A conserved region in intron 1 negatively regulates the expression of the PCNA gene

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ABSTRACT

The Proliferating Cell Nuclear Antigen (PCNA) gene is a growth-regulated gene, whose expression is under the control of both transcriptional and post-transcriptional mechanisms. In previous work, it was shown that the 73 bp immediately upstream of the CAP site and intron 4 are major regulatory elements. We show here that intron 1 also plays a role in determining the levels of PCNA mRNA. Specifically, we show: 1) deletion of intron 1 increases the expression of PCNA mRNA in serum-deprived cells; 2) a 35 bp sequence in intron 1, containing a reverse CCAAT element specifically binds proteins from nuclear extracts; 3) this intron 1 sequence inhibits the expression of a co-transfected human PCNA gene in transient expression assays suggesting that it competes for positive transcription factors; 4) mutations in the CCAAT region of the 35bp intron 1 probe abrogate both its protein-binding capacity and its ability to inhibit the expression of a co-transfected wt PCNA gene; and 5) the CCAAT region of human intron 1 is highly conserved in the mouse gene. We conclude that the reverse CCAAT region of intron 1 is a negative regulatory element of PCNA gene expression, and hypothesize that its inhibitory effect is abolished when certain protein(s) bind to it and that inhibition is restored if these proteins are competed out by an homologous sequence.

INTRODUCTION

As the co-factor of DNA polymerase delta, (1–3) the product of the PCNA gene is required for DNA synthesis (4,5) and cellular proliferation (6,7). The PCNA mRNA levels are growth regulated (8–10) and this regulation is under the control of both transcriptional and post-transcriptional mechanisms (11–13).

Ottavio et al. (14) have reported that deletion of intron 4 increases PCNA mRNA levels in serum-deprived cells. We report here that intron 1 also exerts a negative regulatory effect on the expression of the PCNA gene. The negative regulatory

effect can be localized to a 35 bp sequence in intron 1, conserved in the mouse PCNA gene, that contains a reverse CCAAT element, and binds protein(s) from nuclear extracts.

MATERIALS AND METHODS

Cell Lines

TK⁻ts13 cells, a thymidine kinase-deficient cell line (15) derived from a G₁-specific temperature sensitive (ts) mutant of the cell cycle, (16) are fibroblast cells of Syrian hamster origin. They are grown at 34°C in Dulbecco's minimal essential medium (DMEM) supplemented with 10% calf serum. Growth-arrest in these cells is obtained at the restrictive temperature of 39.6°C. Quiescence is induced by 3 days of serum deprivation. Balb/c 3T3 cells, previously described (9) were made quiescent in 0.5% serum for 4 days (growth medium: DMEM supplemented with 5% calf serum and 5% fetal calf serum).

T98G cells, a human cell line originally derived from a glioblastoma multiforme (17) were grown in Earle's minimal essential medium containing 10% (vol/vol) fetal calf serum (GIBCO) at 37°C, and were kept in 0.5% fetal calf serum for 3 days to make them quiescent. For stimulation, cells were treated with trypsin and replated at a 1:4 split ratio in medium containing 10% fetal calf serum.

Expression plasmids

The full-length human PCNA gene plasmid has been described (18) as well as the human PCNA gene driven by a 73 bp promoter (19). In order to remove intron 1, the full-length PCNA gene subcloned in the pGEM 3 vector (18) was cut with Eco RV resulting in a 1.9 kb and a 9 kb fragment. The 1.9 kb fragment was digested with NcoI and filled in with Klenow. The resulting 1.1 kb fragment was ligated to the 9 kb fragment leading to the PCNA gene without intron 1.

In co-transfection experiments in which the intron 1 probe or the mutated intron 1 probe were used as competitors for the two PCNA genes (full length or minus intron 1) we found that better results were obtained, when the probes were co-transfected

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subcloned in a vector, rather than as synthetic fragments. For this purpose, two 41 oligodeoxynucleotides were synthesized and annealed. This double stranded fragment containing the 35bp intron 1 probe of the human PCNA gene from +441 to +475 with Hind III restriction sites on both ends, was then subcloned in a Bluescript vector. Sequencing confirmed the correct fragment. The same was done with the mutated human PCNA 35bp intron 1 probe (see Results).

