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Transcriptional and Posttranscriptional Regulation of the Proliferating Cell Nuclear Antigen Gene

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The steady-state mRNA levels of the proliferating cell nuclear antigen (PCNA) gene are growth regulated. In a previous paper (L. Ottavio, C.-D. Chang, M. G. Rizzo, S. Travali, C. Casadevall, and R. Baserga, Mol. Cell. Biol. 10:303–309, 1990), we reported that introns (especially intron 4) participate in growth regulation of the PCNA gene. We have now investigated the role of the 5′-flanking sequence of the human PCNA gene stably transfected into BALB/c 3T3 cells. Promoters of different lengths (from −2856 to −45 upstream of the cap site) were tested. All promoters except the *Aat*II promoter (−45), including a short *Hpa*II promoter (−210), were sufficient for a response to serum, platelet-derived growth factor, and to a lesser extent epidermal growth factor. No construct responded to insulin or platelet-poor plasma. The *Aat*II promoter had little detectable activity. Transcriptional activity was also determined in BALB/c 3T3 cells carrying various constructs of the human PCNA gene by two methods: run-on transcription and reverse transcription-polymerase chain reaction (the latter measuring the heterogeneous nuclear RNA [hnRNA] steady-state levels). There was very little difference in the rate of transcription of the PCNA gene between G_0 cells and serum-stimulated cells, although the levels of hnRNA were much higher after stimulation. In G_0 cells carrying a human PCNA gene without introns 4 and 5, both transcription rate and hnRNA levels were high. Together with data on the mRNA half-life, these results suggest a posttranscriptional component in the regulation of PCNA mRNA levels after serum stimulation but a transcriptional regulation by intron 4.

Proliferating cell nuclear antigen (PCNA) is the name for a nuclear protein that has been identified as the cofactor of DNA polymerase delta (2, 27), an important component of cellular DNA replication (12, 43). PCNA is a protein necessary for cellular DNA synthesis (2, 25–27, 44) and for cell cycle progression (14, 21). cDNAs for human PCNA have been cloned by Almendral et al. (1) and Jaskulski et al. (15). More recently, Travali et al. (39) molecularly cloned the gene for human PCNA, sequenced approximately 7 kilobases of it, and showed that a functional promoter is present in the 5′-flanking region. The human PCNA gene localizes to chromosome 20; there is one pseudogene on chromosome X and possibly a second pseudogene on chromosome 6 (17).

The PCNA mRNA is growth regulated (1, 15). In continuously dividing HeLa cells, both the PCNA mRNA and the protein itself vary little in amounts throughout the cell cycle (23). However, in G_0 cells, PCNA mRNA levels are low and rapidly increase when cells are stimulated to proliferate by growth factors, reaching a peak at 16 to 18 h after stimulation (1, 15). In a previous report (24), we showed that intron 4 is necessary for the proper regulation of PCNA mRNA levels in G_0 cells. Removal of intron 4 leads to abnormally high levels of PCNA mRNA in serum-deprived cells (24). In this study, we have attempted to elucidate further the roles of the 5′-flanking region and intron 4 in regulating expression of the human PCNA gene stably transfected into BALB/c 3T3 cells. Specifically, this report addresses three topics: (i) the role of the 5′-flanking sequence of the human PCNA gene in determining the response of the gene to serum, individual growth factors, and a combination of growth factors, (ii) the rate of transcription and the levels of PCNA heterogeneous nuclear RNA (hnRNA) in quiescent cells and in cells stimulated to proliferate, and (iii) the effect of intron 4 on transcription and mRNA stability.

MATERIALS AND METHODS

Plasmids. The full-length human PCNA gene is the *Bam*HI genomic fragment described and sequenced by Travali et al. (39). It includes 2.8 kilobase pairs (kbp) of the 5′-flanking sequence, six exons, five introns, and 400 bp of 3′ untranslated region.

The *Bam*HI PCNA construct without introns 4 and 5 has been described previously (24). It is a fragment that contains the 2.8-kbp PCNA promoter driving the PCNA gene until the *Bgl*II site at +2343 and then ligated to the PCNA cDNA sequence from the *Bgl*II site to the 3′ end. The construct was cloned in the *Bam*HI site of the polylinker of pGEM3. This plasmid contains the full coding sequence of the PCNA gene plus introns 1, 2, and 3 but without introns 4 and 5.

The plasmids carrying the full-length human PCNA gene but with promoters of different lengths (Fig. 1) were constructed by using the plasmids previously described by Travali et al. (39) and Ottavio et al. (24) as follows.

*Eco*RI-Full PCNA is a deletion mutant of the PCNA promoter from the *Eco*RI site (−1257 bp) driving the full-length PCNA gene. The *Bam*HI-Full PCNA was digested with *Sal*I (in the polylinker) and *Nru*I (at position +61) to eliminate the fragment containing the promoter. We then isolated a *Sal*I-*Nru*I fragment from the *Eco*RI minigene of PCNA (24). This fragment, containing the *Eco*RI PCNA promoter, was ligated to the PCNA gene starting at the *Nru*I restriction site.

