferase gene. Lu-

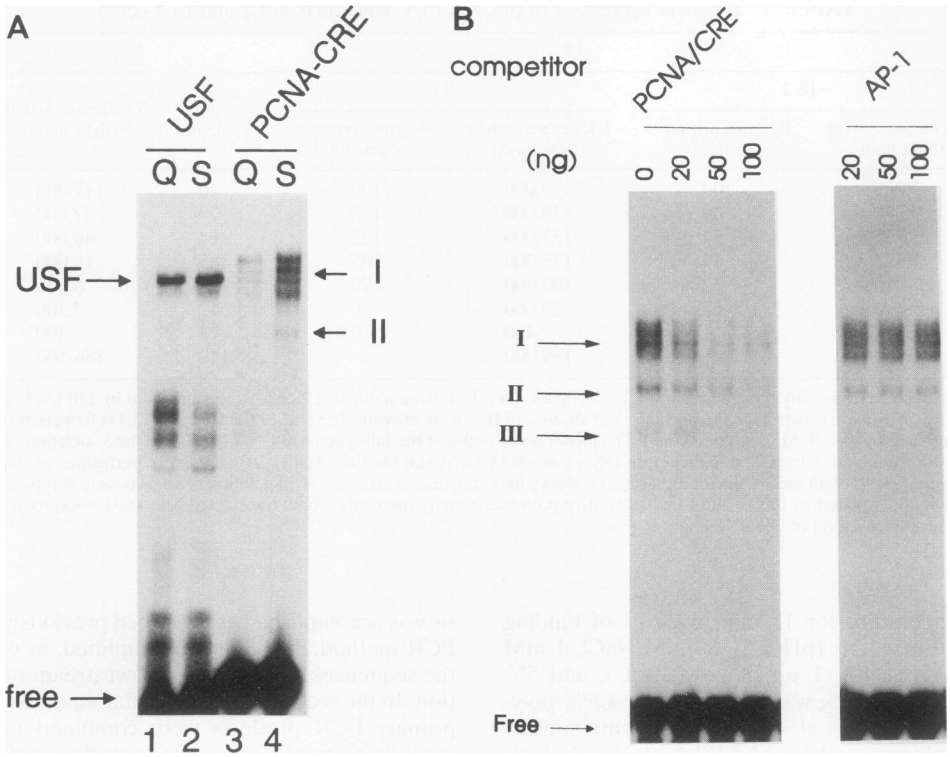


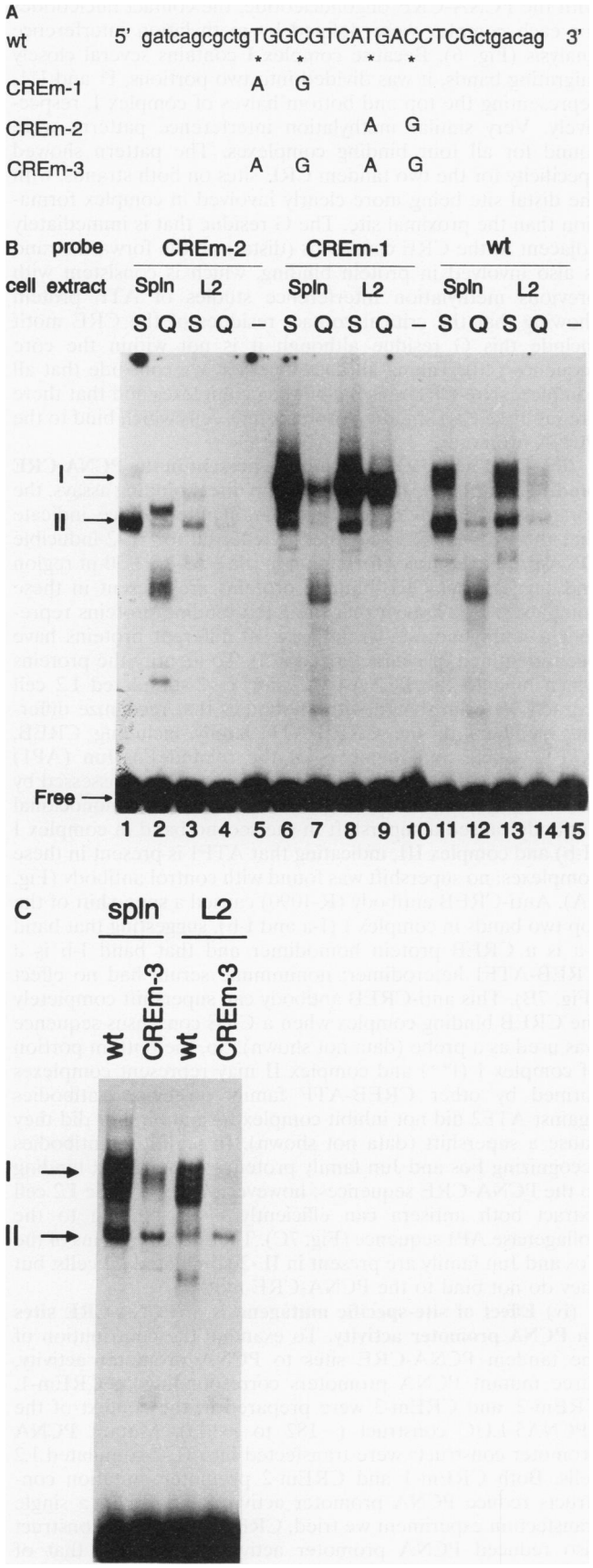

FIG. 3. Binding of IL-2-induced cellular factors to the PCNA promoter sequence (−63 to −30) which contains the tandem CRE binding sites. (A) Gel mobility shift analysis using a $^{32}$P-labeled PCNA-CRE duplex oligonucleotide (−63 to −30 nt) containing the tandem-repeat CRE sites with crude cell extracts (3 μg) from quiescent L2 cells (Q) or 24-h IL-2-stimulated L2 cells (S) (lanes 3 and 4). The arrows indicate the positions of complex I, complex II, and free probe. $^{32}$P-labeled oligonucleotide containing μE3 motif was used as a control. USF binding detected by μE3 probe was constitutively existing in both quiescent and stimulated L2 cells (lanes 1 and 2). (B) Self-competition experiment. Increasing amounts of the nonlabeled PCNA-CRE oligonucleotide were used as competitor for binding proteins in the cell extract from IL-2-stimulated L2 cells. A total of 0, 20, 50, and 100 ng of competitor were added to the binding reaction mixtures. For competition experiments using a nonspecific competitor, 20, 50, and 100 ng of double-stranded oligonucleotide containing the collagenase AP1 sequence were added to the binding reaction mixtures prior to addition of the $^{32}$P-labeled PCNA-CRE oligonucleotide. Complexes I, II, and III are indicated by the arrows.

ciferase activity (in light units) was normalized to the luciferase DNA content as measured by DNA slot blotting with a probe containing the luciferase coding sequence. Deletion of approximately 1,000 bp 5′ of −503 bp did not cause significant change of the luciferase activity (data not shown). The luciferase activity produced by the pPCNA6-LUC construct (−503 to +143) was defined as 100% activity for comparison with that of other constructs.