Transfections

In a transient expression assay, Tk⁻ts13 cells were transfected in suspension as described by Shen et al. (15). The full-length human PCNA gene, used as a positive control, was tested in different concentrations. For co-transfection experiments, 10 μ g of full-length human PCNA was co-transfected with the intron 1 probe or the mutated intron 1 probe in equal amounts. Co-transfection was also carried out with the 73bp promoter driving the human PCNA gene plus the intron 1 probe, or with the human PCNA gene lacking intron 1 plus the intron 1 probe. RNA was extracted 40h after transfection.

Balb/c 3T3 cells were co-transfected with a selectable marker (RSVneo; 20) and either the full-length human PCNA gene or the human PCNA gene lacking intron 1 by the same method. Transfected cells were selected from the second day in G418 (800 μ g/ml)-supplemented medium.

RNA extraction and RNA blots

Total RNA was extracted from cells by the method of Chomczynski and Sacchi (21). RNA blots were carried out by standard procedures (22). Radioactive probes were prepared by the random priming method (23). Two probes were used, a human specific 5'PCNA probe, a 186bp PstI-AvaII fragment of the human PCNA cDNA (12) and the histone H3 probe previously described (11,12). The amount of RNA was compared by densitometry using the Ultro Scan XL (Pharmacia). To monitor the amount of RNA in each lane, we have used the 3A10 probe (24), whose cognate RNA is constantly expressed throughout the cell cycle.

Gel Shift analysis

Nuclear extracts were prepared as described by Stein et al. (25). Gel shift assays were done according to Singh et al. (26). The

Fig. 1. Deregulated expression of the human PCNA gene lacking intron 1. BALB/c 3T3 cells were co-transfected with a selectable marker (RSVneo) and either the full-length human PCNA gene or the human PCNA gene lacking intron 1. RNA was extracted from established mixed population of cells, either from growing cells or quiescent cells after serum deprivation for 4 days. A human specific 5' PCNA probe, the histone H3 probe and the 3A10 probe were used. Lane 1 (quiescent cells) and lane 2 (growing cells): RNA from cells carrying the human PCNA gene without intron 1. Lane 3 (quiescent cells) and lane 4 (growing cells): RNA from cells carrying the full th human PCNA gene.

binding reactions (25 μ l) contained 10mM Tris-HCl (pH 7.5), 50mM NaCl, 1mM dithiothreitol, 1mM EDTA, 5% glycerol, 3 μ g of poly (dI 5 or 10 μ g nuclear extract, and the probe (~0.1ng, 5,000 c.p.m). After 30min incubation at room temperature, the complexes were resolved in a 4% polyacrylamide gel (acrylamide/bis ratio of 29:1). The running buffer was 380mM glycine, 2mM EDTA, and 50mM Tris. First, a 109bp fragment between +361 and +469 (end of exon 1, +380) of the human PCNA gene was used as a probe. Two 20-mer oligodeoxynucleotides were synthesized as primers. The polymerase chain reaction was performed according to the instructions of Perkin Elmer Cetus. For labeling ³²P-dCTP was included in the reaction. After amplification, the probe was purified on a polyacrylamide gel. Subsequently, a 35 bp fragment from +441 to +475 in intron 1 of the human PCNA gene was prepared as a probe. Two 35-mer oligodeoxynucleotides were synthesized, annealed, and end-labeled (27). The synthetic probes used as competitors were synthesized as single strand oligodeoxynucleotides and annealed. All oligodeoxynucleotides were synthesized on an Applied Biosystem, Inc., Model 391 EP DNA synthesizer using β -cyanoethyl phosphoramidite chemistry.

Methylation interference analysis

Methylation interference analysis was performed essentially as described (28). Two 35-mer oligodeoxynucleotides (+441 to +475) were synthesized, one of them endlabeled, annealed, and used as probes.