*Eco*RV-Full PCNA, *Hin*dIII-Full PCNA, and *Pvu*II-Full PCNA are deletion mutants of the PCNA promoter from the *Eco*RV (−789), *Hin*dIII (−557), and *Pvu*II (−395) sites, respectively, driving the full PCNA gene. We prepared the

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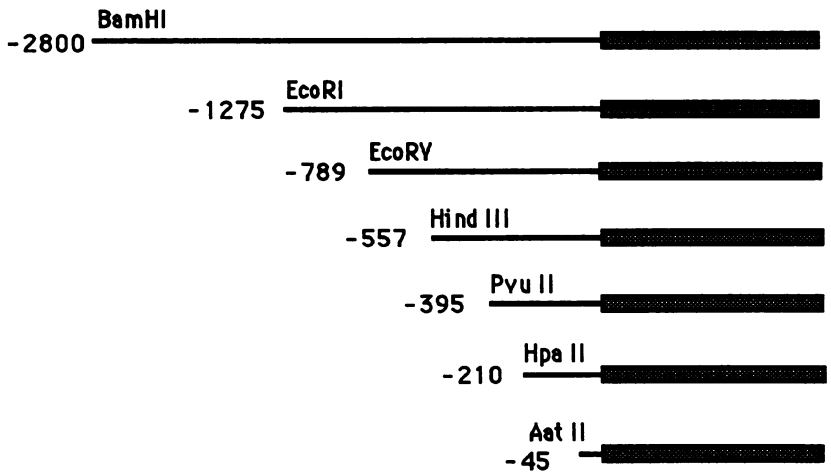

FIG. 1. PCNA promoters used to drive the human PCNA constructs.

same *Nru*I-*Sal*I fragment from the *Bam*HI-Full PCNA plasmid as in the *Eco*RI-Full PCNA construct and ligated it to *Sal*I-*Nru*I fragments containing various lengths of promoter from the *Eco*RV, *Hin*dIII, or *Pvu*II minigene (24). These minigene constructs were all derived from the *Eco*RI minigene by elimination of the *Xho*I-*Aat*II fragment, which contains the PCNA promoter. The *Xho*I-*Aat*II fragments containing various lengths of promoters from the *Eco*RV-thymidine kinase (TK), *Hin*dIII-TK, or *Pvu*II-TK construct (39) were ligated to it, creating the minigene constructs with promoters of different lengths.

*Hpa*II-Full PCNA is a deletion mutant of the PCNA promoter starting from the *Hpa*II site (−210) and driving the full PCNA gene. We isolated the same *Nru*I-*Sal*I fragment from the *Bam*HI-Full PCNA plasmid and ligated it to the *Xho*I-*Nru*I fragment from the *Hpa*II-TK plasmid described below.

The *Aat*II-Full PCNA is a deletion mutant of the PCNA promoter from the *Aat*II site (−45) driving the full-length PCNA gene. The *Bam*HI-Full PCNA plasmid was digested with *Aat*II (one site is in the promoter; the other site is on the vector), the fragment containing the promoter upstream of the *Aat*II site was eliminated, and the remaining plasmid was religated.

Cell lines. BALB/c 3T3 cells were grown as previously described (14, 15). The various derivative cell lines were obtained by transfecting BALB/c 3T3 cells in suspension (32) with one of the desired constructs. Each construct was cotransfected with plasmid pLHL4 (10), which contains the bacterial gene for hygromycin B resistance. The cells were then selected in hygromycin, and the resultant clones were analyzed for the presence of human PCNA mRNA and then investigated as described in Results. The cell lines carrying the *Bam*HI full-length PCNA gene and *Bam*HI PCNA gene without introns 4 and 5 are the same as described by Ottavio et al. (24). Cells were made quiescent with 1% fetal calf serum (FCS) for 4 days. The individual growth factors were added directly to the low-serum medium.

RNA extraction and RNA blots. Total RNA was extracted from cells by the method of Chomczynski and Sacchi (3), and RNA blots were carried out by standard procedures (38). Radioactive probes were prepared by the random priming method (7). The following probes were used: (i) the full-length PCNA cDNA (15), (ii) a human-specific 5′ PCNA probe extending from the *Bam*HI at the cDNA cap site to the *Ava*II site at +178 of the cDNA (24) (it includes the 5′ untranslated region plus 20 bp of coding sequence), and (iii) the histone H3 probe previously described (13). To monitor the amount of RNA in each lane, we used a mouse RNA probe that is not growth regulated. Since the RNA amounts in different lanes did not vary by more than 10 to 20%, this control has been omitted from the results.

Run-on transcription. Run-on transcription was carried out exactly as described by Groudine et al. (11). After autoradiography, the slots were counted in a liquid scintillation counter. Lambda bacteriophage DNA was used as a background. The probes have been described above.