Without IL-2, relatively small amounts of luciferase activity were measured, and the activity decreased progressively with the loss of 5′ sequence. A 40- to 80-fold increase in luciferase activity was found in IL-2-stimulated L2 cells transfected with PCNA-LUC constructs (Table 1). There was also a 140-fold increase in luciferase activity of the pRSV-LUC construct. The first deletion (pPCNA5) resulted in an increase in IL-2-stimulated promoter activity, but not basal promoter activity (promoter activity without IL-2), suggesting the removal of a negative regulatory element which functions in the presence of IL-2. In contrast to L2 cells, in EL4 cells this deletion (pPCNA5) resulted in a dramatic reduction in luciferase activity (to 36%), indicating that the sequence from −503 to −183 contributes significantly to PCNA promoter activity in EL4 cells. In L2 cells, the next three deletions (pPCNA5 to pPCNA2, removal of 102 bp) caused small decreases individually which, cumulatively, resulted in a 43% decrease in promoter activity. The largest single decrease in promoter activity (46%) was observed with removal of the sequence from −80 to −39 (pPCNA2 to pPCNA1), which contains the tandem CRE sequences; in addition, there was a twofold decrease in IL-2 responsiveness with this deletion. However, substantial PCNA promoter activity and IL-2 responsiveness remained for the pPCNA1-LUC construct (−39 to +143). Notably, within this region there is no canonical TATAA box, but there are the following: a sequence similar to the TdT initiator sequence around +1, a putative PEA3 site (5′-AGGAAGC-3′) at −13, and an E2F-like site (5′-TTGCG GCGC-3′) at +10 (Fig. 2).

These results indicate that (i) there is differential promoter utilization in continuously proliferating T cells and IL-2-induced T cells, (ii) optimal PCNA promoter activity requires several *cis* elements, and (iii) IL-2 responsiveness requires a sequence between −80 and +143. Taken together, these results demonstrate that the IL-2-stimulated growth responsiveness of PCNA promoter in L2 cells is regulated at least in part by its 5′-flanking sequence.

**(ii) Characterization of DNA-binding proteins that interact with the PCNA-CRE sequences.** The promoter deletion analysis suggested that the tandem CRE sequences located between −40 and −80 were the single largest contributor to PCNA promoter activity in IL-2-stimulated L2 cells. To characterize the cellular proteins that interact with this region, PCNA-CRE, a 34-nt double-stranded oligonucleotide span-




ning −63 to −30, was radiolabeled and used in gel mobility shift assays with cell extracts from resting and stimulated L2 cells and splenocytes. L2 cells were rested for at least 24 h and then stimulated to proliferate by the addition of IL-2 for 24 h. Splenocytes were freshly isolated from normal mouse spleen and stimulated with ConA for 72 h. Primary cultures of splenocytes contain normal T cells that remain in $G_0$ until stimulated with ConA. Several DNA-protein binding complexes were observed, including two major complexes (complex I and complex II) and a faster-migrating complex (complex III) (Fig. 3A). The electrophoretic patterns (complexes) formed with extracts from L2 cells and splenocytes were very similar. Complex I contained three to four distinguishable bands with a smeared background. Complex III appears to be unstable, and it can be detected only in small amounts in some L2 cell extracts. In L2 cells and splenocytes (data not shown), all of these complexes were detected only at very low levels in quiescent cells; levels increased markedly after stimulation with either IL-2 or ConA. In contrast to PCNA-CRE, when the same cell extract was used with a $^{32}$P-labeled oligonucleotide containing an immunoglobin heavy chain enhancer μE3 motif (39), the USF binding (22) detected by the μE3 probe did not change significantly during IL-2 stimulation; thus, USF binding served as a control for extract protein integrity in unstimulated cells. To determine the specificity of these binding complexes for the PCNA-CRE sequence, a competition binding assay was performed in the presence of unlabeled PCNA-CRE oligonucleotides or unlabeled oligonucleotides containing the collagenase AP1 motif. The formation of all three complexes was inhibited in a dose-dependent manner by excess unlabeled PCNA-CRE oligonucleotide but not by unlabeled AP1 oligonucleotide (Fig. 3B). Therefore, all three complexes represent specific DNA-protein interactions for the PCNA sequence spanning −63 to −30.

The 34-nt PCNA sequence (−63 to −30) includes two adjacent putative CRE binding sites (25). The proximal sequence (5′-TGACCTCG-3′) differs from the somatostatin CRE consensus sequence TGACGTCA by 2 nt; the distal sequence (5′-TGGC GTCA-3′) differs by only 1 nt. The AP1 consensus sequence (5′-TGACTCA-3′) which is recognized by Fos-Jun family proteins differs by only a single nucleotide from the CRE consensus sequence. Because several complexes were detected in the gel shift assays, it is possible that various protein complexes may be binding selectively to the tandem CRE binding sites or adjacent sequence. Thus, three mutant PCNA-CRE oligonucleotides, CREm-1 (distal mutation), CREm-2 (proximal mutation), and CREm-3 (double mutation), were synthesized to determine the contribution of each sequence to specific complex formation (Fig. 4A) (15). The

FIG. 4. Effect of CRE binding site mutations on the binding of IL-2-induced cellular factors. (A) Sequence of synthetic oligonucleotide PCNA-CRE (wt) which is derived from −63 to −30 of the murine PCNA promoter and three CRE binding site mutations: CREm-1, CREm-2, and CREm-3. The mutated bases are indicated by asterisks. (B) Gel mobility shift assay using the probes shown in panel A. $^{32}$P-labeled oligonucleotide PCNA-CRE (wt), CREm-1, or CREm-2 was mixed with buffer (−) or 3 μg of cell extract prepared from either quiescent L2 cells (Q) or 24-h IL-2-stimulated L2 cells (S) or with cell extracts from nonstimulated (Q) splenocytes (Spln) and ConA-stimulated splenocytes (S). The types of probe and cell extracts added to the reaction mixtures are indicated above the lanes. (C) Gel mobility shift assay using PCNA-CRE (wt) and CREm-3 probes with cell extracts from 24-h IL-2-stimulated L2 cells (L2) or ConA-stimulated splenocytes (spln).