Fig. 2. Gelshift analysis of the human PCNA intron 1 sequence, incubated with nuclear extracts from TK⁻ts13 cells. Gelshift assay with a 109 bp fragment, from residue +361 to residue +469 straddling the end of exon 1 and beginning of intron 1 (+380/381) of the human PCNA gene. Two 20 oligodeoxynucleotides were synthesized as primers to amplify by PCR the 109 bp fragment. The binding assay was performed as described by Singh et al., (26,45). The lower bands (F) represent unbound DNA and the bands indicated by B represent the major complexes formed. Lanes:(1) No added nuclear extracts; (2-4) 10 μ g nuclear extracts of quiescent cells, cells stimulated for 12h at permissive temperature, and cells stimulated for 12h at restrictive temperature, respectively; (5-7) same as 2-4, but competed with the same 109 bp non-radioactive fragment in a 50 times excess; (8-10) same as 2l competed with a sequence adjacent to the 109 bp fragment (100 bp from +281 to +380 of the PCNA gene) in a 50-fold excess; (11 same as 2-4, but in the presence of an unrelated competing sequence containing a CCAAT element (102 bp from -137 to -238 of the promoter region of the PCNA gene) in a 50-fold excess.

Sequencing of murine PCNA intron 1

A genomic murine PCNA was isolated by screening a Balb/c liver genomic library (29). A 3.1 kb Hind III-Xba I fragment of a genomic mouse PCNA clone was subcloned and used for sequencing murine PCNA intron 1 by the chain termination method (30) following the Sequenase protocol.

RESULTS

Deletion of Intron 1 Deregulates PCNA Expression

In preliminary experiments with Tk⁻ts 13 cells, we had noticed that deletion of intron 1 jK in a transfected PCNA gene resulted in overexpression of the PCNA mRNA. Tk⁻ts are not as exquisitely growth-regulated as BALB/c 3T3 cells (31); we therefore repeated the experiment by transfecting the 2 PCNA genes (wt and minus intron 1) into BALB/c 3T3, together with a selectable marker (see Materials and Methods). Mixed populations for each transfected gene were obtained. Fig. 1 shows the RNA blot of a typical experiment in which RNA was extracted from exponentially growing or quiescent cells. A human-specific PCNA probe was used which does not hybridize to mouse PCNA mRNA (12). In cells carrying the wt human PCNA gene, the expression of human PCNA mRNA is growth-regulated, being low in G₀ (lane 3) and high in serum-stimulated cells (lane 4). In cells carrying the minus intron 1 gene, human PCNA is equally expressed in G₀ and S phase cells (lanes 1 and 2) indicating a loss of growth regulation. The absence of histone H3 expression in lanes 1 and 3 confirms that the serum-deprived cells were not cycling. In addition, when the same blot was hybridized to a mouse-specific probe, it showed that the endogenous mouse PCNA gene was growth-regulated as the transfected wt human gene (not shown). The 3A10 probe detects a mRNA, whose expression is constant throughout the cell cycle and is independent of cell proliferation (24).

Fig. 3. Gelshift assay with a 35 bp fragment from residue +441 to residue +475 in intron 1 # of the human PCNA gene incubated with nuclear extracts from the human cell line T98G. To prepare the probe, two 35-mer oligodeoxynucleotides were synthesized, annealed, and endlabeled. The lower bands (F) represent unbound DNA and the bands indicated by B represent the major complexes formed. (a) Lanes: (1) No added nuclear extracts; (2) 10 μ g nuclear extracts of T98G cells serum-stimulated for 8h; (3) same as (2), but competed with the same nonactive 35bp fragment in a 50 times excess; (4) same as (2), but competed with the mutated 35bp fragment shown in Fig. 5b.

Gel Shift Analysis of PCNA Intron 1

Intron 1 is 709 bp long from nucleotide +381 to nucleotide +1088 of the PCNA gene following the numbering of Travali et al. (18). It was desirable to identify a shorter sequence whose effect on PCNA mRNA levels could then be investigated. We elected to search for this sequence by gel shift and methylation interference analysis. Several probes were synthesized spanning the whole length of intron 1 (data not shown) and we eventually concentrated on probes straddling exon 1 and intron 1, from nucleotide +281 to nucleotide +560. These probes were incubated with nuclear extracts (24) from TK⁻ts13 cells and analyzed by gel shift retardation (26). One probe was retarded on gel shifts; all others failed to show retardation bands (data not shown). The probe retarded on gel shift (Fig. 2, lanes 2–4) was a PCR-prepared probe extending from +361 to +469; the binding was competed by an excess of identical cold probe (lanes 5–7), but not by unrelated probes (lanes 8–13). Although we have designated the retarded band as a single complex (called B), careful examination shows that there are two major bands, and possibly two minor bands. There is a decrease of the second major retardation band and the appearance or at least an increase