RT-PCR. The reverse transcription (RT) and polymerase chain reaction (PCR) amplification techniques used were similar to published methods (28–30). Details of the reaction as used in our laboratory are given by Lipson and Baserga (18). DNA was rigorously excluded from the RT-PCR by centrifugation of the RNA in a cesium chloride gradient (18). The amplimers used for the amplification were as follows: HC-PCNA-1 (primer from the 5′ end of exon 1 of the PCNA gene), AAACTAGCTAGACTTTCCTC; HC-PCNA-2 (primer from intron 1 of the PCNA gene), CTTTCTCACTTTG GCGCGAA; HC-PCNA-3 (primer from the 3′ end of exon 1 of the PCNA gene), CAACCTGGCCATGGGCGTGA; HC-PCNA-5 (primer from exon 5), GCACTGAGGTACCT GAACTT; and HC-PCNA-6 (from exon 6), TCTTCA TCCTCGATCTTGGG. The amplification products were then subjected to Southern blotting (35), and the transferred DNA was hybridized to the desired probes.

RT-PCR probes. The intron-specific probe was a *Sma*I fragment in the middle of intron 1 of the human PCNA gene (39). For intron 5, we used the 30-mer sequence GAACCT TGTTTTGATGGTAGTCATATGTGA. In one experiment, we also used as a probe the full-length PCNA cDNA. The specific activities of the probes were approximately 10^9 dpm/μg.

RESULTS

In a previous report (24), we showed that the human PCNA gene transfected into BALB/c 3T3 cells is correctly regulated in different growth conditions provided that (i) a 5′-flanking sequence is present extending to the *Bam*HI restriction site (−2856 bp from the preferred cap site) and (ii) intron 4 is present. Removal of intron 4 results in abnormally

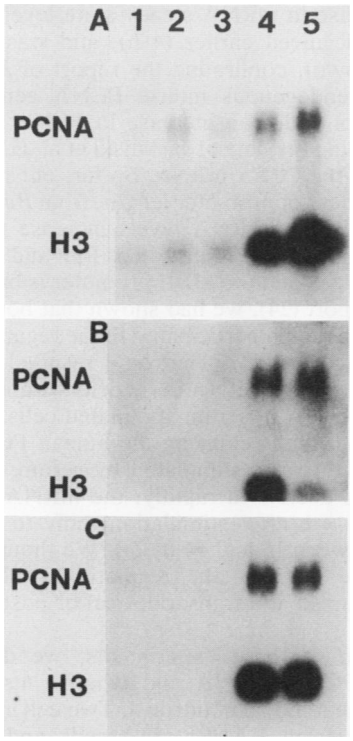

FIG. 2. Composite RNA blots from BALB/c 3T3 cells transfected with the human PCNA gene driven by promoters of different lengths. The cells were made quiescent by serum deprivation and subsequently stimulated with 10% FCS. Lanes: 1, G_0 cells; 2 to 5, cells serum stimulated for 4, 8, 16, and 24 h, respectively. The RNA blots were hybridized to a histone H3 probe and to a 5′ PCNA probe that recognizes only the human PCNA mRNA (see text). (A) Cells carrying a full-length human PCNA gene driven by the *Bam*HI promoter; (B) 3T3 cells carrying a similar construct but with a *Pvu*II promoter; (C) cells with a *Hpa*II promoter. Lengths of the promoters are given in Fig. 1.

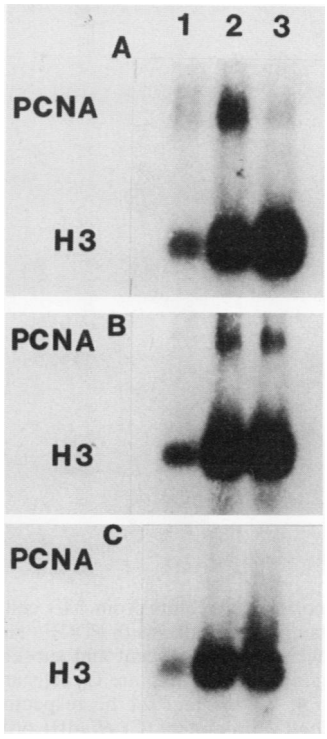

FIG. 3. Composite RNA blots from 3T3 cells carrying the human PCNA gene driven by promoters of different lengths. The experiments were carried out as for Fig. 2 except that the cells carried different constructs of the human PCNA gene. The 0- and 4-hr results have been omitted. Lanes 1 to 3 are RNAs from cells stimulated for 8, 16, and 24 h, respectively, with serum. (A) *Eco*RV promoter and full-length human PCNA gene; (B) the same gene driven by the *Hin*dIII promoter; (C) the same gene driven by the *Aat*II promoter. Lengths of the promoters are shown in Fig. 1.

high levels of PCNA mRNA in G_0 cells. The first series of experiments addresses the role of the 5′-flanking region of the human PCNA gene in the response to growth factors. For this purpose, we have used promoters of different lengths to drive the full-length human PCNA gene (six exons and five introns; 39).

Serum stimulation and the 5′-flanking region of the human PCNA gene. Cell lines (mixed populations) were established from BALB/c 3T3 cells by cotransfection of the desired construct (human PCNA gene under the control of different-length promoters) with a selectable marker (the bacterial gene for hygromycin B resistance;10). The various promoters have been described previously (24, 39) and above. The lengths of the promoters are summarized in Fig. 1. Cells carrying the different constructs were made quiescent in 1% serum and subsequently stimulated with 10% FCS. Total RNA was extracted, and the RNA blots were hybridized to two probes: the histone H3 probe, which is specific for S-phase cells (13), and the *Bam*HI-*Ava*II probe of the 5′ end of the human PCNA cDNA, which is specific for human PCNA and does not recognize the mouse PCNA mRNA (24).