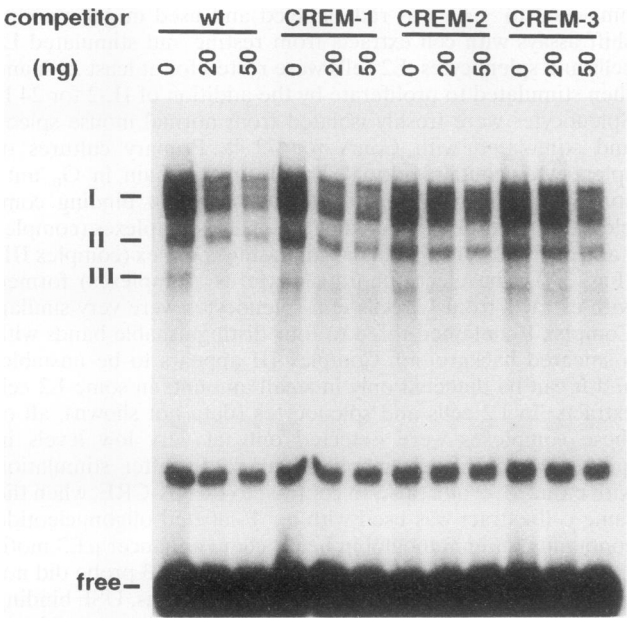



FIG. 5. Inhibition of complex formation by mutated CRE oligonucleotides. End-labeled probe spanning the wild-type −63 to −30 region (PCNA-CRE) was mixed with L2 cell extract prepared 24 h after IL-2 stimulation. Complexes formed were subjected to competition with increasing amounts (20 or 50 ng) of the unlabeled homologous oligonucleotide (wt) or oligonucleotides containing CRE site mutations (CREm-1, CREm-2, and CREm-3). The sequences of these oligonucleotides are shown in Fig. 4A. The types and amounts of competitors used are indicated above the lanes.

relative binding affinity of these three mutated CRE sequences for lymphocyte DNA-binding proteins was determined by either direct binding with labeled mutant oligonucleotides (Fig. 4B and C) or by measuring the ability of mutant sequences to compete for the binding of complexes to the wild-type PCNA-CRE sequence (Fig. 5).

Direct binding studies show that mutation of the distal sequence (CREm-1) appears to enhance complex I formation (compare wild type with CREm-1 in Fig. 4B). In contrast, mutation of the proximal site (CREm-2) or both CRE sequences (CREm-3) markedly inhibits formation of all three complexes (Fig. 4). Similar results were obtained for either IL-2-stimulated L2 cells or ConA-stimulated splenocytes. In the competition analysis, homologous competition was more efficient than heterologous competition with the following relative order: wild type > CREm-1 > CREm-2 = CREm-3 (Fig. 5). The fast-migrating band below complex III (Fig. 5) was detected only in some cell extract preparations; it represented a nonspecific binding because it was not inhibited by either PCNA-CRE wild type or the CRE mutant oligonucleotides. Similar results were obtained for both L2 cell and splenocyte extracts. From the direct binding experiment, one might expect CREm-1 to compete better than the wild-type sequence; however, it did not inhibit protein binding to the wild-type sequence as effectively as the self-competitor. CREm-1 may bind to some of the proteins found in complex I, or CREm-1 may represent a new protein complex not observed with the native PCNA-CRE. These results suggest that both CRE sites contribute to protein binding and formation of the three complexes.

To characterize better the protein-DNA complexes formed with the PCNA-CRE oligonucleotide, the contact nucleotides for each complex were defined by methylation interference analysis (Fig. 6). Because complex I contains several closely migrating bands, it was divided into two portions, I* and I**, representing the top and bottom halves of complex I, respectively. Very similar methylation interference patterns were found for all four binding complexes. The pattern showed specificity for the two tandem CRE sites on both strands, with the distal site being more clearly involved in complex formation than the proximal site. The G residue that is immediately adjacent to the CRE consensus (distal) on the forward strand is also involved in protein binding, which is consistent with previous methylation interference studies of ATF protein showing that the critical contact residues of the CRE motif include this G residue although it is not within the core sequence (40). Taking all data together, we conclude that all complexes are CRE-specific binding complexes and that there are multiple CRE-binding proteins in T cells which bind to the PCNA promoter.

**(iii) ATF1 and CREB protein are present in the PCNA-CRE binding complexes.** The results of the direct binding assays, the competition analyses, and methylation interference indicate that the PCNA-CRE sequence is required for IL-2-inducible DNA-protein complex formation in the −63- to −30-nt region and suggest that CRE-binding proteins are present in these complexes. It is known that the CRE-binding proteins represent a multigene family. At least 10 different proteins have been identified or characterized (23). To identify the proteins which bind to the PCNA-CRE site, IL-2-stimulated L2 cell extracts were incubated with antibodies that recognize different members of the CREB-ATF family including CREB, ATF1, ATF2, and members of the related Fos-Jun (AP1) family. DNA-protein complex formation was then assessed by gel mobility shift assay (Fig. 7). An anti-ATF1 monoclonal antibody caused a supershift in the second band in complex I (I-b) and complex III, indicating that ATF1 is present in these complexes; no supershift was found with control antibody (Fig. 7A). Anti-CREB antibody (R-1090) caused a supershift of the top two bands in complex I (I-a and I-b), suggesting that band I-a is a CREB protein homodimer and that band I-b is a CREB-ATF1 heterodimer; nonimmune serum had no effect (Fig. 7B). This anti-CREB antibody can supershift completely the CREB binding complex when a CRE consensus sequence was used as a probe (data not shown). So, the bottom portion of complex I (I**) and complex II may represent complexes formed by other CREB-ATF family proteins. Antibodies against ATF2 did not inhibit complex formation, nor did they cause a supershift (data not shown). In addition, antibodies recognizing Fos and Jun family proteins do not affect binding to the PCNA-CRE sequences; however, with the same L2 cell extract both antisera can efficiently block binding to the collagenase AP1 sequence (Fig. 7C). Therefore, proteins of the Fos and Jun family are present in IL-2-stimulated L2 cells, but they do not bind to the PCNA-CRE motif.

**(iv) Effect of site-specific mutagenesis of PCNA-CRE sites on PCNA promoter activity.** To examine the contribution of the tandem PCNA-CRE sites to PCNA promoter activity, three mutant PCNA promoters corresponding to CREm-1, CREm-2, and CREm-3 were prepared in the context of the pPCNA5-LUC construct (−182 to +143). Mutant PCNA promoter constructs were transfected into IL-2-stimulated L2 cells. Both CREm-1 and CREm-2 promoter mutation constructs reduce PCNA promoter activity (Fig. 8). In a single transfection experiment we tried, CREm-3 mutation construct also reduced PCNA promoter activity to 80% of that of pPCNA5 (data not shown). These data are consistent with the

