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Importance of Introns in the Growth Regulation of mRNA Levels 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. We have begun to identify the elements in the human PCNA gene that participate in its growth regulation by transfecting appropriate constructs in BALB/c3T3 cells. The results can be summarized as follows. (i) The 400 base pairs of the 5′-flanking sequence of the human PCNA gene upstream of the preferred cap site are sufficient for directing expression of a heterologous cDNA (S. Travali, D.-H. Ku, M. G. Rizzo, L. Ottavio, R. Baserga, and B. Calabretta, J. Biol. Chem. 264:7466–7472, 1989). (ii) 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, although the shortened PCNA gene with its own promoter is still responsive to serum stimulation. (iii) The presence of introns also increases the steady-state levels of PCNA mRNA in proliferating cells. These results are especially interesting for two reasons: (i) because of the extensive sequence similarities among introns and between introns and exons of the human PCNA gene, and (ii) because, usually, the presence of introns leads to increased expression, whereas in this case, removal of intron 4 caused an increase in mRNA levels, and this occurred only in quiescent cells.

The proliferating cell nuclear antigen (PCNA) is a nuclear protein of molecular mass 29,000 whose synthesis correlates with the proliferative state of the cells (5, 38). The protein itself is part of the DNA-synthesizing machinery, since it has been identified as the cofactor of DNA polymerase delta (6, 31). Almendral et al. (1) were the first to report the cloning and sequencing of a cDNA for human PCNA. Subsequently, we isolated in our laboratory (19) a slightly longer cDNA, while the cDNA from rat cells was isolated by Matsumoto et al. (25). More recently (41), we molecularly cloned the full gene for human PCNA and sequenced approximately seven kilobases of it. The cloned gene included extensive 5′- and 3′-flanking sequences, and a functional promoter was identified in the 5′-flanking sequence (41). The human PCNA gene localizes to chromosome 20; there is a pseudogene on chromosome X and possibly a second pseudogene on chromosome 6 (21).

The PCNA mRNA is growth regulated. It was shown to be induced in quiescent 3T3 cells by stimulation with fetal calf serum, becoming detectable 12 h after stimulation and reaching a maximum at about 16 to 18 h, which is the maximum of DNA synthesis, as also shown by hybridization of the same filter to a histone probe (1). Jaskulski et al. (19) confirmed that the levels of mRNA are growth regulated in BALB/c3T3 cells, being very low in G_0 cells and increasing markedly when the cells are stimulated to proliferate by serum or growth factors. In this study, we have begun to localize the elements that regulate the growth-related expression of the human PCNA gene. We have limited ourselves, because of the complexity of the problem, to elements that regulate the steady-state levels of PCNA mRNA, leaving to a subsequent investigation the determination of whether these levels are regulated at the transcriptional or posttranscriptional level.

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MATERIALS AND METHODS

Plasmid construction. The full sequence of the human PCNA gene has been previously reported (41). The sequence of the human PCNA cDNA has also been reported by Almendral et al. (1) and Jaskulski et al. (19). By using these sequences, i.e., the human PCNA gene and the human PCNA cDNA, we have constructed the various plasmids that have been used in these experiments (see Fig. 1 and 5). The construction of the various plasmids is described below.

(i) PCNA gene. The full-length human PCNA gene is the *Bam*HI fragment described and sequenced by Travali et al. (41). It includes 2.8 kilobase pairs (kbp) of 5′-flanking sequence, 6 exons, 5 introns, and 400 base pairs (bp) of 3′ untranslated region.

(ii) *Bam*HI-m-gene. The *Bam*HI-m-gene contains the PCNA promoter (2.8 kbp) driving the PCNA gene until the *Eco*RI site (+1343) and cloned in the *Kpn*I and *Eco*RI sites of the polylinker of Bluescript KS. To this was added the 3′ untranslated region of PCNA from the *Xmn*I site (+4903), cloned in the *Sma*I and *Bam*HI sites of the same polylinker.

(iii) *Eco*RI-m-gene. The *Eco*RI-m-gene contains the PCNA promoter from *Eco*RI (−1257) driving the PCNA gene until the *Eco*RI site (+1343 on the 3rd exon), cloned at the *Eco*RI site of the polylinker of Bluescript KS plus the 3′ untranslated region of PCNA from *Xmn*I (+4903), cloned in the *Sma*I and *Bam*HI sites of the same polylinker.

In these two constructs, we truncated the PCNA translated region at the *Eco*RI site (+1373 on the 3rd exon) and we eliminated the last 400 bp of the coding sequence, the first 50 bp of the untranslated region, and the 3rd, 4th, and 5th introns.

(iv) *Bam*HI-m-cDNA and *Eco*RI-m-cDNA. The *Bam*HI-m-cDNA and *Eco*RI-m-cDNA fragments are like the *Bam*HI-m-gene and the *Eco*RI-m-gene, with the promoters driving the PCNA cDNA truncated at *Eco*RI (+540) plus the 3′ untranslated region (from *Xmn*I +991 of the cDNA).