With all constructs used, the results were qualitatively similar (Fig. 2 and 3). The levels of human PCNA mRNA were negligible in G_0 cells and increased in serum-stimulated cells, reaching a peak at 16 to 24 after serum stimulation. The levels of expression were quantitated by densitometry readings on the 16-h samples and are summarized in Table 1, where they are standardized with respect to the levels of histone H3 expression, which is a reasonable measure of the response of the cells to stimulation. The *Bam*HI and *Eco*RI promoters were the strongest, followed closely by the *Pvu*II, *Hpa*II, and *Eco*RV promoters. As reported previously (39), the *Hin*dIII promoter was less efficient, and the *Aat*II promoter was barely active, although after prolonged overexposure, it could still be shown to be growth regulated. The *Eco*RI promoter is the only promoter not shown in Fig. 2 and 3. In repeated experiments, the *Eco*RV promoter showed a

TABLE 1. Densitometry measurements of PCNA expression 16 h after serum stimulation[a]

Promoter	PCNA RNA
*Bam*HI	4.2
*Eco*RI	3.8
*Eco*RV	2.2
*Hin*dIII	0.7
*Pvu*II	3.0
*Hpa*II	2.8
*Aat*II	<0.1

[a] 3T3 cells carrying full-length human PCNA genes under the control of different PCNA promoters were used as described in the legends to Fig. 2 and 3. The hybridization bands were quantitated by linear densitometry, taking as a reference the intensity of the histone H3 band. The numbers for PCNA mRNA are density units, with the value for histone H3 made equal to 9.

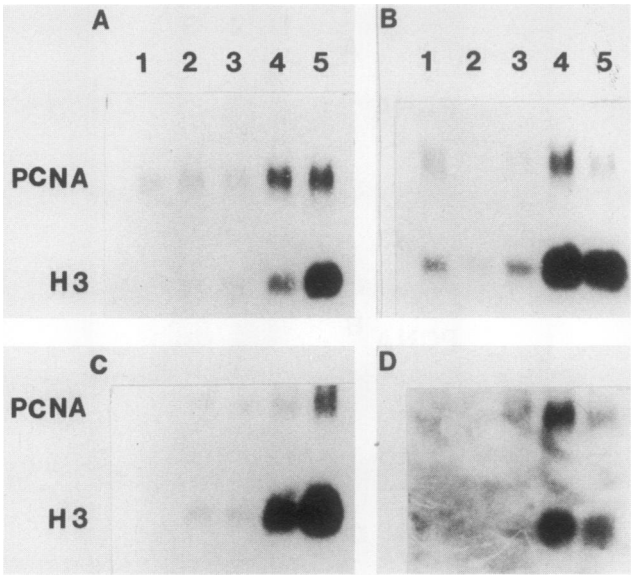

FIG. 4. Composite RNA blots from 3T3 cells carrying human PCNA constructs and stimulated with PDGF. The cell lines shown in Fig. 2 and 3 were made quiescent and subsequently stimulated with PDGF (1 ng/ml). Lanes 1 to 5 are G_0 cells and cells stimulated with PDGF for 4, 8, 16, and 24 h, respectively. (A) *Bam*HI promoter; (B) *Eco*RV promoter; (C) *Hin*dIII promoter; (D) *Hpa*II promoter.

marked decline in expression from 16 to 24 h after stimulation (compare this result with those for the other promoters in Fig. 2 and 3). We have not further investigated this peculiarity of the *Eco*RV promoter. We can only note that hnRNA levels were also lower at 24 than at 16 h after stimulation (see below).

The experiments shown in Fig. 2 and 3 were repeated with use of platelet-poor plasma (PPP) instead of FCS to stimulate quiescent cells. None of the constructs responded to PPP (data not shown), confirming the report by Jaskulski et al. (15) that the mouse PCNA gene is not inducible by PPP.

Response of the PCNA promoters to individual growth factors. The cell lines described above were then tested for their ability to respond to individual growth factors or combinations of growth factors. For instance, BALB/c 3T3 cells carrying the various constructs were made quiescent and then stimulated with platelet-derived growth factor (PDGF) at 1.0 ng/ml (Fig. 4). As before, the RNA blots were hybridized with both probes, histone H3 and the *Bam*HI-*Ava*II 5′ fragment of the human PCNA cDNA. All constructs responded to PDGF as efficiently as they responded to FCS (Fig. 4). Again, there was a sharp decline at 24 h in PCNA (but not in H3) expression in cells carrying the *Eco*RV promoter. The human PCNA mRNA was not detectable in cells carrying the construct with the *Aat*II promoter (not shown). However, when the same filter was probed with a full-length PCNA cDNA (which recognizes both human and mouse PCNA mRNA), hybridization bands were present (not shown), indicating that the cells were stimulated and the endogenous mouse PCNA gene had been expressed.

