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RNA polymerase III promoter elements enhance transcription of RNA polymerase II genes

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ABSTRACT

Using transient expression assays in cultured human cells we have observed that RNA Polymerase III promoter sequences exert a positive *cis*-acting enhancer effect on RNA Polymerase II transcription. A DNA segment containing a copy of the Alu repeated element enhances transcription of the liver specific Haptoglobin related (Hpr) promoter in Hepatoma cell lines but not in HeLa cells. A $tRNA^{pro}$ gene acts as enhancer of the SV40 promoter both in Hepatoma and in HeLa cell lines. Transcription from the SV40 promoter is also enhanced by DNA segments containing only the box A or the box B of the $tRNA^{pro}$ promoter.

INTRODUCTION

In all eukaryotes there are three distinct RNA Polymerases responsible for transcription of different classes of genes (1). Purified preparations of RNA Polymerase I, II or III are not capable of correct initiation of transcription from natural promoters (2, 3). For this purpose it is necessary to supplement the purified RNA Polymerase with additional factors which are apparently essential for the formation of a stable transcriptional complex (3). RNA Polymerase II *cis*-acting regulatory elements have been found mainly in the 5' flanking sequences of genes (4), while the RNA Polymerase III regulatory sequences so far characterized are mostly internal to the transcribed genes (4) although the 5' flanking region seems to play a role at least in a subset of RNA Polymerase III genes (5). Recently it has been reported that the octamer binding protein, a well characterized *trans*-acting factor essential for transcription of several promoters by RNA Polymerase II, is also necessary for transcription of the U6 gene by RNA Polymerase III (8, 9). Conversely it has been reported that ID elements, which

are transcribed by RNA Polymerase III, act as enhancers of RNA Polymerase II promoters in cell lines that express these RNAs (10). A contribution of RNA Polymerase III transcriptional signals to the efficiency of transcription from RNA Polymerase II promoters was suggested by the observation that, in transfection experiments in the human hepatoma cell line Hep G2, chimeric constructs of the Hpr promoter, which contain an Alu repeated element 183 nucleotides upstream from the transcriptional start site, are 3-4 fold more active than constructs driven by the highly homologous Hp 1 promoter (11). Here we report that either an Alu repeated element or a $tRNA^{pro}$ promoter enhance the accurate transcription of a downstream RNA Polymerase II promoter.

MATERIALS AND METHODS

Cell culture

Human hepatoma Hep G2 (12) and HeLa cells were cultured as monolayers in Dulbecco's Modified Eagle's medium supplemented with 10% fetal calf serum, glutamine (final concentration 5mM) and penicillin/streptomycin (100 U/ml).

Plasmid construction

Hpr-CAT plasmid results from the subcloning of the Xba I-Hind III DNA fragment from the Hpr 5' flanking sequence in the Xba I-Hind III sites of pEMBL8-CAT (11). Hpr Δ Alu-CAT was obtained by oligonucleotide directed deletion in the Xba I-Hind III fragment(see text) as previously described (13). Hpr-183-CAT was obtained from Hpr Δ Alu-CAT by Bam HI-Kpn I digestion, treatment with T4 Polymerase and religation at low concentration of the vector.

$tRNA^{pro}$-CAT constructions were obtained by inserting the blunt ended 263 base pairs Eco RI-Eco RI fragment from mcet1 (14) into the Sma I site of the vector pUC19-CAT_2 (11). In the same site of pUC19-CAT_2 were also inserted the Eco RI-Sma I fragment deriving from the same mcet1 plasmid containing either the box A or B in both orientations. All constructions were verified by DNA sequencing (15). To obtain probes for the S1 mapping, the Sac I-Eco RI fragment deriving from Hpr-CAT and containing the Hpr promoter sequence fused to 250 bases from CAT

gene was subcloned into the Sac I-Eco RI sites of mp19. The S1 probe for the $tRNA^{pro}$-CAT and pSV2-CAT constructions was obtained by cloning the Bam HI-Eco RI fragment from pSV2-CAT (16) into mp19.

DNA transfections

DNA transfections were performed by the calcium phosphate precipitation technique (17). 10 µg of plasmid DNA for each 60 mm dishes (Falcon) were used. The cells were harvested 36 hours after transfection either for CAT assays (17) or for the RNA extraction with guanidine thiocyanate (11).