(v) *Bam*HI PCNA without 4th and 5th intron. The *Bam*HI

PCNA without introns 4 and 5 is a fragment containing the 2.8-kbp PCNA promoter driving the PCNA gene until the *Bgl*II site (+2343) plus PCNA cDNA from the *Bgl*II site until the 3′ end, cloned in the *Bam*HI site of the polylinker of pGEM 3. To prepare this construct, we digested the *Bam*HI promoter driving the full PCNA gene and the PCNA cDNA with *Bgl*II. There are two *Bgl*II sites on the PCNA gene: +2348 on the 4th exon and +4848 on the 3′ untranslated region (+607 and 1136 of cDNA). We then substituted the *Bgl*II fragment containing the 4th and 5th introns with the *Bgl*II fragment from the PCNA cDNA.

(vi) *Bam*HI PCNA without 5th intron. The *Bam*HI PCNA without intron 5 is a fragment containing a 2.8-kbp PCNA promoter driving the PCNA gene until the *Kpn*I site (+4419 on the 5th exon) plus PCNA cDNA from the *Kpn*I site until the 3′ end. We digested the *Bam*HI promoter driving the full PCNA cDNA with *Kpn*I and *Hpa*I. On the PCNA gene there is only one *Kpn*I site (+4419 on the 5th exon, +793 on the cDNA) and only one *Hpa*I site (+4862 on the 3′ untranslated region, +1150 on the cDNA). We substituted the *Kpn*I-*Hpa*I fragment of the gene with the *Kpn*I-*Hpa*I fragment of the cDNA.

(vii) *Bam*HI PCNA without 5th intron and truncated. The *Bam*HI PCNA without intron 5 and truncated is a fragment containing a 2.8-kbp PCNA promoter driving the PCNA gene truncated in the *Kpn*I site (+4419 on the 5th exon) plus part of the 3′ untranslated region. We digested the *Bam*HI promoter driving the full PCNA gene with *Kpn*I and *Hpa*I (see *Bam*HI PCNA without 5th intron), eliminated the *Kpn*I-*Hpa*I fragment (~450 bp), made blunt ends with T4 DNA polymerase, and religated the extremities. In this construct, we truncated the translated region of the PCNA gene in the 5th exon and eliminated the last 150 bp of translated region and the 5th intron and 207 bp of the 3′ untranslated region.

(viii) *Bam*HI PCNA without introns 1, 2, and 3. The full-length human PCNA gene was digested with *Stu*I and religated. This digestion removes part of exon 1, introns 1 and 2, exons 2 and 3, and most of intron 3 (41).

Cell lines. BALB/c3T3 cells were grown in our laboratory as previously described (19). 3T3 cells were transfected in suspension (34) with one of the desired constructs. Each construct was cotransfected with plasmid pLHL4 (15), which contains the bacterial gene for hygromycin B resistance. The cells were then selected in hygromycin; the resultant clones were analyzed for the presence of the PCNA construct and then investigated as described in Results.

RNA extraction and RNA blots. Total RNA was extracted from cells by the method of Chomczynski and Sacchi (10), and RNA blots were carried out by standard procedures (40). Radioactive probes were prepared by the random-priming method (14). The following probes were used: (i) the full-length PCNA cDNA (19), cloned in pGEM-3; (ii) the 5′ portion of the human PCNA cDNA (18) extending from the *Bam*HI (cap site) to the *Ava*II site at +178 of the cDNA (it includes the 5′ untranslated region plus 20 bp of coding sequence); and (iii) the histone H3 probe, previously described (19). To monitor the amounts of RNA in each lane, we used as a probe a mouse cDNA insert (isolated from a differential screening of a cDNA library and designated as L4) whose expression is not growth regulated. Since the amounts of RNA in the different lanes did not vary by more than 10 to 20%, this control has been omitted from Results.

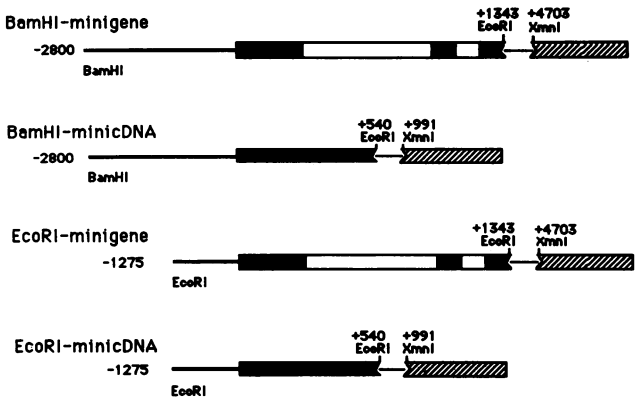

FIG. 1. Diagram of the minigenes and mini-cDNA of human PCNA transfected into BALB/c3T3 cells. The diagram shows only two promoters, the *Bam*HI and the *Eco*RI promoters, but similar constructs were also made with shorter promoters. Thin lines, 5′-Flanking sequences; black boxes, coding sequences; empty boxes, introns; striped boxes, 3′-flanking sequences, including the 3′ untranslated region.

RESULTS

Regulation of the human PCNA gene in BALB/c3T3 cells. In a previous study (41), we had shown that approximately 400 bp of 5′-flanking sequence (*Pvu*II restriction site) was sufficient for the full expression of a linked reporter, in that case the cDNA of human thymidine kinase.