A combination of PDGF, epidermal growth factor (EGF), and insulin gave the same results as PDGF only regardless of whether the *Bam*HI or the *Hpa*II promoter was used (not shown).

EGF by itself induced expression of the transfected human PCNA gene regardless of the length of the promoter, but the increase in mRNA steady-state levels was of small magnitude, occurred earlier (4 h), and was not sustained (data not shown), confirming the report of Jaskulski et al. (15) on the endogenous mouse PCNA gene. Insulin (10 μg/ml) did not cause an increase in PCNA mRNA levels, again confirming the data of Jaskulski et al. (15) (not shown).

Stability of the PCNA mRNA. So far, our results indicate that (i) regardless of promoter length, from *Bam*HI to *Hpa*II, steady-state PCNA mRNA levels increase in response to FCS and PDGF but not to insulin or PPP and (ii) the *Hin*dIII promoter is weak and the *Aat*II promoter is barely active. In a previous report (24), we had shown that both intron 4 and the 5′-flanking region participated in the regulation of PCNA mRNA levels. Removal of intron 4 resulted in abnormally high levels of PCNA mRNA in G_0 cells without affecting the amounts of mRNA in serum-stimulated cells. Furthermore, when these 3T3 cells carrying the human PCNA construct without intron 4 were stimulated by serum, human PCNA mRNA levels decreased rapidly, the mRNA becoming undetectable by 4 h after stimulation, only to increase again markedly between 16 and 24 h (24). We thought it desirable to investigate whether the regulation of PCNA mRNA amounts occurred at a transcriptional or posttranscriptional level.

In the first of these experiments, we determined the stability of PCNA mRNA and whether its stability was affected by the removal of intron 4. Two cell lines were used, both derived from BALB/c 3T3 cells and described by Ottavio et al. (24). One had the human *Bam*HI promoter driving the full-length human PCNA gene; the second had a similar construct from which introns 4 and 5 had been removed (intron 5 is not important in the regulation of PCNA expression; 24). Serum-stimulated (S-phase) cells were incubated with dactinomycin (5 μg/ml). Total RNA was extracted at various times and blotted, and the blots were hybridized to the histone and PCNA probes described above. PCNA mRNA decayed with a half-life of 7 to 8 h, and it was no longer detectable 16 h after addition of dactinomycin (Fig. 5A and B). By densitometry, the intensity of the hybridization bands in cells with the full-length gene fell from 24 U at time zero to 14 U at 4 h after addition of dactinomycin to 10 U at 8 h. In cells carrying the construct without introns 4 and 5, the densitometry measurements were, respectively, 23, 17, and 6. Essentially, there was very little difference in mRNA stability regardless of whether intron 4 was removed. These results, however, do not explain the rapid disappearance of PCNA mRNA in serum-stimulated cells carrying the construct without intron 4 (24). We therefore determined mRNA stability in these latter cells in G_0. Cells were made quiescent and, without stimulation or after 1 h of serum stimulation, were exposed to dactinomycin (5 μg/ml). RNA was collected at various times, and the RNA blots are shown in Fig. 5 C and D. Clearly, the half-life of the human PCNA mRNA was shorter in G_0 than in S-phase cells. For instance, densitometry readings (with an integrated densitometer) decreased from 566 at time zero to 323 at 2 h to 93 at 4 h after addition of dactinomycin, giving a half-life of 2.5 h (Fig. 5C). Furthermore, when these cells were serum stimulated, the PCNA mRNA turned over even more rapidly, with a half-life of less than 1 h (again by densitometry, readings were, respectively, 523, 151, and 28).

No mRNA stability could be determined in G_0 cells carrying the full-length human PCNA gene (with all introns), because in these cells mRNA is not detectable in the G_0 state.

Run-on transcription. Using the method of Groudine et al.