Fig. 4. Methylation interference analysis of the human PCNA intron 1-B probe. Two 35-mer oligodeoxynucleotides were synthesized, one of them end-labeled, annealed, and used as probes. Preparative binding reactions were done with partially methylated probe essentially as described in Gilman et al. (28). Lanes: (c) Maxam and Gilbert G sequencing reaction. (b) DNA recovered from the bound fraction. (f) DNA recovered from the free fraction. Numbers indicate points of nucleotide-protein contact: open circles, weak interaction; full circles, strong interaction (measured by densitometry). Nuclear extracts of TK⁻ts13 cells were used.

in the minor bands when nuclear extracts are used from growth-arrested cells (lanes 2–4), but lanes 11 to 13 (from which non specific binding has been completely eliminated) are even more convincing showing the differences in the different physiological conditions. We have reported previously that in growth arrested cells (stimulation at the restrictive temperature), PCNA mRNA levels are sharply decreased (9, 32). Of particular interest is the finding that there is no competition (lanes 11–13) from a probe extending from –137 to –238 of the 5' flanking sequence of the human PCNA gene (18). This probe includes a reverse CCAAT (33); its failure to compete will become important in co-transfection experiments (see below).

We have further reduced the protein-binding sequence to a 35 bp fragment extending from +441 to +475. For this purpose, probes were synthesized, annealed and end-labeled. Fig. 3 shows a gel shift analysis with the 35 bp probe (+441 to +475) after incubation with nuclear extracts from T98G cells (17). In lane 3, the probe was competed with itself (50× excess); in lane 4, the probe was competed with a mutant sequence. The sequences of the wt probe and its mutant are given below. The wt 35bp probe will be called intron 1-B probe.

Methylation Interference Analysis

Figure 4 shows a methylation interference analysis of intron 1 centered on the sequence of the intron 1-B probe. The protein-nucleotide contacts can be identified at the following residues: A458, G461 and G462 on the coding strand, and G453, A454,

(a) Human PCNA 35bp intron 1-B probe.

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+441                ○ ●●                +475
CTTCGAGCCTAGCCCTCATTGGCTGGCGTGGGCAT
GAAGCTCGGATCGGGAGTAACCGACCGCACCCGTA
                ●● ○● ○○

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(b) Mutated human PCNA 35 bp intron 1-B probe.

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                **
CTTCGAGCCTAGAACTAATTTTATGGCGTGGGCAT
GAAGCTCGGATCTTGATTAAATACCGCACCCGTA
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(c) Comparison between mouse and human intron 1-B probe.

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TGTGACTCTGCACGCTCATTGGCTGGCTTGCCAT
|      |      | | | | | | | | | | | | |
CTTCGAGCCTAGCCCTCATTGGCTGGCGTGGGCAT
+441                +475

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Fig. 5. Sequences of the human PCNA intron 1 probe, its mutant, and comparison with the corresponding sequence in the PCNA gene of mouse (29, 35).

A456, G457, A459 and A460 on the non coding strand, still using the nucleotide numbering of Travallo et al (18). The sequence of the intron 1-B probe with its contact points is shown in Fig. 5. Note that the sequence contains a reverse CCAAT box on the non-coding strand, precisely in the area of the contact points. The sequence of the mutant probe used in Fig. 3, is also shown in Fig. 5, where we also make a comparison with the corresponding sequence in the mouse PCNA gene (see below).

Competition by Other Probes

In these experiments, we asked whether the intron 1-B probe's gel retardation by nuclear extracts could be competed out by synthetic probes, known to be binding sites for different transcriptional factors. Figure 6 shows a list of these probes, together with their sequences and a gel shift analysis of intron 1-B probe competed with the same probes (cold and in large excess). In the gel shift of intron 1-B probe, the major bands are competed by the proximal CCAAT element of the human TK gene (33, lane 3), by the distal CCAAT element of the same gene (lane 5), by the CCAAT element of the Major Histocompatibility Complex gene (lane 6) and by the CCAAT