We have now investigated the regulation of the human PCNA gene in BALB/c3T3 cells, which are exquisitely growth regulated (33, 37). The constructs used for these experiments are shown in Fig. 1. In order to distinguish the human PCNA mRNA from endogenous mouse PCNA mRNA (which is approximately the same size), we at first prepared constructs in which the human PCNA coding sequence was shortened, thus creating a minigene or a mini-cDNA. The minigene, as Fig. 1 shows, included the first two introns (the PCNA gene has 5 introns). Shortening of the coding sequence resulted in a smaller RNA.

BALB/c3T3 cells were cotransfected with each of the various constructs and a selectable marker, the plasmid pLHL4, which contains the bacterial gene for hygromycin B. Clones were selected, and the established cell lines were then studied for growth regulation. The experiments were carried out in the traditional way for BALB/c3T3, i.e., the cells were made quiescent by decreasing the concentration of serum for 4 to 5 days (G_0 cells), and they were subsequently stimulated with 10% fetal calf serum. Total RNA was extracted from the cells in G_0 and at various times after serum stimulation (Fig. 2). In these experiments the filters were hybridized to a full-length human PCNA cDNA probe, and, as expected, two bands were distinguishable on the filters, the endogenous mouse PCNA product and the (smaller) product of the human PCNA minigene or mini-cDNA. Figure 2 compares the expression of the human PCNA minigene under the control of the *Bam*HI promoter and the mouse PCNA gene. It is clear that the *Bam*HI human PCNA minigene is not correctly regulated. Although it responds to serum efficiently, minigene mRNA is present in similar amounts in G_0 and S-phase cells, while the endogenous mouse PCNA mRNA is barely visible in G_0 cells and strongly expressed in S-phase cells. The same results were obtained with other promoters and with mini-cDNAs (not shown). In all cases, however, in serum-stimulated cells the

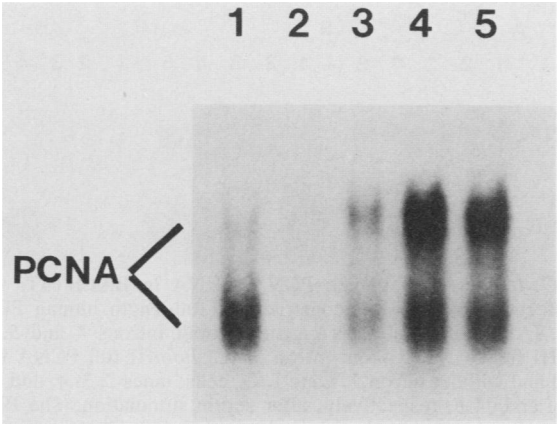

FIG. 2. Levels of endogenous PCNA mRNA and of transfected PCNA minigene RNA in BALB/c3T3 cells. The cells were cotransfected with the *Bam*HI minigene plus a selectable marker (see Materials and Methods). Mixed populations were used for the experiment. Lane 1, Serum-deprived cells; lane 2, 4 h after serum stimulation; lanes 3, 4, and 5, 8, 16, and 24 h, respectively, after serum stimulation. Total RNA was extracted and blotted, and the filter was hybridized to a human full-length PCNA cDNA probe. The higher band represents endogenous PCNA, and the lower band represents the mRNA from the PCNA minigene.

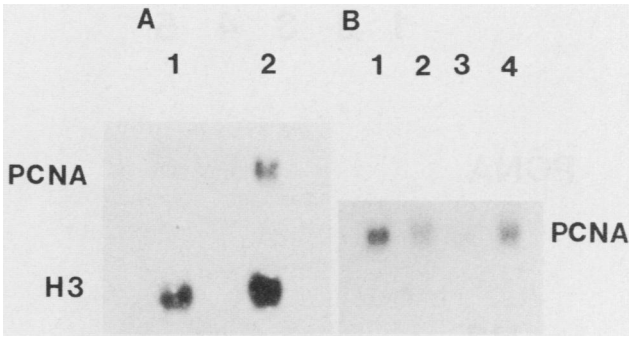

FIG. 3. Composite picture from two Northern (RNA) blots of RNA from BALB/c3T3 cells. (A) Lane 1, BALB/c3T3 cells exponentially growing; lane 2, BALB/c3T3 cells transfected with a *Bam*HI full-length PCNA gene, also exponentially growing. (B) BALB/c3T3 cells carrying the *Eco*RI minigene PCNA were investigated as described in the legend to Fig. 2. The lanes are the same as in Fig. 2, except that the 24-h time is missing. Both filters were hybridized with a *Bam*HI-*Ava*II fragment of the human PCNA cDNA that recognizes only the human PCNA mRNA.

levels of minigene RNAs were higher than those of mini-cDNA RNAs (Table 1), indicating that introns have a favorable effect on the expression of the PCNA gene, as already reported in the literature (13, 43). The deregulation of the *Bam*HI promoter minigene is even more striking when, on the same filter, it is clear that the endogenous mouse PCNA promoter is correctly regulated.