S1 mapping

S1 mapping was performed according to Berk and Sharp (18). All probes were obtained by *in vitro* elongation on M13 single stranded templates. For the synthesis of radioactive probes 400 ng of single stranded phage DNA was incubated with the commercial 17 mer sequence primer (Biolabs) in presence of 50mM NaCl, 10mM Tris pH 7.4, 10mM $MgSO_4$, 1mM DTT, 100µM dGTP, 100µM dTTP, 40µCi [α ^{32}P] dATP and 40µCi [α^{32}P] dCTP (Amersham, sp.act. 3000Ci/mmol) with 10 units of DNA Polymerase I large fragment in a total volume of 20 µl. After 15 min at room temperature, dATP and dCTP were added to a final concentration of 100 µM and the incubation continued for 15 min. The synthesized DNA was digested with either Sac I for the Hpr probe, or Bam HI, for the SV40 probe and the resulting labeled DNA segments were isolated by polyacrylamide gel electrophoresis under denaturing conditions.

RESULTS

The Haptoglobin related gene (Hpr) is highly homologous to the liver specific Haptoglobin gene (Hp). The homology is interrupted at position -183 of the Hpr gene by the presence of an Alu sequence (19-20). In transfection experiments with the hepatoma cell line HepG2 the 5' flanking region of the Hpr gene promotes transcription more efficiently than the equivalent region from the Hp gene. Both promoters are inactive in HeLa cells (11).

To determine whether the Alu sequence present in the Hpr 5' flanking sequence could be influencing the expression of the downstream promoter we performed site-directed *in vitro*

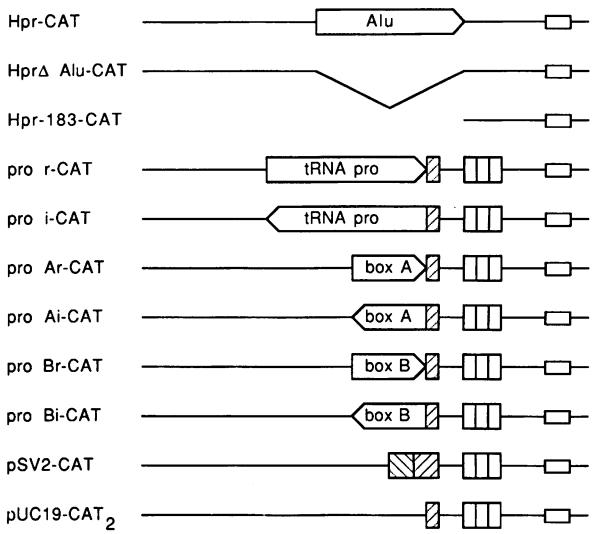

Fig.1 Scheme of plasmid constructions. The horizontal white rectangles indicate the TATA box. The vertical white rectangles indicate the 21 bp repeats of the SV40. The dashed boxes indicate the 72 bp repeats of SV40 while the dashed rectangles indicate only the part till the Sph I site. The orientations of the RNA Polymerase III sequences cloned in front of the RNA Polymerase II promoters are indicated.

mutagenesis on this DNA segment using the oligonucleotide 5'ATTTTCCCGTGGTACCTCGTGAGGGCT3' which anneals at both sides flanking the Alu sequence and therefore permits its selective deletion. The oligonucleotide was designed to insert a new Kpn I site in the mutated plasmid. The new construction differs from

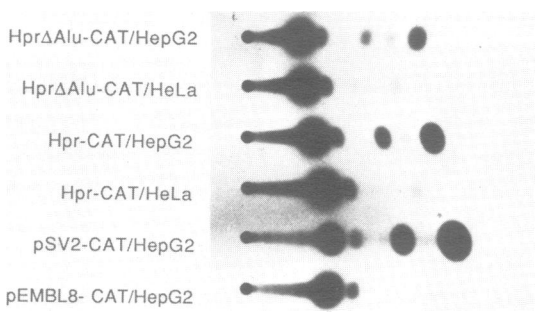

Fig.2 CAT activity in transfected cells. In each lane the construction used and the cell type transfected is indicated.