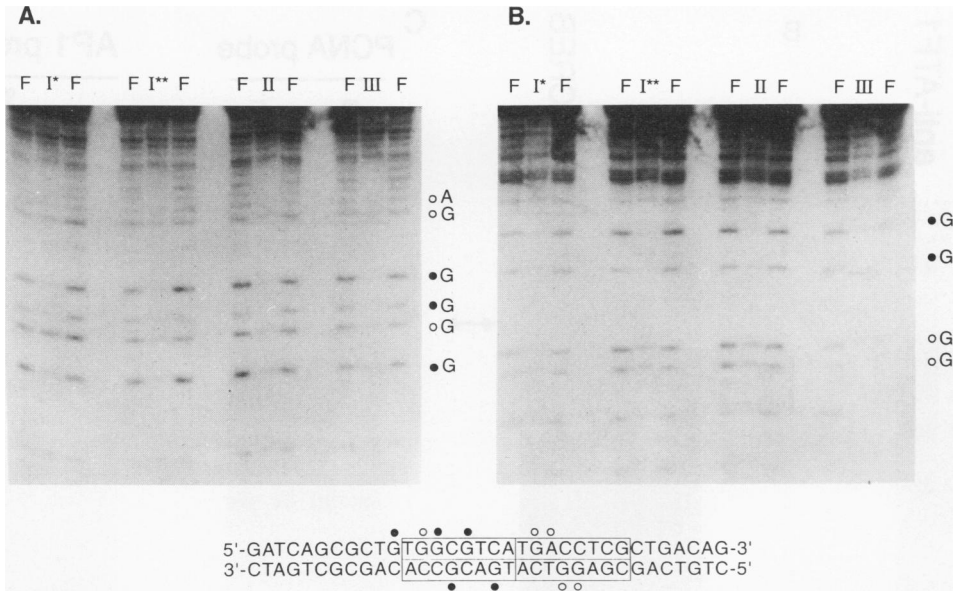



FIG. 6. Methylation interference analysis of the PCNA-CRE binding complexes. PCNA-CRE oligonucleotides of top (A) and bottom (B) strands were 5′ end labeled and annealed and then partially methylated with dimethyl sulfate. These probes were used in binding reactions with IL-2-stimulated L2 cell nuclear extract and electrophoresed on 4.5% polyacrylamide gels. Shifted bands corresponding to complexes I* (top half of complex I), I** (bottom half), II, and III were excised from the gels, as well as bands corresponding to the free-probe DNA. DNA eluted from the bands was cleaved with piperidine and resolved on 15% sequencing gels. Lanes F are free DNA, and lanes I*, I**, II, and III are bound DNAs from various CRE binding complexes. Summary of methylation interference analysis is shown at the bottom. Both the top and the bottom strand of PCNA-CRE oligonucleotides are shown, and the tandem CRE binding sites are boxed. G and A residues that interfere strongly or weakly with complex formation when methylated are indicated by solid or open circles, respectively.

results from in vitro binding data determined by competition binding experiments and methylation interference indicating that the tandem CRE sites are necessary for optimal promoter activity.

## DISCUSSION

Several putative binding sites for transcription factors can be identified in the 5′-flanking sequence of the murine PCNA gene (Fig. 2). There is significant homology between murine and human PCNA genes in this region (71); e.g., both contain CCAAT, CRE, AP2, octamer, and a cluster of Sp1 sites at similar locations within the first 500 bp upstream of the transcription start site. Both genes lack a canonical TATAA box, and both contain a region 3′ to the cap site which has homology to the TdT initiator sequence (45, 72). The conservation of these potential protein binding sequences between species suggests that they may regulate PCNA transcription during cellular proliferation.

**Cell type-specific utilization of the PCNA promoter.** Using continuously proliferating cells, Morris and Mathews showed that 249 nt of 5′-flanking sequence of the human PCNA gene are sufficient for full promoter activity in HeLa cells, whereas only 172 nt are needed in 293 cells in which adenovirus E1A is expressed constitutively (51). EL4 cells appear to be similar to HeLa cells in that they require more than 182 nt of murine 5′-flanking sequence for full promoter activity. When promoter activity in EL4 cells is compared with activity in IL-2-stimulated L2 cells, additional differences are observed. In L2 cells, pPCNA6-LUC (−503 to +143) resulted in only 72% of the luciferase activity of pPCNA5 (−182 to +143), suggesting that a negative regulatory element exists between −503 and −182. This is consistent with the results obtained by Travali et al. (73) with BHK (baby hamster kidney) cells transfected with the human PCNA gene. They observed a negative effect on PCNA promoter activity between nt −560 and −397. Taken together, these data argue for differential PCNA promoter utilization in both continuously dividing and growth factor-stimulated cells.

**Regulation of growth factor-stimulated PCNA expression.** Previous studies have shown that PCNA RNA and protein are barely detectable in quiescent lymphocytes and fibroblasts; stimulation of lymphocytes with IL-2 and of fibroblasts with serum-derived growth factors induces a rapid increase in PCNA expression (30, 69). The increase in PCNA RNA levels in IL-2-stimulated lymphocytes results from an increase in transcription and an increase in RNA stability (30, 70). In contrast, using a human PCNA promoter in murine 3T3 cells, Chang et al. (12) found that the human PCNA promoter was as active in serum-deprived cells as in serum-stimulated cells, leading them to conclude that serum-derived growth factor-stimulated increases in PCNA RNA levels are posttranscriptionally regulated (56). Additional studies by Baserga and coworkers have suggested a transcriptional pause or splicing block as a potential mechanism for regulating PCNA RNA accumulation in 3T3 cells (1, 12). Results reported herein demonstrate that the 182-bp murine PCNA promoter confers IL-2-stimulated growth responsiveness upon a heterologous reporter gene. These data confirm our previous finding that transcriptional regulation is, in part, responsible for IL-2-induced PCNA expression in lymphocytes and suggest that different regulatory mechanisms might be adopted in controlling PCNA gene expression in different cell types, such as lymphocytes and fibroblasts.

At least two *cis*-acting elements are required for IL-2-induced PCNA promoter activity within the 182-bp promoter, on the basis of the fold increase observed between unstimulated and IL-2-stimulated L2 cells (Table 1). The increase in