There are essentially three possible reasons why the *Bam*HI minigene construct is deregulated in G_0 cells, namely: (i) the *Bam*HI PCNA promoter is an incomplete promoter that is not correctly regulated; (ii) the lack of intron 3, 4, or 5, or all of them; and (iii) the fact that the minigene and the mini-cDNA produce a truncated PCNA protein. The last possibility is somewhat unlikely, since the endogenous mouse PCNA gene is producing a normal protein; therefore, either both gene and minigene should be properly regulated or deregulated. At any rate, to solve this problem we proceeded with the investigation of growth regulation with different PCNA constructs.

Regulation with *Bam*HI PCNA promoter driving the full PCNA gene. We elected to try first the *Bam*HI PCNA promoter driving a full-length human PCNA gene. In order, however, to distinguish the product of the human PCNA gene from the product of the mouse PCNA gene, it was necessary first to find a probe that could distinguish them. The 5′ untranslated regions of the PCNA cDNA sequence are different in mice and humans, and we therefore took a probe that extends from essentially the cap site to +178 of the human cDNA. We then tested this probe for its ability to distinguish mouse and human PCNA mRNAs. Under the conditions used in our experiments, it was clear that the human 5′ PCNA probe recognizes only the human sequence and does not recognize the mouse sequence (Fig. 3). No signal was detectable in BALB/c3T3 cells, whereas the signal was clearly detectable in the same cells carrying a human PCNA construct (A). Figure 3B shows that this probe recognizes only the human PCNA minigene. It also shows that the EcoRI construct, like the *Bam*HI construct, is not correctly regulated, mRNA levels being high in G_0 cells. It is therefore possible to identify the expression of the human PCNA full-length gene in BALB/c3T3 cells carrying the desired construct. Figure 4 shows that when the *Bam*HI promoter drives the full-length human PCNA gene, it is correctly regulated. No human PCNA mRNA is detectable in G_0 cells, although it becomes clearly detectable in cells stimulated to proliferate with serum. Similar results were obtained with other, shorter promoters (not shown). The results, therefore, show that the *Bam*HI promoter can be properly regulated like the endogenous PCNA promoter, provided that all five introns are present.

TABLE 1. Relative expression of PCNA minigenes or PCNA mini-cDNA in BALB/c3T3 cells[a]

Promoter (restriction site and bp from the cap site)	PCNA minigene	PCNA mini-cDNA
*Bam*HI (2,800)	4.0	1.4
*Eco*RI (1,257)	4.0	1.6
*Eco*RV (789)	2.0	1.0
*Pvu*II (395)	3.1	1.2
*Hpa*II (210)	3.2	0.9

[a] BALB/c3T3 cells were cotransfected with one of the human PCNA constructs and a selectable marker (see Materials and Methods). Established cells lines were made quiescent and then were stimulated with serum for 16 h. RNA blots were hybridized to both human PCNA cDNA and a histone H3 probe. The densitometry readings of the autoradiographs represent the ratios of the PCNA bands to the H3 bands (in order to normalize for the fraction of cells in S phase).

Necessity of intron 4 for correct regulation of the human PCNA gene. In the next experiment we decided to investigate the effect on the regulation of mRNA levels from the human PCNA promoter when either both 4th and 5th introns were removed or only the 5th intron was removed. In addition, we made a construct in which the first 4 introns, but not the 5th one, were present and the coding sequence was truncated. These three last constructs, diagrammed in Fig. 5, were cotransfected as usual with a selectable marker, and cell lines were established. The same experiments already described were then carried out with these new cell lines. The results are shown in Fig. 6, and they clearly indicate that when both introns 4 and 5 are removed, the expression of the human PCNA gene is deregulated,

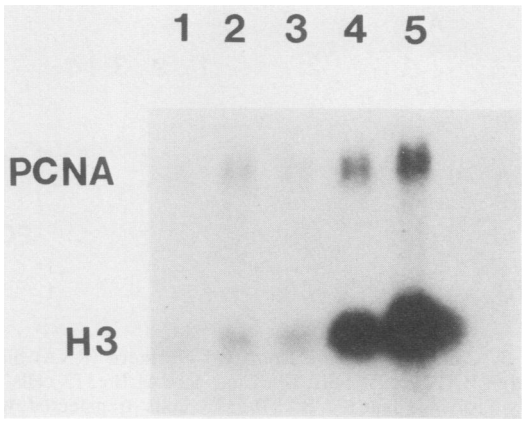

FIG. 4. PCNA mRNA levels in BALB/c3T3 cells transfected with a full-length human PCNA gene. BALB/c3T3 cells were cotransfected with a full-length human PCNA gene plus a selectable marker (see Materials and Methods). Mixed populations were then selected, and the cells were made quiescent and were subsequently stimulated with serum. Lane 1, G_0 cells; lanes 2, 3, 4, and 5, the same cells 4, 8, 16, and 24 h, respectively, after serum stimulation. The RNAs were isolated and blotted, and the filter was hybridized to a *Bam*HI-*Ava*II fragment of human PCNA cDNA and, also, to a human histone H3 probe. The full-length human gene had a promoter extending to the *Bam*HI restriction site.

whereas when only the 5th intron is removed, the construct is perfectly regulated. No mRNA is detectable in G_0 cells, although it does appear clearly in serum-stimulated cells. Finally, Fig. 6 also shows that the truncation of the coding sequence has no effect on the regulation of the *Bam*HI PCNA promoter in G_0 cells, provided that intron 4 is present. The absence of a histone H3 signal indicates that the cells in lane 1, panel A, were not in S phase, but we confirmed it further by labeling cells in a separate experiment with [^{3}H]thymidine for 24 h. About 2% of the cells became labeled, while in S phase >90% of the cells incorporated [^{3}H]thymidine.