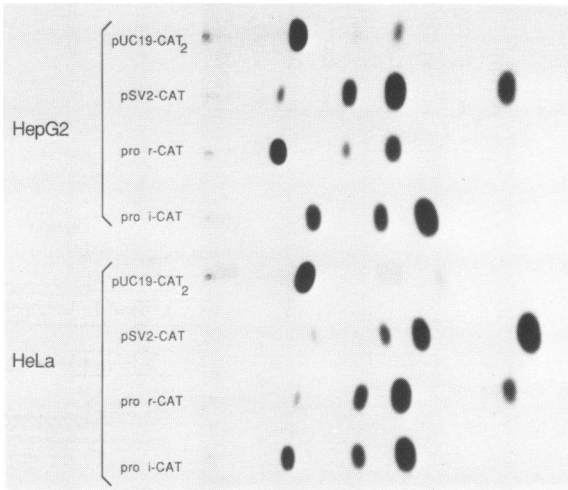

Fig.3 CAT activity in transfected cells.

the Hpr 5' flanking region by the absence of the Alu sequence (fig. 1) and the insertion of a T residue necessary to generate a Kpn I site.

Both the Hpr 5' flanking sequence and the mutagenized Hpr Δ Alu were cloned in front of the bacterial chloramphenicol acetyl transferase gene (CAT). The resulting plasmid were transfected into both the hepatoma cell line Hep G2 and HeLa cells. As shown in fig.2, both constructions are expressed in Hep G2 cells (lanes 1 and 3). The two constructions show a quantitative difference: the Hpr-CAT construction is expressed about 3 times more than Hpr Δ Alu-CAT. The transfection experiment has been repeated several times with different DNA preparations and we always observed the same difference in expression. Neither construction is expressed in HeLa cells, showing that the Alu sequence in front of the Hpr promoter is not required for the tissue specificity of the downstream promoter.

A new construction generated by deleting all the upstream sequences from position -183 is expressed in Hep G2 cells at the same level as Hpr Δ Alu-CAT. This shows that in this experimental system further upstream sequences do not contribute to the efficiency of transcription and do not encode ancillary transcription start sites. As shown by the results of an S1

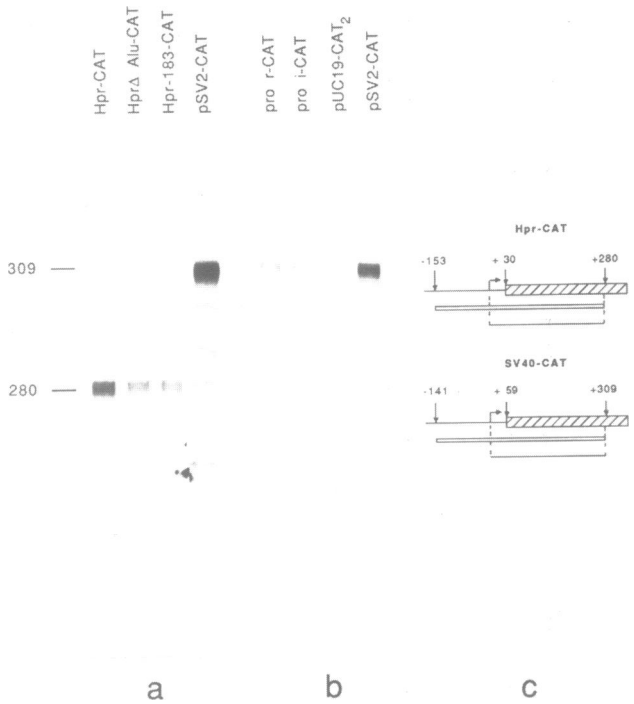

Fig.4 S1 mapping of the CAT mRNAs. Panel A: S1 mapping of Hpr-CAT constructions transfected into HepG2 cells. Panel B: S1 mapping of the tRNA-CAT constructions transfected into HeLa cells.Panel C: Schematic draw with the position of the probes (open lane) and the protected band with respect to the CAT transcription units.

mapping experiment (fig. 4a), Hpr-CAT, Hpr Δ Alu-CAT and Hpr-183-CAT transcripts start at the expected cap site.