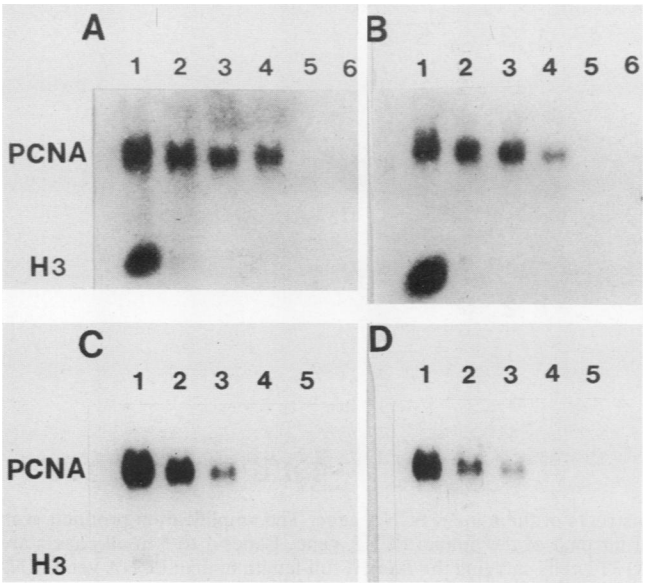

FIG. 5. Stability of the human PCNA mRNA. BALB/c 3T3 cells carrying the *Bam*HI full-length PCNA gene and BALB/c 3T3 cells carrying the same construct but without introns 4 and 5 (see text) were used. (A and B) Serum-stimulated (S-phase) cells exposed to dactinomycin (5 μg/ml). Lanes: 1, cells serum stimulated for 16 h; 2 to 6, cells 2, 4, 8, 16, and 24 h, respectively, after addition of dactinomycin. The 5′ human specific probe of the PCNA cDNA and the histone H3 probe were used for hybridization to the RNA blots. (A) Cells carrying the *Bam*HI full-length human PCNA gene; (B) cells carrying the *Bam*HI PCNA gene without introns 4 and 5. (C and D) Cells carrying the human PCNA gene without introns 4 and 5. (C) G_0 cells treated with dactinomycin at time zero; (D) same cells stimulated with serum for 1 h before addition of dactinomycin. Other conditions were as for panels A and B.

(11), we investigated the transcriptional rates of the human PCNA gene stably transfected into BALB/c 3T3 cell. Again, two cell lines were used, the one carrying the full-length human PCNA gene and the one carrying the same gene minus introns 4 and 5. Transcriptional rates were determined in G_0 cells and in cells stimulated with serum for 24 h. There was very little difference in transcriptional activity between G_0 cells and serum-stimulated cells regardless of whether intron 4 was present (290 and 320 cpm per slot, respectively, for cells carrying the full-length gene; 610 and 640 cpm per slot, respectively, for cells lacking intron 4). At the same time, transcriptional activity was higher in G_0 cells lacking intron 4 than in G_0 cells carrying the full-length human PCNA gene (610 versus 290 cpm per slot). This experiment was repeated twice, with the same results.

Levels of PCNA hnRNA under different growth conditions. RT-PCR (28, 29) allows a quantitative evaluation of the steady-state amounts of specific hnRNAs in cells under different physiological conditions (18). The amplimers and the probes we used for the human PCNA gene are diagrammed in Fig. 6 and described in Materials and Methods. We first determined the levels of PCNA hnRNA in BALB/c 3T3 cells carrying the human PCNA gene (Fig. 7). Two cell lines were used: the one carrying the full-length gene with a *Bam*HI promoter (Fig. 7A and B) and the one with a similar construct but without introns 4 and 5 (Fig. 7C). Cells were made quiescent and subsequently stimulated as usual. A moderate amount of PCNA hnRNA was detectable in G_0 cells (Fig. 7A, lane 1), and this amount increased sharply (16-fold) at 16 h but then decreased by 24 h, when most of the cells were still in S phase. Figure 7C shows the same experiment but with cells carrying the human PCNA gene from which introns 4 and 5 had removed. Clearly, PCNA hnRNA was present in high amounts in G_0 cells (at almost the same level as at 16 h after stimulation), and it increased (1.5-fold above the level of G_0 cells) only at 24 h. In these two experiments, the amplimers used were for exon 1 and intron 1, and the probe was also from intron 1. Figure 7B shows essentially the same experiment as in Fig. 7A except that the amplimers were from exons 5 and 6 and the probe was from intron 5. Again, there was some PCNA hnRNA even in quiescent cells, and the maximum was reached at 16 h after stimulation. Lane 6 in Fig. 7B is from exponentially growing, untransfected BALB/c 3T3 cells; confirming the specificity of the RT-PCR reaction, no signal was detectable. With the amplimers and probes used, no PCNA hnRNA was detectable in BALB/c 3T3 cells that did not carry the human PCNA gene (Fig. 7D). The same filter was subsequently hybridized to a full-length human PCNA cDNA (which can

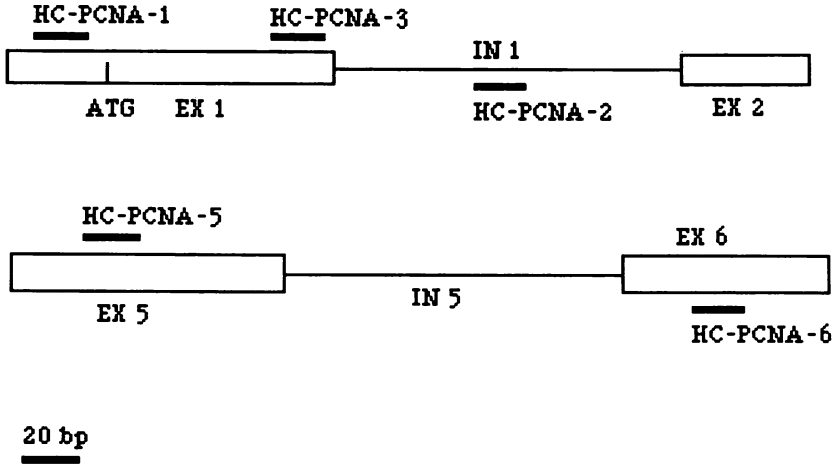

FIG. 6. Amplimers and probes used for RT-PCR. Shown is a partial diagram of the human PCNA gene which include exon 1, intron 1, and the beginning of exon 2. The probes used as amplimers are shown with their respective numbers. The sequences are given in Materials and Methods.