First three introns not necessary for correct regulation of PCNA mRNA levels in G_0 cells. We finally made a construct from which we removed introns 1 and 2 and most of intron 3 (see Materials and Methods) and established with it the usual cell lines from BALB/c3T3 cells. RNA was extracted from quiescent and serum-stimulated cells, and the resultant blot

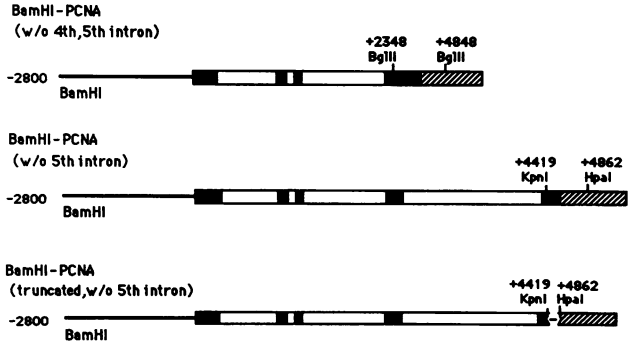

FIG. 5. Constructs used to determine the role of different introns in the regulation of G_0 mRNA levels of human PCNA gene. Thin lines, 5′-Flanking sequences; solid boxes, coding sequences; open boxes, introns; striped boxes, 3′ untranslated sequences and flanking regions.

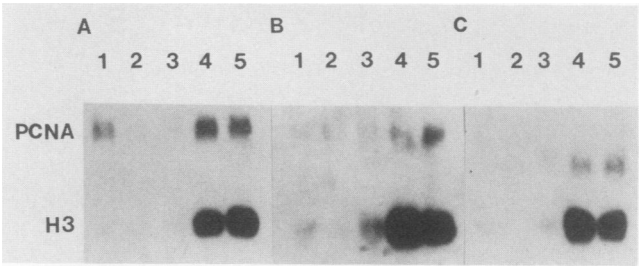

FIG. 6. Levels of human PCNA mRNA in BALB/c3T3 cells transfected with different constructs of full-length human PCNA gene. (A) *Bam*HI full PCNA gene without introns 4 and 5. (B) *Bam*HI full PCNA without intron 5. (C) *Bam*HI full PCNA truncated and without intron 5. Lane 1, G_0 cells; lanes 2, 3, 4, and 5, 4, 8, 16, and 24 h, respectively, after serum stimulation. The RNAs were isolated and blotted, and the filters were hybridized to both the human histone H3 probe and the *Bam*HI-*Ava*II fragment of human PCNA cDNA.

is shown in Fig. 7. The absence of the first three introns had no effect on the correct regulation of PCNA mRNA levels in G_0 cells; the truncated human PCNA gene was expressed as the endogenous mouse gene.

DISCUSSION

The PCNA protein is the auxiliary factor for DNA polymerase delta and is necessary for cellular DNA synthesis (6, 29, 31, 44). The PCNA gene product seems to occupy an important position in DNA replication and cell cycle progression (18, 23, 30). The encoded protein product of the PCNA gene is probably present in a multimeric form (39), and its synthesis correlates with the proliferative state of the cell (5, 38). It has been suggested that PCNA inhibits an inhibitor of DNA replication (22) and that its amount is critical to the onset of DNA synthesis (18, 23). The PCNA protein is induced in fibroblasts by purified growth factors such as platelet-derived growth factor and fibroblast growth factor (5, 7). It is induced in lymphocytes by interleukin-2

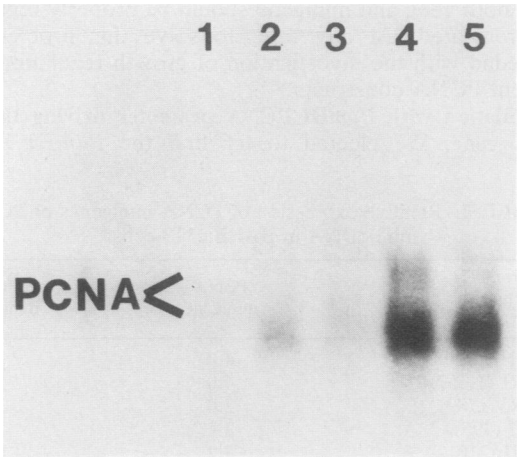

FIG. 7. Levels of PCNA mRNA in BALB/c3T3 cells transfected with a construct of the human PCNA gene lacking introns 1, 2, and most of 3. RNA was extracted from G_0 cells (lane 1), and cells were serum stimulated for 4, 8, 16, and 24 h (lanes 2 through 5). Blots were hybridized with a full-length human PCNA cDNA. Two bands are distinguishable: the upper one is the endogenous mouse mRNA, and the lower one is the truncated mRNA from the human gene.