A $tRNA^{pro}$ gene in front of the SV40 promoter enhances its transcription

The observation that an Alu sequence inserted in front of an RNA Polymerase II promoter increases the accurate transcription of a downstream promoter led us to test whether any gene transcribed by RNA Polymerase III could, when inserted in front of an RNA Polymerase II promoter, behave in the same way. As a model RNA Polymerase III gene we used a $tRNA^{pro}$ gene which has been previously characterized (14). We constructed two new plasmids by insertion of the $tRNA^{pro}$ gene, in both orientations,

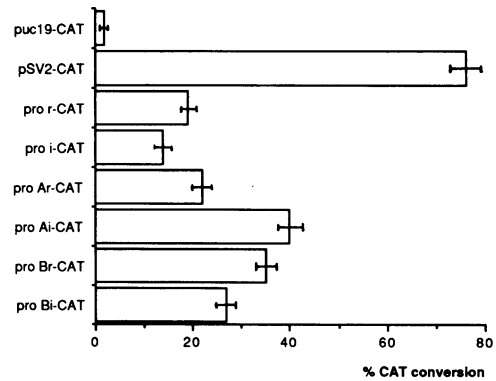

Fig.5 CAT activity in transfected HeLa cells. The results of three transfection experiments are reported. Bars indicate acetylated over non acetylated form of chloramphenicol.

in front of the SV40 promoter present in the previously decribed plasmid pUC19-CAT_2 (11), which contains the SV40 promoter fused to the CAT gene (see fig. 1 for a scheme). These constructions were transfected into HepG2 and HeLa cells. The results are shown in fig.3. The expression of the CAT gene is enhanced between 8 and 10 times when $tRNA^{pro}$ gene is inserted in front of the SV40 promoter in both orientations. Transcription from these constructions starts at the accurate RNA Polymerase II starting point and no longer transcripts are present (fig. 4b).

Box A and box B enhance RNA Polymerase II transcription

It has previously been shown that for active transcription the $tRNA^{pro}$ promoter must contain at least two transcriptional signals, box A and box B. Deletion of either boxes inactivates the promoter (4). We show here that the enhancement of an RNA polymerase II promoter does not require an intact RNA Polymerase III promoter and DNA segments containing only box A or box B are active. This is shown by the results obtained with a new set of plasmids in which DNA segments carrying either the box A or the box B were cloned in front of the SV40 promoter. In fig. 5 are shown the results of three independent transfections in HeLa cells. DNA segments carrying either box A or box B in both orientations enhance between 10 and 20 times the SV40 promoter transcription.

DISCUSSION

Different RNA Polymerase III genes put in front of RNA Polymerase II promoters are able to increase their transcriptional levels. In the case of the Alu sequence the enhancement observed is about three times the basal level of the downstream promoter. This enhancement does not affect the tissue specificity of the RNA Polymerase II promoter. Comparable results can be obtained with constructions where the $tRNA^{pro}$ gene is cloned in front of the SV40 promoter. The enhancement due to the $tRNA^{pro}$ promoter sequences is observed in HeLa as well as in Hepatoma cells.

It is very unlikely that transcripts starting within the RNA Polymerase III gene continue reading through the RNA Polymerase II promoter because i) the initiation of transcription of all the constructs is at the expected Polymerase II cap site; ii) the Alu sequence of the Hpr promoter in injected Xenopus oocytes, directs transcription and also efficient termination at the stretch of four T that is present in its 3' sequence (Ciliberto and Cortese, unpublished observations); iii) we do not observe CAT activity in constructs where the Alu sequence in front of CAT gene is not followed by RNA Polymerase II promoters (data not shown).

In experiments performed with ID sequences (10), enhancer activity has been observed only in cells where the ID sequences are expressed, but it was not established whether transcription of the RNA Polymerase III gene was important for enhancement of transcription of the adjacent RNA Polymerase II promoter. The experiments where segments of DNA carrying either the box A or box B are transfected show that the enhancement is independent of RNA Polymerase III transcription. Box A or box B alone can in fact activate the RNA Polymerase II transcription even better than the whole RNA Polymerase III gene does, suggesting that *trans*-acting factors responsible for activating RNA Polymerase III genes are also able to interact directly or indirectly with the RNA Polymerase II transcriptional machinery.

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