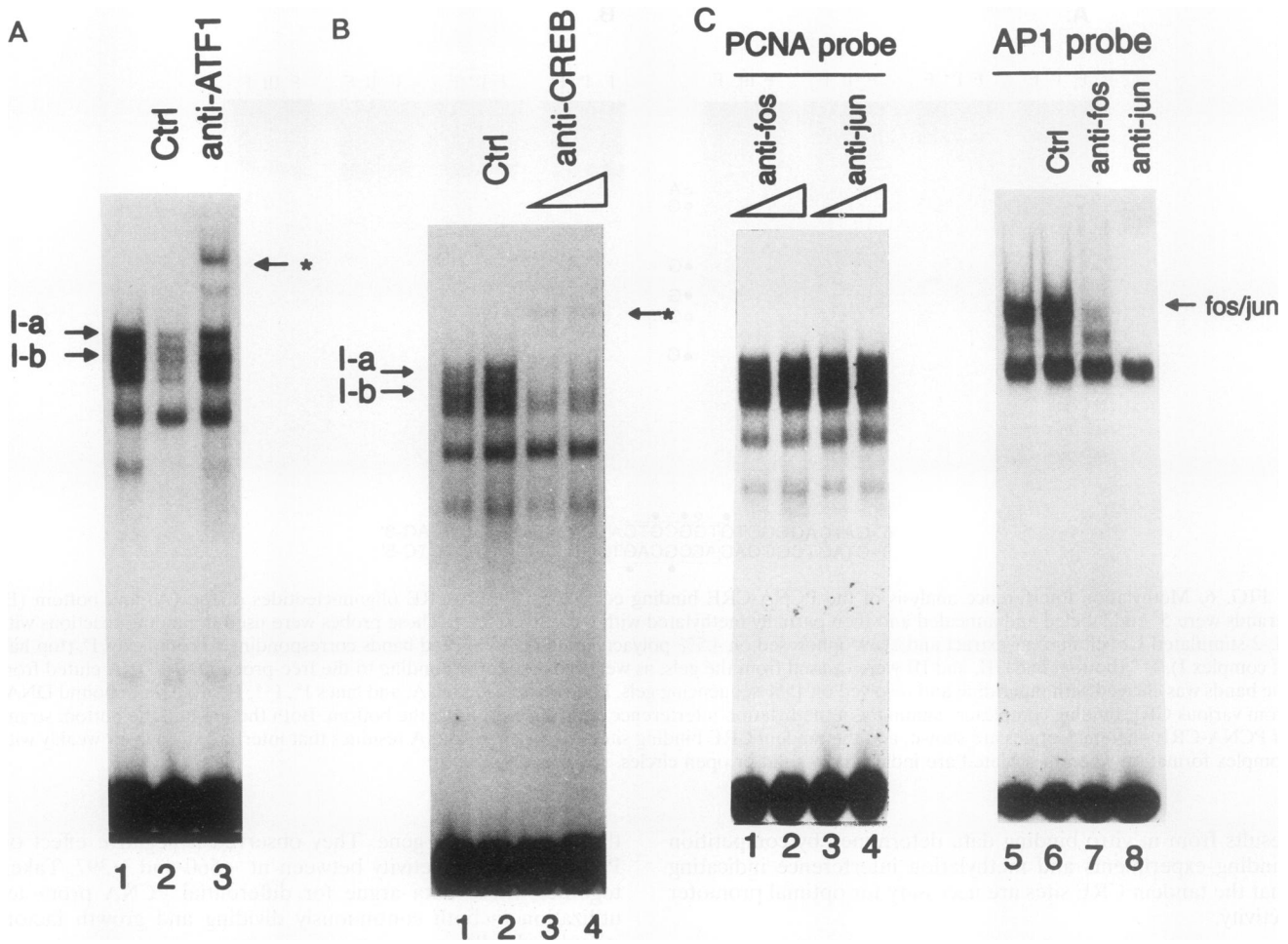



FIG. 7. CREB and ATF1 are present in PCNA-CRE binding complex I. (A) Gel mobility shift assay with L2 cell extract and monoclonal antibody against ATF1 protein. Ctrl, control ascites fluid containing a nonrelated monoclonal antibody (anti-Thy1) (lane 2). The second band in complex I (I-b) and complex III were specifically supershifted by the anti-ATF1 antibody (lane 3) but not by the control antibody (lane 2) in comparison with the complexes detected with extracts alone (lane 1). The position of the supershifted complex is indicated by an asterisk. (B) Gel mobility shift assay with L2 cell extract and anti-CREB antiserum. One or three microliters of anti-CREB antiserum (R-1090) was added (lanes 3 and 4). Normal rabbit serum was used as a control (Ctrl). Both of the top two bands (I-a and I-b) were supershifted (indicated by asterisk) (lanes 3 and 4) in comparison with the complexes detected with L2 cell extract alone (lane 1). (C) Gel mobility shift assay with L2 cell extracts and antiserum against Fos and Jun family proteins. One or three microliters of anti-Fos or anti-Jun antiserum was added (lanes 1 to 4). AP1 probe was used to show the existence of Fos-Jun binding complexes (lanes 5 to 8) in the same L2 cell extract preparation. Normal rabbit serum was used as a control (Ctrl).

promoter activity dropped from 85-fold to 42-fold when the CRE motifs located between −80 and −40 were deleted, indicating that these elements contribute to IL-2 responsiveness. Since promoter activity of pRSV-LUC also increases in response to IL-2, it appears that the induced promoter activity (including PCNA and Rous sarcoma virus) may not be IL-2 specific but growth dependent. Because there was a 42-fold increase in promoter activity with pPCNA1-LUC (−40 to +143), there should be an element(s) in this region that confers IL-2-stimulated growth responsiveness. Within this region, there are three recognizable elements: (i) an initiator-like element at the transcription start site, (ii) a PEA3 binding site (5′-AGGAAGT-3′) immediately adjacent to the transcription initiation site between −13 and −7 (6 of 7 nt match), and (iii) a sequence similar to an E2F binding site (5′-TT TCGCGC-3′ [52]) at +10 to +17. The PEA3 motif was first identified in the polyomavirus enhancer. It has been shown to be the primary component of the polyomavirus late transcription initiator element (80) and has been found in and around the transcription initiation sites of many cellular and viral promoters lacking the TATAA motif. The E2F site has been found in several genes which encode proteins that are essential for S phase, such as dihydrofolate reductase, thymidine kinase, and DNA polymerase α. The E2F motif of the dihydrofolate reductase gene has been shown to be the initiator element and to be required for efficient expression of the dihydrofolate reductase gene in vivo (6, 46). It has been postulated that E2F may function as a transcription factor that regulates coordinate expression of these DNA synthesis genes and may represent a major regulator of cellular proliferation during the $G_1$/S transition. Recent experiments show that E2F is able to complex with another key cell cycle control protein, the retinoblastoma susceptibility gene product (pRB). It is thought that pRB suppresses E2F function as a transcription factor by direct interaction with E2F (13, 53). Thus, during DNA tumor virus infection, viral oncoproteins activate viral transcription by

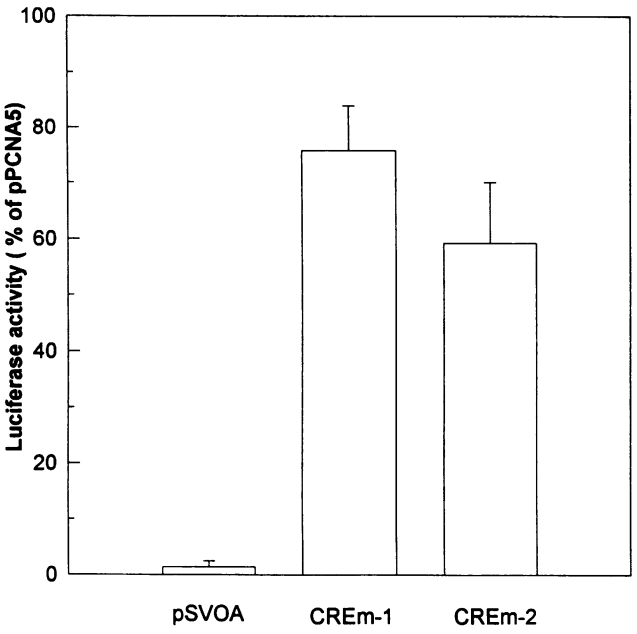



FIG. 8. Effect of mutations at CRE binding sites on PCNA promoter activity. pSVOA-LUC, pPCNA5-LUC, CREm-1, and CREm-2 constructs were transiently transfected into L2 cells by the DEAE-dextran method. The experiments were performed in duplicate and repeated at least three times. Luciferase activity has not been normalized to luciferase DNA content. Luciferase activities of CRE mutation constructs were compared with pPCNA5-LUC (designated as 100%) and expressed as relative promoter activity (percent).