(26, 35), and it is also induced by the E1A protein of adenovirus (46). PCNA mRNA was shown by Almendral et al. (1) and by Jaskulski et al. (19) to be induced in quiescent cells by stimulation with 10% fetal calf serum or other growth factors in the same way as the thymidine kinase gene (12, 42). Platelet-poor plasma, which is essentially serum without platelet-derived growth factor, does not cause an increase in the steady-state level of PCNA mRNA.

The fact that its expression is growth regulated and the fact that it codes for a protein of the DNA-synthesizing machinery make the PCNA gene a very attractive gene for regulatory studies. The onset of cellular DNA synthesis is certainly one of the critical steps in the regulation of cell cycle progression. A study of the regulation of the PCNA gene (as well as other genes coding for proteins of the DNA-synthesizing machinery) could be of considerable interest in our understanding of how cellular proliferation is regulated in animal cells. In this study, we have attempted to identify the elements that regulate the steady-state levels of human PCNA mRNA. No attempt was made here to determine whether these levels are regulated at a transcriptional or a posttranscriptional level. The use of mixed populations and the similarity of results with different established cell lines seem to rule out a position effect as an explanation of our results.

The results can be summarized as follows. (i) Although the longer promoters are just as strong, the *Pvu*II promoter, with only 395 bp, is sufficient to confer a high level of expression to a heterologous cDNA (41). (ii) The presence of introns, in general, increases the levels of PCNA mRNA in proliferating cells. (iii) When intron 4 is absent, the PCNA gene does respond to serum but does not turn off when serum is withdrawn, so that the steady-state levels of PCNA mRNA remain high also in G_0 cells.

The last point is, of course, the most interesting one and is discussed further.

Our data seem to indicate that the PCNA gene is correctly regulated in terms of steady-state levels of mRNA, provided that all the introns are present and provided that the promoter up to the *Bam*HI restriction sites is present. However, the omission of introns 4 and 5 results in deregulated levels of mRNA in G_0 cells. Omission of only intron 5 restores the PCNA gene to its proper regulation, from which it is inferred that the presence of intron 4 is necessary for regulation. Removal of introns 1, 2, and most of 3 (leaving in intron 4) generates a construct that is correctly regulated. We emphasize that the presence of intron 4 seems necessary only for the regulation of steady-state levels in G_0 cells. We also emphasize that at this point we can only say that the presence of intron 4 is necessary for correct regulation of PCNA mRNA levels. We do not know yet if it is necessary and sufficient; the possibility still exists that another intron (any one) may be necessary for a cooperative effect with intron 4. Truncation at the 3′ end does not lead to deregulation, provided that intron 4 is present. The response to serum seems to be largely located in the 5′-flanking sequence, although introns seem to have a general role in increasing the levels of steady-state mRNA in proliferating cells. This seems to go along the same lines as the recently described gene for ribosomal protein L32 in which introns exert two different types of control, a general control in terms of levels of mRNA and a more specific control which is located in that particular gene in intron 1 (11). Although there are reports that introns have no effect on the mRNA levels of specific genes, there are also reports of introns functioning as enhancers or, at any rate, increasing mRNA levels (8, 9, 11, 13, 16, 17, 20, 28, 36, 45). In the case of the PCNA gene, the situation is somewhat different: on one hand, there is, as already mentioned, a general effect on mRNA levels, but, on the other hand, intron 4 (by itself or in cooperation with other elements) regulates the levels of PCNA mRNA in G_0 cells, while after serum stimulation, the presence of intron 4 is less critical. It is intriguing that in our case, the lack of an intron results not in lower but in higher levels of mRNA. Negative elements in an intron have been reported in the case of the human alpha 1 (type I) collagen gene (2, 32). The uniqueness of the human PCNA gene is that the removal of intron 4 alters the regulation of the mRNA levels in G_0 cells.

The presence of a regulatory element in an intron of the human PCNA gene assumes a particular interest because of the peculiarity of this gene. As previously reported (41), there are unusual and extensive similarities among the introns of the human PCNA gene and, indeed, even between introns and exons. These sequence similarities are not casual, since they can extend to approximately 50 to 60% sequence similarity over stretches of 100 to 400 nucleotides. These sequence similarities have been described in the previous paper (41), but for the purpose of the present paper, it seems to us worthwhile to emphasize the similarities that intron 4 has with other sequences of the PCNA gene. Thus, intron 4 has more than 60% sequence similarity over a stretch of almost 300 nucleotides with the 3′ end of the human PCNA cDNA. It also has more than 60% sequence similarities with stretches of intron 1 (over almost 200 nucleotides), intron 2 (64 nucleotides), intron 3 (approximately 460 nucleotides), and intron 5 (41 nucleotides). Intron 4 is the longest of the human PCNA introns, 1,885 bp, while intron 5, 86 bp, is the shortest. As mentioned above, these sequence similarities among introns in genes are somewhat unusual. Similarity has been reported in the introns of heat shock genes (27) and also in a ribosomal protein of *Xenopus* spp., the L1 ribosomal protein (24). The latter one is particularly interesting because it displays, when the gene is microinjected into frog oocyte nuclei, a splicing block (3). This leads us to a working hypothesis which can be formulated as follows. When BALB/c3T3 cells are made quiescent, a splicing block occurs in the PCNA transcripts, leading to a decrease in the steady-state levels of mature mRNA. Somehow the absence of intron 4 removes the splicing block, so that mature mRNA remains easily detectable also in G_0 cells. This seems to be an attractive hypothesis which can be tested, although it is clear that other possibilities should be kept in mind. Indeed, no clear explanation of the role of intron 4 in the regulation of steady-state levels of PCNA mRNA can be formulated until determinations of transcriptions are carried out in detail. Another very attractive possibility would be that intron 4 contains a silencer (4) that is functional only in G_0. Tentatively, it seems that intron 4 functions as a *cis*-acting element, since a *trans*-acting factor in our cell lines ought to be provided by the endogenous PCNA gene (hamster or mouse). More complicated scenarios would otherwise have to be invoked, such as species-specific *trans*-acting factor or a combination of a *trans* effect on a longer promoter. For the moment, however, we can conclude that the steady-state levels of PCNA mRNA, although clearly regulated at the 5′-flanking sequence by serum, are also regulated by the presence of intron 4, whose absence leads to an increase of mRNA levels in G_0 cells. Further studies are directed at identifying the serum-responsive elements in the 5′-flanking sequence and