Fig. 6. Gelshift assay with the intron 1-B probe incubated with nuclear extracts of T98G cells and competed by synthetic probes. 5 μg nuclear extracts were used in all lanes and the competitors were in a 100 times excess. The lower bands (F) represent unbound DNA and the bands indicated by B represent the major complexes formed. Lanes: 1: No added nuclear extracts; 2: 5 μg nuclear extracts of T98G cells stimulated for 8h; 3: Competition with PTK: GCGGGGCCGGC-TCGTGATTGGCCAGCAGCCGT, proximal CCAAT element of the human TK gene (46); 4: PTKM1: GCGGGGCCGGCTCGTGATCAGCCAGCAG-CCGT, mutated proximal CCAAT element of TK; 5: DTK: CAGCGGCCGG-TCGTGATTGGCCCATGGCGGC, distal CCAAT element of the human TK gene; 6: MHC: CGTAAACATTTTCTGATTGGTTAAAAGTTGAG, CCAAT of major histocompatibility complex (47); 7: H1: CTAGGTGATGCACCA-ATCAGCGCGCCCTAC, CCAAT of histone H1 (48); 8: HSV: CCCAGC-GTCTTGTCATTGGCGAATTCGAACA, 31bp (–70 to –100) of the HSV-1 thymidine kinase promoter including an inverted CCAAT (49); 9: alpha-G: GCGCGCTCATTTGGCTGGCGCGGAGCCCG, CCAAT of alpha-globulin (50); 10: beta-G: GTGTGAGCAGATTGGCCCTTACCAGGGTGT, CCAAT of beta-globulin (50); 11: NF-1: ATTTTGGATTGAAGCCAATATG, 22bp of the CTF/NF-1 binding site in the upstream region of the human ras-1 (Ha-ras) gene including a CCAAT (51); all the following sequences are in the 5' flanking regions of the respective genes; 12: AP3 or C/EBP (52): CTAGTGGGACTTCCAC-AGATC; 13: SP1 (53): GATCGATCGGGGCGGGGCGATC; 14: AP1 (54): CTAGTGATGAGTCAGCCGGATC; 15: Oct1 (45): AGTCGACATTTGCA-TCCATGGT.

elements of histone H1 (lane 7) and HSV (lane 8). It is not competed by a mutant of the TK proximal CCAAT and by the other probes, including 2 other CCAAT-containing sequences.

The binding affinity of the intron 1-B probe was also determined by using different concentrations of the competing probes (Fig. 7). The MHC and H1 probes were good competitors even at low concentrations (lanes 9–11 and 12–14, respectively). The other competing probes were effective only at higher concentrations.

Co-transfection with Intron 1-B Probe Inhibits the Expression of the PCNA Gene

For these experiments we used a transient expression assay. The full length human PCNA gene was co-transfected with equal amounts of intron 1-B probe into TK⁻ts13 cells, that are highly transfectable (15). The expression of human PCNA mRNA was detected with a human specific PCNA probe (located in the 5' untranslated region of the PCNA gene) that does not detect hamster or mouse PCNA mRNA (12,14). The RNA blots of one such experiment are shown in Fig. 8. No human PCNA mRNA is detectable in mock transfected cells (lane 5), but is clearly detectable (and concentration dependent) in cells transfected with 5, 10, or 15 μ g of the full length human PCNA gene (lanes 2 to 4). Co-transfection with equal amounts of intron 1-B probe markedly decreases the expression of human PCNA mRNA (lane 6). If a mutated intron 1 probe (with mutation in the reverse CCAAT box, see Fig. 5) is co-transfected with the human PCNA gene, no inhibition occurs (lane 7), indeed, there may even be a modest increase in expression. Comparison of densitometry measurements (lane 3=100) gave the following readings: lane 1 12.3%, lane 2 64%, lane 3 100%, lane 4 137%, lane 6 14.3%, and lane 7 259.5%. The 3A10 probe was again used to monitor RNA amounts per cell (24).