releasing E2F from the inactive E2F-pRB complex and cause the cellular transforming phenotype. While the functional importance of the PEA3 and E2F binding sites in the PCNA promoter needs to be determined, these elements may function to assist the formation of the transcription initiation complex and contribute to IL-2-stimulated PCNA transcriptional activation.

**ATF1 and CREB bind to the CRE motif in the mouse PCNA promoter.** Our data suggest that CRE binding sites in the 5′-flanking sequence are important for optimal PCNA promoter activity and IL-2-stimulated transcriptional activation since promoter activity is markedly decreased when the CRE sequences are removed or mutated. In addition, ATF1 and CREB protein bind specifically to the PCNA-CRE binding sites as shown by a gel mobility shift assay. The ability to form these specific complexes is induced during IL-2-stimulated T-cell proliferation. Mutation of either of the two CRE sites in the mouse PCNA promoter caused a reduction in PCNA promoter activity and altered DNA-protein interaction, further supporting their role in IL-2-induced PCNA promoter activity. Morris and Mathews showed that the corresponding CRE binding site in the human PCNA gene is required for adenovirus E1A-driven transactivation (50, 51). In addition, a sequence (PERE) immediately upstream of the CRE site was shown to be important for promoter activity and activation by E1A (37, 50, 51). Using an oligonucleotide derived from the murine PCNA promoter which contains both the CRE sequences and the PERE sequence, we did not detect binding to the PERE site in a methylation interference assay. Furthermore, we have shown recently that anti-ATF1 antibody blocks in vitro transcription from the murine PCNA promoter with HeLa cell extract (55), indicating that CREB-ATF family proteins are involved in the regulation of PCNA transcription.

The CRE binding site is found in a variety of cellular or viral genes and is required for basal promoter activity as well as inducible promoter activity (64). ATF1 and CREB are members of the CREB-ATF family of proteins, which is a multigene family composed of at least 10 different proteins (23). All of these proteins contain a basic region and a leucine zipper as their DNA binding domain. CREB is the best-characterized member in the family and encodes a 43-kDa nuclear protein (26, 48). CREB activates transcription of cyclic AMP (cAMP)-inducible genes in response to the change in cellular cAMP levels by binding to the CRE motif. The binding and transcriptional activation functions of CREB are modulated by phosphorylation catalyzed predominantly by cAMP-dependent protein kinase A (48, 79). In addition to homodimer formation, CREB can form heterodimers with other members of the family including ATF1, a 38-kDa protein with 75 to 80% sequence homology to CREB (24), or even with members of the Fos-Jun family (28). Although all ATF proteins can bind to CRE sites, they may be functionally distinguishable. For example, while ATF2 cannot support a cAMP-inducible transcription response like CREB, it can support E1A transcriptional activation (41). ATF2 also has been shown to mediate the pRb regulation of the expression of transforming growth factor β2 (33). Despite the homology of ATF1 to CREB, it has been reported that ATF1 responds poorly to cAMP and activation by protein kinase A and does not mediate E1A activation (19, 27). In addition to ATF1 and CREB, there appear to be other proteins which form PCNA-CRE-specific complexes, suggesting that other members of the CREB-ATF family may be involved in PCNA transcriptional regulation.

Activation of CREB-ATF family members by non-cAMP-protein kinase A pathways has been demonstrated (68). These alternative activation pathways may be extremely important in lymphocytes, since it is well known that cAMP or analogs of cAMP suppress T-lymphocyte proliferation (32). Also, the suppression by cAMP appears to be mediated by inhibiting IL-2-driven T-cell activation at a discrete point in $G_1$ (31, 75).

In summary, our data show that 182 bp of 5′-flanking sequence of the mouse PCNA gene are sufficient for full IL-2-inducible promoter activity in the cloned T lymphocyte L2. The tandem CRE elements are required for optimal IL-2-stimulated growth responsiveness, and two CREB-ATF family members, CREB and ATF1, and perhaps other unidentified CRE-binding proteins are present in IL-2-induced DNA-protein complexes. It is known that cell cycle progression from $G_1$ to S phase involves multiple steps and multiple regulatory components (58). One approach to study this complex system is the reverse approach, namely, working backwards from regulated events to the mechanisms that regulate them. In this report, CRE-binding proteins have been identified as potential regulators of PCNA transcription. Future studies will determine the mechanisms by which CREB-ATF family transcription factors are activated and mediate IL-2 signaling during $G_1$ activation and cell cycle progression in T lymphocytes.