the mechanism by which introns can alter the steady-state levels of PCNA mRNA in quiescent cells.

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LITERATURE CITED

1. **Almendral, J. M., D. Huebsch, P. A. Blundell, H. MacDonald-Bravo, and R. Bravo.** 1987. Cloning and sequence of the human nuclear protein cyclin: homology with DNA-binding proteins. Proc. Natl. Acad. Sci. USA **84**:1575–1579.
2. **Bornstein, P., J. McKay, J. K. Morishima, S. Devarayalu, and R. E. Gelinas.** 1987. Regulatory elements in the first intron contribute to transcriptional control of the human 1(I) collagen gene. Proc. Natl. Acad. Sci. USA **84**:8869–8873.
3. **Bozzoni, I., P. Fragapane, F. Annesi, P. Pierandrei-Amaldi, F. Amaldi, and E. Beccari.** 1984. Expression of two Xenopus laevis ribosomal protein genes in injected frog oocytes. A specific splicing block interferes with the L1 RNA maturation. J. Mol. Biol. **180**:987–1005.
4. **Brand, A. H., G. Micklem, and K. Nasmyth.** 1987. A yeast silencer contains sequences that can promote autonomous plasmid replication and transcriptional activation. Cell **51**:709–719.
5. **Bravo, R.** 1986. Synthesis of the nuclear protein cyclin (PCNA) and its relationship with DNA replication. Exp. Cell Res. **163**:287–293.
6. **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.
7. **Bravo, R., and H. MacDonald-Bravo.** 1985. Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication. EMBO J. **4**:655–661.
8. **Buchman, A. R., and P. Berg.** 1988. Comparison of intron-dependent and intron-independent gene expression. Mol. Cell. Biol. **8**:4395–4405.
9. **Callis, J., M. Fromm, and V. Walbot.** 1987. Introns increase gene expression in cultured maize cells. Genes Dev. **1**:1183–1200.
10. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
11. **Chung, S., and R. P. Perry.** 1989. Importance of introns for expression of mouse ribosomal protein gene rpL32. Mol. Cell. Biol. **9**:2075–2082.
12. **Coppock, D. L., and A. B. Pardee.** 1987. Control of thymidine kinase mRNA during the cell cycle. Mol. Cell. Biol. **7**:2925–2932.
13. **Deng, T., Y. Li, and L. F. Johnson.** 1989. Thymidylate synthase gene expression is stimulated by some (but not all) introns. Nucleic Acids Res. **17**:645–658.
14. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
15. **Gritz, L., and J. Davies.** 1983. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in Escherichia coli and Saccharomyces cerevisiae. Gene **25**:179–188.
16. **Gruss, P., and G. Khoury.** 1980. Rescue of splicing defective mutant by insertion of an heterologous intron. Nature (London) **286**:634–637.
17. **Hamer, D. H., and P. Leder.** 1979. Splicing and the formation of stable RNA. Cell **18**:1299–1302.
18. **Jaskulski, D., J. K. deRiel, W. E. Mercer, B. Calabretta, and R. Baserga.** 1988. Inhibition of cellular proliferation by antisense oligodeoxynucleotides to PCNA cyclin. Science **240**:1544–1546.
19. **Jaskulski, D., G. 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.
20. **Karpinski, B. A., L. H. Yang, P. Cacheris, G. D. Morle, and J. M. Leiden.** 1989. The first intron of the 4F2 heavy-chain gene contains a transcriptional enhancer element that binds multiple nuclear proteins. Mol. Cell. Biol. **9**:2588–2597.
21. **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. **15**:297–307.
22. **Lee, S. H., Y. Ishimi, M. K. Kenny, P. Bullock, F. B. Dean, and J. Hurwitz.** 1988. An inhibitor of the in vitro elongation reaction of simian virus 40 DNA replication is overcome by proliferating cell nuclear antigen. Proc. Natl. Acad. Sci. USA **85**:9469–9473.
23. **Liu, Y. C., R. L. Marraccino, P. C. Keng, R. A. Bambara, E. M. Lord, W. G. Chou, and S. B. Zain.** 1989. Requirement for proliferating cell nuclear antigen expression during stages of the Chinese hamster ovary cell cycle. Biochemistry **28**:2967–2974.
24. **Loreni, F., I. Ruberti, I. Bozzoni, P. Pierandrei-Amaldi, and F. Amaldi.** 1985. Nucleotide sequence of the L1 ribosomal protein gene of Xenopus laevis: remarkable sequence homology among introns. EMBO J. **4**:3483–3488.
25. **Matsumoto, K., T. Moriuchi, T. Koji, and P. K. Nakane.** 1987. Molecular cloning of cDNA coding for rate proliferating cell nuclear antigen (PCNA)/cyclin. EMBO J. **6**:637–642.
26. **Moore, K., 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.
27. **Munn, T. Z., and G. I. Mues.** 1988. Highly conserved repeats in heat-shock introns. Nature (London) **332**:789.
28. **Pellegrino, R., and B. DeCrombrugghe.** 1987. Identification of a cell-specific transcriptional enhancer in the first intron of the mouse alpha (type I) collagen gene. Proc. Natl. Acad. Sci. USA **84**:5590–5594.
29. **Prelich, G., M. Kostura, D. R. Marshak, M. B. Mathews, and B. Stillman.** 1987. The cell-cycle regulated proliferating cell nuclear antigen is required for SV40 DNA replication in vitro. Nature (London) **326**:471–475.
30. **Prelich, G., and B. Stillman.** 1988. Coordinated leading and lagging strand synthesis during SV40 DNA replication in vitro requires PCNA. Cell **53**:117–126.
31. **Prelich, G., C. K. Tan, M. Kostura, M. B. Mathews, A. G. So, K. M. Downey, and B. Stillman.** 1987. Functional identity of proliferating cell nuclear antigen and a DNA polymerase delta auxiliary protein. Nature (London) **326**:517–520.
32. **Rippe, R. A., S.-I. Lorenzen, D. A. Brenner, and M. Breindl.** 1989. Regulatory elements in the 5′-flanking region and the first intron contribute to transcriptional control of the mouse alpha 1 type I collagen gene. Mol. Cell. Biol. **9**:2224–2227.
33. **Scher, C. D., R. C. Shephard, N. H. Antoniades, and C. D. Stiles.** 1979. Platelet derived growth factor and the regulation of the mammalian fibroblasts cell cycle. Biochim. Biophys. Acta **560**: 217–241.
34. **Shen, Y. M., R. R. Hirschhorn, W. E. Mercer, E. Surmacz, Y. Tsutsui, K. J. Soprano, and R. Baserga.** 1982. Gene transfer: DNA microinjection compared with DNA transfection with a very high efficiency. Mol. Cell. Biol. **2**:1145–1154.
35. **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.
36. **Slater, E. P., O. Rabenau, M. Karin, J. D. Baster, and M. Beato.** 1985. Glucocorticoid receptor binding and activation of a heterologous promoter by dexamethasone by the first intron of the human growth hormone gene. Mol. Cell. Biol. **5**:2984–2992.
37. **Stiles, C. D., G. T. Capone, C. D. Scher, N. H. Antoniades, J. J. Van Wyk, and W. J. Pledger.** 1979. Dual control of cell growth by somatomedins and platelet derived growth factor. Proc. Natl. Acad. Sci. USA **76**:1279–1283.
38. **Takasaki, J., D. Fischwild, and E. M. Tan.** 1984. Characterization of proliferating cell nuclear antigen recognized by autoantibodies in lupus sera. J. Exp. Med. **159**:981–992.