Since the human PCNA gene contains other CCAAT elements in its 5' flanking sequence (18,33,34), it is possible that the intron 1-B probe competition obtained in co-transfection experiments

Fig. 7. Gelshift binding affinity assay of the intron 1-B probe using different concentrations of some of the competing probes shown in Fig. 6. 5 μ g nuclear extracts were used in all lanes and the concentrations of the non active competitors were in a 100, 50, 10 excess, respectively. Lanes: (1) No added nuclear extracts; (2) 5 μ g nuclear extracts of T98G cells stimulated for 8h; (3–5) Competition with PTK in a 100, 50, 10 times excess, respectively; (6–8) TK; (9–11) MHC; (12–14) H1; (15–17) HSV; (18–20) SP1.

could be due to competition with the CCAAT elements in the 5' flanking region. We therefore repeated the experiments with the human PCNA gene driven by an abbreviated promoter, a 73 bp promoter that contains an enhancer, no CCAAT elements and is as effective in promoting transcription as the full length promoter (19). The results are shown in Fig. 9. Co-transfection with the intron 1-B probe again inhibits the expression of the human PCNA gene driven by the –73bp promoter (lanes 3 and 4), the expression drops 60% according to densitometry measurements, indicating that the inhibitory effect is not due to CCAAT sequences in the 5' flanking region of the PCNA gene (see also Fig. 2). When the minus intron 1 gene is used for co-transfection, the levels of human PCNA mRNA are the same in the presence or absence of intron 1-B probe (data not shown). In these experiments, we could not use the endogenous mouse PCNA gene as a control. Although it is possible that the intron 1-B probe may have also inhibited the expression of the endogenous gene, it would be impossible to detect it, because of the small number of cells that actually become transfected. However, these co-transfection experiments have been repeated 3 times with essentially the same results.

The CCAAT Element in Intron 1 is Conserved in the Mouse PCNA Gene

If the intron 1 probe plays an important role in regulating PCNA mRNA levels, it should be conserved in different species. The mouse PCNA gene (29) has been sequenced by Yamaguchi et al. (35) and by our laboratory (data not shown). Fig. 5 includes a comparison of the human and mouse sequences corresponding to our intron 1 probe. While the sequence similarity is only about 40% throughout intron 1, in the reverse CCAAT region (from +451 to +475) it is 80%, (20 out of 25 nucleotides), which is the same degree of sequence similarity that is obtained in the

Fig. 8. Transient expression assay of the full-length human PCNA gene co-transfected with the intron 1-B probe into Tk⁻ts13 cells. A human specific PCNA probe was used to detect the human mRNA. RNA was extracted 40h after transfection. Lanes: (1) 2.5 μ g of full-length human PCNA DNA transfected; (2) 5 μ g; (3) 10 μ g; (4) 15 μ g; (5) Mock transfected cells; (6) 10 μ g of full-length human PCNA DNA co-transfected with 10 μ g of the intron 1-B probe; (7) 10 μ g of full-length human PCNA DNA co-transfected with 10 μ g of the mutated intron 1-B probe (see Fig.5).

Fig. 9. Transient expression assay of the 73bp promoter driving the PCNA gene, co-transfected with the intron 1-B probe into Tk⁻ts13 cells. A human specific PCNA probe was used to detect the human mRNA. RNA was extracted 40 h after transfection. Lanes: (1) 10 μ g of full length human PCNA DNA transfected; (2) Mock transfected cells; (3) Transfection of 10 μ g of 73 bp promoter driving the PCNA gene DNA. (4) Same as 3 but co-transfected with 10 μ g of the intron 1-B probe.

respective PCNA coding sequences. Actually, in the binding region (see Fig. 5) which comprises nucleotides +453 to +462, the sequence similarity is 90% (9 out of 10).

DISCUSSION

In a previous paper, Ottavio et al. (14) had shown that intron 4 played a role in the regulation of PCNA mRNA levels. Deletion of intron 4 results in abnormally high levels of PCNA mRNA in G₀ cells. Elements in the 5' flanking sequence of the human PCNA gene also play a role in the control of mRNA levels (19,34). Specifically, Pietrzowski et al. (19) have shown that a -73bp promoter (the 73 bp immediately upstream of the CAP site) is a very efficient promoter, contains an enhancer between -73 and -45, and is still growth-regulated. We report here that intron 1 also exerts a negative regulatory effect on the expression of the PCNA gene. This statement is supported by the following findings: 1) deletion of intron 1 results in de-regulated expression of a transfected human PCNA gene in two different cell lines, TK-ts13 and BALB/c 3T3 cells. In the TK-ts13 cells, PCNA mRNA levels are increased in both G₀ cells and cells serum-stimulated at the permissive temperature; in 3T3 cells, the human PCNA mRNA levels are increased in serum-deprived cells, while in serum-stimulated cells, the results are ambiguous; 2) in transient expression assays, the co-transfection of a 35 bp fragment from intron 1 inhibits the expression of the transfected human PCNA gene. Co-transfection of a mutant 35 bp fragment fails to inhibit the expression of the human PCNA gene.