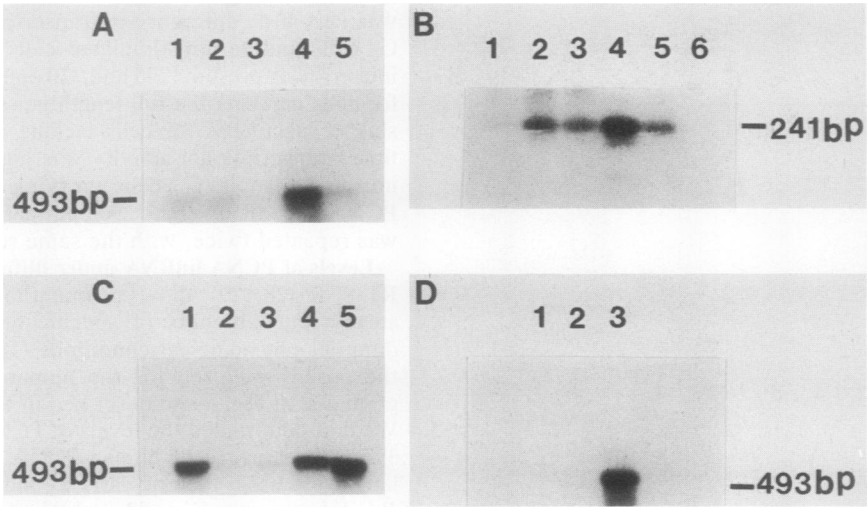

FIG. 7. RT-PCR of PCNA hnRNA in 3T3 cells carrying different constructs of the human PCNA gene. The amplification products were displayed on a Southern blot and hybridized with the *Sma*I fragment of intron 1 of the human PCNA gene. Lanes 1 to 5 in all panels are, respectively, G_0 cells and cells serum stimulated for 4, 8, 16, and 24 h. (A) 3T3 cells carrying the *Bam*HI full-length human PCNA gene; RNA amplified with amplimers 1 and 2; amplification products hybridized with a probe for human PCNA intron 1. (B) Same as panel A except that the amplimers were from PCNA exons 5 and 6 and the probe was from intron 5. Lane 6 is RNA from exponentially growing, untransfected BALB/c 3T3 cells treated in the same way as the RNAs from cells carrying the human PCNA construct. (C) Cells carrying the human PCNA gene with the *Bam*HI promoter but without introns 4 and 5. The probe is the same as for panel A. (D) Hybridization of the amplification products to the same probe as in panels A and C. Lane 3 is the same RNA sample as in lane 4 of panel B. Lanes 1 and 2 contain RNA from untransfected BALB/c 3T3 cells. In lane 1, the amplimers were 1 and 2; in lane 2, the amplimers were 3 and 2.

detect a mouse transcript), but no bands were visible in nontransfected 3T3 cells (not shown), indicating that the amplimers used were specific for human PCNA transcripts.

DISCUSSION

The mRNA levels of the PCNA gene are growth regulated (1, 15), as are those of other genes coding for proteins of the DNA-synthesizing machinery, such as the genes for TK (6, 15, 20, 37), DNA polymerase alpha (41), RNA primase (40), and thymidylate synthase (16). In continuously dividing cells such as HeLa cells, the mRNA levels of some of these genes (those encoding TK [33], DNA polymerase alpha [41], RNA primase [40], and PCNA [23]) vary little throughout the cell cycle. However, in quiescent fibroblasts or fibroblastlike cells in culture, the mRNA levels of these genes are very low or undetectable and increase sharply when serum-stimulated cells reach the G_1/S boundary. The same is true of mitogen-stimulated peripheral blood mononuclear cells (22, 34). This study is concerned with the regulation of expression of the PCNA gene in G_0 cells stimulated to proliferate; to analyze the mechanism, we chose to transfect the human PCNA gene (and its deletion mutants) in BALB/c 3T3 cells, which are exquisitely growth regulated (31, 36).

In previous reports, we had shown that (i) the PCNA *Pvu*II promoter at −395 from the cap site is sufficient to derive the expression of a heterologous gene (39) and (ii) intron 4 is necessary for the correct regulation of PCNA mRNA levels in G_0 cells. Removal of intron 4 leads to abnormally high levels of PCNA in G_0 cells (24).