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## REFERENCES


1. **Alder, H., M. Yoshinouchi, M. B. Prystowsky, P. Appasamy, and R. Baserga.** 1992. A conserved region in intron 1 negatively regulates the expression of the PCNA gene. Nucleic Acids Res. **20:**1769–1775.
2. **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.
3. **Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonhat, P. Herrlich, and M. Karin.** 1987. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell **49:**729–739.
4. **Ausubell, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, and J. G. Seidman.** 1992. Current protocols in molecular biology. John Wiley & Sons, New York.
5. **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.
6. **Blake, M. C., and J. C. Azizkhan.** 1989. Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene in vitro and in vivo. Mol. Cell. Biol. **9:**4994–5002.
7. **Brasier, A., J. E. Tate, and J. F. Habener.** 1989. Optimalization use of the firefly luciferase assay as a reporter gene in mammalian cell line. BioTechniques **7:**1116–1122.
8. **Bravo, R., and J. E. Celis.** 1980. A search for differential polypeptide synthesis throughout the cell cycle of HeLa cells. J. Cell Biol. **84:**795–802.
9. **Bravo, R., R. Frank, P. A. Blundell, and H. Macdonald-Bravo.** 1987. Cyclin/PCNA is the auxiliary protein of DNA polymerase delta. Nature (London) **326:**515–517.
10. **Bravo, R., and H. Macdonald-Bravo.** 1987. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. J. Cell Biol. **105:**1549–1554.
11. **Cantrell, D. A., and K. A. Smith.** 1984. The interleukin-2 cell system: a new cell growth model. Science **224:**1312–1316.
12. **Chang, C. D., L. Ottavio, S. Travali, K. E. Lipson, and R. Baserga.** 1990. Transcriptional and posttranscriptional regulation of the proliferating cell nuclear antigen gene. Mol. Cell. Biol. **10:**3289–3296.
13. **Chellappan, S., S. Hiebert, M. Mudryj, J. M. Horowitz, and J. R. Nevins.** 1991. The E2F transcription factor is a cellular target for the RB protein. Cell **65:**1053–1061.
14. **Clevenger, C., S. W. Altmann, and M. B. Prystowsky.** 1991. Requirement of nuclear prolactin for interleukin 2 stimulated proliferation of T lymphocytes. Science **253:**77–79.
15. **Deutsch, P. J., J. P. Hoeffler, J. L. Jameson, J. C. Lin, and J. F. Habener.** 1988. Structural determinants for transcriptional activation by cAMP-responsive DNA elements. J. Biol. Chem. **263:**18466–18472.
16. **de Wet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7:**725–737.
17. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
18. **Fathman, C. G., and F. W. Fitch.** 1982. Isolation, characterization, and utilization of T lymphocyte clones. Academic Press, New York.
19. **Flint, K. J., and N. C. Jones.** 1991. Differential regulation of three members of the ATF/CREB family of DNA binding proteins. Oncogene **6:**2019–2026.
20. **Fujita, T., H. Shibuya, T. Ohashi, K. Yamanishi, and T. Taniguchi.** 1986. Regulation of human interleukin-2 gene: functional DNA sequences in the 5′ flanking region for the gene expression in activated T lymphocytes. Cell **46:**401–407.
21. **Garner, N. M., and A. Rezvin.** 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose operon regulatory system. Nucleic Acids Res. **9:**3047–3060.
22. **Gregor, P. D., M. Sawadogo, and R. G. Roeder.** 1990. The adenovirus major late transcription factor USF is a member of the helix-loop-helix group of regulatory proteins and binds to DNA as a dimer. Genes Dev. **4:**1730–1740.
23. **Habener, J. F.** 1991. Cyclic AMP response element binding proteins: a cornucopia of transcription factors. Mol. Endocrinol. **4:**1087–1094.
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. **Hardy, S., and T. Shenk.** 1988. Adenovirus control region activated by E1A and cAMP response element bind to the same factor. Proc. Natl. Acad. Sci. USA **85:**4171–4175.
26. **Hoeffler, J. P., T. E. Mayer, Y. Yun, J. L. Jameson, and J. F. Habener.** 1988. Cyclic AMP-responsive DNA binding protein: structure determined from a cloned placental cDNA. Science **242:**1430–1433.
27. **Hurst, H. C., N. F. Totty, and N. C. Jones.** 1991. Identification and functional characterization of the cellular activating transcription factor 43 (ATF43) protein. Nucleic Acids Res. **19:**4601–4609.
28. **Ivashkiv, L. B., H.-C. Liou, C. J. Kara, W. W. Lamph, I. M. Verma, and L. H. Glimcher.** 1990. mXBP/CRE-BP2 and c-Jun form a complex which binds to the cyclic AMP, but not to the 12-*O*-tetradecanoylphorbol-13-acetate, response element. Mol. Cell. Biol. **10:**1609–1621.
29. **Jaskulski, D., J. K. DeRiel, W. E. Mercer, B. Calabretta, and R. Baserga.** 1988. Inhibition of cellular proliferation by antisense oligonucleotides to PCNA/cyclin. Science **240:**1544–1546.
30. **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.
31. **Johnson, K. W., B. H. Davis, and K. A. Smith.** 1988. cAMP antagonizes interleukin 2-promoted T-cell cycle progression at a discrete point in early $G_1$. Proc. Natl. Acad. Sci. USA **85:**6072–6076.
32. **Kammer, G. M.** 1988. The cyclic-AMP-protein kinase A pathway and regulation of the immune response. Immunol. Today **9:**222–229.
33. **Kim, S. J., S. Wagner, F. Liu, M. A. O'Reilly, P. D. Robbins, and M. R. Green.** 1991. Retinoblastoma gene product activates expression of the human TGF beta gene through transcription factor ATF-2. Nature (London) **6:**2019–2026.
34. **Kovary, K., and R. Bravo.** 1991. The Jun and Fos protein families are both required for cell cycle progression in fibroblasts. Mol. Cell. Biol. **11:**4466–4472.
35. **Kronke, M., W. J. Leonard, J. M. Depper, and W. C. Greene.** 1985. Sequential expression of genes involved in human T lymphocyte growth and differentiation. J. Exp. Med. **161:**1593–1598.
36. **Ku, D.-H., S. Travali, B. Calabretta, K. Huebner, and R. Baserga.** 1989. Human gene for proliferating cell nuclear antigen has pseudogenes and localizes to chromosome 20. Somatic Cell Mol. Genet. **14:**297–307.
37. **Labrie, C., G. F. Morris, and M. B. Mathews.** 1993. A complex promoter element mediates transcription of the human proliferating cell nuclear antigen promoter by the 243-residue adenovirus E1A oncoprotein. Mol. Cell. Biol. **13:**1697–1707.
38. **Larsson, E.-L., N. N. Iscove, and A. Coutinho.** 1980. Two distinct factors are required for induction of T-cell growth. Nature (London) **283:**664–666.
39. **Lenardo, M., J. W. Pierce, and D. Baltimore.** 1987. Protein-binding sites in Ig gene enhancers determine transcriptional activity and inducibility. Science **236:**1573–1577.
40. **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.
41. **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.
42. **Madsen, P., and J. E. Celis.** 1985. S-phase patterns of cyclin (PCNA) antigen staining resemble topographical patterns of DNA synthesis. FEBS Lett. **193:**5–11.
43. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular

cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **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.
45. **Means, A. L., and P. J. Farnham.** 1990. Transcription initiation from the dihydrofolate reductase promoter is positioned by HIP1 binding at the initiation site. Mol. Cell. Biol. **10**:653–661.
46. **Means, A. L., J. E. Slansky, S. L. McMahon, M. W. Knuth, and P. J. Fernham.** 1992. The HIP binding site is required for growth regulation of the dihydrofolate reductase gene promoter. Mol. Cell. Biol. **12**:1054–1063.
47. **Miyachi, K., M. J. Fritzler, and E. M. Tan.** 1978. Autoantibody to a nuclear antigen in proliferating cells. J. Immunol. **121**:2228–2234.
48. **Montminy, M. R., and L. M. Bilezikjian.** 1987. Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature (London) **328**:175–178.
49. **Moore, K. S., K. Sullivan, E. M. Tan, and M. B. Prystowsky.** 1987. Proliferating cell nuclear antigen/cyclin is an interleukin 2-responsive gene. J. Biol. Chem. **262**:8447–8450.
50. **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.
51. **Morris, G. F., and M. B. Mathews.** 1990. Analysis of the proliferation cell nuclear antigen promoter and its response to adenovirus early region 1. J. Biol. Chem. **265**:16116–16125.
52. **Mudryj, M., S. W. Hiebert, and J. R. Nevins.** 1990. Role for the adenovirus inducible E2F transcription factor in a proliferation dependent signal transduction pathway. EMBO J. **9**:2179–2184.
53. **Nevins, J. R.** 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science **258**:424–429.
54. **Ohashi, Y., Y. Sawada, T. Moriuchi, and K. Fujimaga.** 1992. Analysis of the 5′ flanking region of the rat proliferation cell nuclear antigen (PCNA) gene. Biochim. Biophys. Acta **1130**:175–181.
55. **Orten, D. J., S. H. Hinrichs, and D. Sanderson.** Unpublished data.
56. **Ottavio, L., C.-D. Chang, M. G. Rizzo, S. Petralia, S. Travali, and R. Baserga.** 1990. The promoter of the proliferating cell nuclear antigen (PCNA) gene is active in serum-deprived cells. Biochem. Biophys. Res. Commun. **169**:509–516.
57. **Ottavio, L., C.-D. Chang, M. G. Rizzo, S. Travali, C. Casadevall, and R. Baserga.** 1990. Importance of introns in the growth regulation of mRNA levels of the proliferating cell nuclear antigen gene. Mol. Cell. Biol. **10**:303–309.
58. **Pardee, A. B.** 1989. G1 events and regulation of cell proliferation. Science **246**:603–608.
59. **Prelich, G., and B. Stillman.** 1988. Coordinated leading and lagging strand synthesis during SV40 DNA replication in vitro requires PCNA. Cell **53**:117–126.
60. **Prelich, H. A.** 1993. PCR technology: principles and applications for DNA amplification. Stockton Press, New York.
61. **Prystowsky, M. B.** 1989. Interleukin-2-driven cloned T-cell proliferation, p. 95–104. *In* R. Baserga (ed.), Cell growth and division. IRL Press, Oxford.
62. **Prystowsky, M. B., J. M. Ely, D. I. Beller, L. Eisenberg, J. Goldman, M. Goldman, E. Goldwasser, J. Ihle, J. Quintans, H. Remold, S. N. Vogel, and F. W. Fitch.** 1982. Alloreactive cloned T cell lines VI. Multiple lymphokine activities secreted by helper and cytolytic cloned T lymphocytes. J. Immunol. **129**:2337–2344.
63. **Prystowsky, M. B., G. Otten, S. K. Pierce, J. Shay, J. Olshan, and F. W. Fitch.** 1985. Lymphokine production by cloned T lymphocytes. Lymphokines **12**:13–38.
64. **Roesler, W. J., G. R. Vandenbark, and R. W. Hanson.** 1988. Cyclic AMP and induction of eukaryotic gene transcription. J. Biol. Chem. **263**:9063–9066.
65. **Rosenberg, S. A., E. A. Grimm, M. McGrogan, M. Doyle, E. Kawasaki, K. Koths, and D. F. Mark.** 1984. Biological activity of recombinant human interleukin-2 produced in Escherichia coli. Science **223**:1412–1415.
66. **Sabath, D. E., D. S. Monos, S. C. Lee, C. Deutsch, and M. B. Prystowsky.** 1986. Cloned T-cell proliferation and synthesis of specific proteins are inhibited by quinine. Proc. Natl. Acad. Sci. USA **83**:4739–4743.
67. **Sabath, D. E., P. Podolin, P. G. Comber, and M. B. Prystowsky.** 1990. cDNA cloning and characterization of interleukin 2-induced genes in a cloned T helper lymphocyte. J. Biol. Chem. **265**:12671–12678.
68. **Sheng, M., M. A. Thompson, and M. E. Greenberg.** 1992. CREB: a $Ca^{2+}$-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science **252**:1427–1430.
69. **Shipman, P. M., D. E. Sabath, A. H. Fischer, P. G. Comber, K. Sullivan, E. M. Tan, and M. B. Prystowsky.** 1988. Cyclin mRNA and protein expression in recombinant interleukin 2-stimulated cloned murine T lymphocytes. J. Cell. Biochem. **38**:189–198.
70. **Shipman-Appasamy, P., K. Cohen, and M. B. Prystowsky.** 1990. Interleukin 2-induced expression of proliferating cell nuclear antigen is regulated by transcriptional and post-transcriptional mechanisms. J. Biol. Chem. **265**:19180–19184.
71. **Shipman-Appasamy, P. M., K. S. Cohen, and M. B. Prystowsky.** 1991. Nucleotide sequence of murine PCNA: interspecies comparison of the cDNA and the 5′ flanking region of the gene. J. DNA Sequencing Mapping **2**:181–191.
72. **Smale, S. T., and D. Baltimore.** 1989. The "initiator" as a transcription control element. Cell **57**:103–113.
73. **Travali, S., D.-H. Ku, M. G. Rizzo, and L. Ottavio.** 1989. Structure of the human gene for the proliferating cell nuclear antigen. J. Biol. Chem. **264**:7466–7472.
74. **Waeber, G., T. E. Meyer, M. Lesieur, H. L. Hermann, N. Gerard, and J. F. Habener.** 1991. Developmental stage specific expression of cyclic adenosine 3′,5-monophosphate response element-binding protein CREB during spermatogenesis involves alternative exon splicing. Mol. Endocrinol. **91**:1418–1430.
75. **Wang, T., J. R. Sheppard, and J. E. Foker.** 1991. Rise and fall of cyclic AMP required for onset of lymphocyte DNA synthesis. Science **201**:155–157.
76. **Wong, R. L., M. E. Katz, K. Ogata, E. M. Tan, and S. Cohen.** 1987. Inhibition of nuclear DNA synthesis by an autoantibody to proliferating cell nuclear antigen/cyclin. Cell. Immunol. **110**:443–448.
77. **Xiong, Y., H. Zhang, and D. Beach.** 1992. D type cyclins associated with multiple protein kinases and DNA replication and DNA repair factor PCNA. Cell **71**:505–514.
78. **Yamaguchi, M., Y. Nishida, T. Moriuchi, F. Hirose, C.-C. Hui, Y. Suzuki, and A. Matsukage.** 1990. *Drosophila* proliferating cell nuclear antigen (cyclin) gene: structure, expression during development, and specific binding of homeodomain proteins to its 5′-flanking region. Mol. Cell. Biol. **10**:872–879.
79. **Yamamoto, K. K., G. A. Gonzalez, W. H. Biggs, and M. R. Montminy.** 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature (London) **334**:494–498.
80. **Yoo, W., M. E. Matin, and W. R. Folk.** 1991. PEA1 and PEA3 enhancer elements are primary components of the polyomavirus late transcription initiator element. J. Virol. **65**:5391–5400.