These results can be explained with the hypothesis that intron 1 in quiescent cells down-regulates the levels of PCNA mRNA; that, in growing cells, more proteins bind to intron 1 and suppress its inhibitory effect; and that the inhibitory effect is maintained if these protein(s) are kept from binding to intron 1 by co-transfection with equal amounts of the 35 bp fragment.

This 35 bp intron 1 fragment binds nuclear protein(s) in a gel shift assay. We have used nuclear extracts from different cellular sources and they all gave retardation bands with what we call the intron 1-B probe (=35bp fragment). The binding to the intron 1-B probe was effectively competed by the probe itself but not by a mutant (see Fig. 2 and 3). The amount of protein(s) binding to the intron 1-B probe, by gel shift, is higher in growing cells than in growth-arrested cells. It seems therefore that some protein(s) do bind to the intron 1 sequence even in quiescent cells, when PCNA mRNA levels are very low, and that the additional protein(s) binding to it are those that repress its inhibitory effect.

The 35 bp fragment contains an inverted CCAAT element, which is probably crucial to its function for 3 reasons: 1) when the CCAAT is mutated, the mutant probe no longer competes for binding with the wt sequence; 2) by methylation interference analysis, the nucleotide residues that bind nuclear proteins center around and in the CCAAT element; and 3) a 35 bp fragment mutated in the CCAAT element fails to compete with a co-transfected human PCNA gene in transient expression assays (Fig 8 and 9). The importance of the CCAAT element in the negative regulatory action of intron 1 finds indirect support in the fact that it is well conserved in the mouse genome, as conserved as the PCNA coding sequence itself.

It is now known that CCAAT-binding proteins constitute a family of proteins that bind with different specificities to various CCAAT containing sequences (36-39). It was therefore important to determine the relationship of our CCAAT element to other CCAAT elements in the 5' flanking region of the PCNA

gene and other sequences known to bind transcription factors. It is clear from Fig. 6 and 7, that the intron 1-B probe is not competed out by unrelated transcription factors binding sequences like those for Octamer 1, AP1 and SP1, C/EPB and also by some sequences containing direct or reverse CCAAT, like NF-1 and the alpha and beta globins. The best competitors were CCAAT containing sequences from histone H1 and MHC genes; CCAAT sequences of human and HSV TK were competing, but less effectively.

Since the 5' flanking sequence of the human PCNA gene also contains 2 CCAAT elements (18,33,34) we also had to rule out the possibility, especially in co-transfection experiments, that other CCAAT sequences in the human PCNA gene could compete with the intron 1-B probe. Fig. 2 shows that the CCAAT element in the 5' flanking sequence, from -137 to -238, fails to compete. Furthermore, in co-transfection experiments, the intron 1-B probe inhibits the expression of the human PCNA gene, even when this gene is under the control of the -73bp promoter, thus excluding the CCAAT elements that are present in the upstream sequence of the 5' flanking region of the human PCNA gene (18,33,34).

A negative regulation by a CCAAT element in intron 1 is especially attractive because: 1) protein binding to an inverted CCAAT box has been shown to induce termination of RNA pol II transcription (40, 41); 2) homologous sequences are found within apparent termination regions downstream of the poly (A) site in several vertebrate gene (42); 3) one CCAAT-binding protein, C/EBP, inhibits cell proliferation and promotes terminal cell differentiation (43). The obvious question is whether the inverted CCAAT in intron 1 of the PCNA gene acts as a signal for transcriptional pause. Regrettably, we cannot give a prompt answer to this question, because of the many artifacts displayed in run-on assays by the PCNA gene, from antisense transcription to the activation of crypto promoters (Sell et al. in preparation).

It is known that a mutation in an intron of the Ha-c-ras gene increases its expression (44). Thus, negative regulatory elements located in introns are known; it will be now important to identify the protein(s) that bind to these negative elements, modifying their function. In preliminary experiments, we have found that at least 4 different proteins bind to the 35 bp intron 1 probe (Ferber et al. in preparation). We do not have any information, though, on whether the number of binding proteins change from growing to growth-arrested cells.

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