The study presented here focused on the role of the 5′-flanking region (from *Bam*HI at −2856 to *Hpa*II at −210) and how growth factors and intron 4 affect the levels of PCNA mRNA and hnRNA. We will discuss first the regulation of the full-length human PCNA gene, i.e., the gene with all exons and introns. For growth factor regulation of mRNA levels, the *Hpa*II promoter (−210 from the cap site) is sufficient. Constructs with this promoter respond to serum, PDGF, and to a lesser extent EGF as does the endogenous mouse gene or the transfected human PCNA gene driven by its longest (molecularly cloned) 5′-flanking sequence, the *Bam*HI promoter (−2856). The *Aat*II promoter (−45) is essentially inactive. Interestingly, centered at about −60 in the human PCNA promoter is a sequence GGGCTCCGA CAG in which 8 of 11 nucleotides line up with the core sequence of the simian virus 40 enhancer (8). The strength of the *Hpa*II promoter (as measured by mRNA levels at 16 h after serum stimulation; Table 1) is 60% that of the *Bam*HI promoter but the same as that of the *Pvu*II promoter (−395) and higher than that of the *Eco*RV (−789) and *Hin*dIII (−557) promoters (Fig. 1 and Table 1). None of the PCNA constructs studied (as well as the endogenous mouse gene; 15) responded to insulin or PPP. The PCNA gene differs from the TK gene in that it is inducible by PDGF only (this paper; 15), whereas the TK gene requires at least a second growth factor, insulin, or insulinlike growth factor 1, or EGF (15). Both genes, however, are inhibited in G_1-specific temperature-sensitive mutants of the cell cycle stimulated at the restrictive temperature (15, 19, 20) and by low concentrations of cycloheximide (6, 15, 19). The transient expression of PCNA mRNA induced by EGF (without concomitant DNA synthesis) suggests that stability of PCNA mRNA may play a role in activation of the DNA-synthesizing machinery.

Run-on transcription experiments clearly show that the full-length human PCNA gene is transcribed at the same rates in G_0 cells and in serum-stimulated cells. Two other findings seem to confirm a posttranscriptional regulation of PCNA mRNA levels: hnRNA levels and mRNA stability.

To determine hnRNA levels, we used RT-PCR (28, 29), which allows us to determine hnRNA levels quantitatively (18). Since mRNA precursors are transient intermediates, their concentration at any given time reflects rates of transcription, processing, and degradation. Therefore, increases

in hnRNA levels reflect increases in transcription or decreases in processing or degradation. Thus, RT-PCR is not intended to replace run-on transcription (9, 11); it measures something different, i.e., the steady-state levels of mRNA precursors, analogous to the steady-state levels of mature mRNA by Northern blots (see below). The advantages of this technique (ability to detect signals too difficult to detect by other ways, small number of cells needed, and in vivo formation of hnRNA), its quantitation, and the necessity to exclude rigorously any DNA are described by Lipson and Baserga (18). There are even more rigorous methods to quantitate RT-PCR (42), but for the present purpose, a comparison of hnRNA levels, our method is sufficient. Our data show that hnRNA levels increase in cells stimulated to proliferate (Fig. 7A) regardless of whether the amplification products include the first or the last intron (Fig. 7A and B). The amplimers used were specific for human PCNA hnRNA and did not give any signal with untransfected BALB/c 3T3 cells.

We could not determine PCNA mRNA stability in G_0 cells carrying the full-length construct, since PCNA mRNA is not detectable in truly quiescent cells, but we could determine it in G_0 cells with the construct lacking intron 4. It seems that mRNA stability increases between G_0 and S phase. By taking into consideration run-on transcription, hnRNA levels, mRNA levels, and mRNA half-life, a reasonable model is that the full-length PCNA gene is transcribed at the same rate from G_0 to S and that the increase in mRNA levels is largely due to increased stability of both hnRNA and mRNA. Incidentally, we have been unable to determine the half-life of hnRNA. At least in our hands, dactinomycin by itself causes a marked increase in hnRNA levels (not shown). We do not know whether this result reflects a true effect of dactinomycin on hnRNA processing or is an artifact. In any case, until this question can be resolved, we must forgo the determination of hnRNA half-life. However, if transcription rate is unchanged and both hnRNA and mRNA levels go up, it is reasonable to assume that both products have become more stable.

The situation is slightly different in cells carrying the construct without introns 4 and 5. First, run-on transcription in these cells indicates higher transcription rates than in cells carrying the full-length human PCNA gene (with intron 4). Furthermore, the hnRNA levels are also high in BALB/c 3T3 cells carrying the human PCNA gene without introns 4 and 5. hnRNA levels drop at 4 and 8 h after stimulation and increase again at 16 and 24 h. Exactly the same time-related changes occur in mRNA levels (24). If hnRNA levels are high in G_0 cells and very low 4 h after stimulation, one is prompted to explain this finding as reflecting release from a splicing block occurring in G_0 cells. But this release should lead to an increase in mRNA levels, which, instead, are also lower. A reasonable explanation can be found as follows. The half-life of PCNA mRNA in cells carrying the construct without introns 4 and 5 is shorter in cells briefly stimulated with serum than in unstimulated cells. Thus, stimulation (at least in these cells) would cause a transient increase in stability of both hnRNA and mRNA, leading to a decrease in their levels. Subsequently, increased stability of the products, coupled with continuous transcription, leads again to increased levels of both hnRNA and mRNA.

These data can be summarized in the following hypothesis, which is compatible with all results obtained so far. The PCNA gene (full length) is transcribed at the same rate in G_0 cells and in serum-stimulated cells, but in G_0 cells, both hnRNA and mRNA are less stable than in growing cells. Intron 4 reduces transcription of the PCNA gene without altering the stability of either hnRNA or mRNA; its removal leads to increased transcription and increased levels of hnRNA and mRNA. It will be interesting to see whether PCNA intron 4 acts similarly to the last intron of the H-*ras* proto-oncogene, in which a mutation causes a marked increase in expression of the gene (4, 5).

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