39. **Tan, E. M., K. Ogata, and Y. Takasaki.** 1987. PCNA/cyclin: a lupus antigen connected with DNA replication. J. Rheumatol. **14**(Suppl. 13):91–96.
40. **Thomas, P. S.** 1983. Hybridization of denatured RNA transferred on dotted nitrocellulose paper. Methods Enzymol. **100:** 255–266.
41. **Travali, S., D.-H. Ku, M. G. Rizzo, L. Ottavio, R. Baserga, and B. Calabretta.** 1989. Structure of the human gene for the proliferating cell nuclear antigen. J. Biol. Chem. **264:**7466–7472.
42. **Travali, S., K. E. Lipson, D. Jaskulski, E. Lauret, and R. Baserga.** 1988. Role of the promoter in the regulation of the thymidine kinase gene. Mol. Cell. Biol. **8:**1551–1557.
43. **Venolia, L., G. Urlaub, and L. A. Chasin.** 1987. Polyadenylation of Chinese hamster dihydrofolate reductase genomic genes and minigenes after gene transfer. Somatic Cell Mol. Genet. **13:** 491–504.
44. **Wold, M. S., D. H. Weinberg, D. M. Virshup, J. J. Li, and T. J. Kelly.** 1989. Identification of cellular proteins required for simian virus 40 DNA replication. J. Biol. Chem. **264:**2801–2809.
45. **Yutzey, K. E., R. L. Kline, and S. F. Konieczny.** 1989. An internal regulatory element controls troponin I gene expression. Mol. Cell. Biol. **9:**1397–1405.
46. **Zerler, B., R. J. Roberts, M. B. Mathews, and E. Moran.** 1987. Different functional domains of the adenovirus E1A gene are involved in regulation of host cell cycle products. Mol. Cell. Biol. **7:**821–829.