Vol. 127, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1985 Pages 239-246

ENHANCER-FACILITATED EXPRESSION OF A HUMAN H4 HISTONE GENE

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Received January 15, 1985

Summary: Cultured mammalian cells were transfected with a recombinant human H4 histone gene. S1 nuclease mapping of cellular RNAs from transfected cells revealed: (i) correct initiation of transcription at the cap site, with some transcripts originating from other sites in the 5′ flanking region of this H4 gene; (ii) cis-linkage of an SV-40 transcriptional enhancer element upstream of the H4 5′-flanking region resulted in about a 50-fold increase in the level of correctly initiated H4 mRNA and (iii) in a heterologous murine system stability of human H4 mRNAs was apparently sensitive to inhibition of DNA-synthesis by hydroxyurea. Our results suggest that certain sequences required for the initiation of a human H4 histone gene transcript reside within the 210 nucleotides immediately upstream from the cap site and that the level of expression is influenced by the introduction of an enhancer element.

Human histone genes represent a family of moderately reiterated sequences arranged in clusters located on several chromosomes (1,2). Most of these clusters contain combinations of core (H2A, H2B, H3 and H4) histone genes with or without H1 histone mRNA coding regions (3,4). In human (HeLa) cells at least 15 different histone genes, are coordinately expressed during the S phase of the cell cycle (5) and appear to be temporally and functionally coupled with DNA replication. Histone genes are similarly expressed (6) in human diploid fibroblasts. Both transcriptional and post-transcriptional components contribute to the regulation of those histone genes expressed in conjunction with DNA replication (5,7).

The isolation and characterization of a human genomic H4 histone gene designated pF0108 (3,9) has enabled us to identify consensus 5′ sequences

0006-291X/85 $1.50

which putatively function as regulatory elements (8). Using 5′ deletion mutants of this human H4 gene, it has been previously demonstrated that sequences upstream from the consensus TATA box (8) are not required for site-specific initiation of transcription *in vitro* by RNA polymerase II (9). As an initial attempt to define 5′ regulatory sequences necessary for human H4 histone gene transcription *in vivo*, we have monitored transient expression of this gene directed by its own promoter region either with or without the influence of a viral enhancer element.

Enhancers are cis-acting DNA sequences that stimulate the transcription of genes, natural or cloned, in an orientation-independent manner (10,11). A number of enhancers have now been identified, both of viral and cellular origins (10,11). The archetypal enhancer, the 72 bp tandem repeat of simian virus 40 (SV40) DNA, was originally found to be cis-essential for the activation of early gene expression of T antigen which is in turn required for viral DNA replication (12). The activity of enhancers is not however, restricted to their natural gene partners. For example, the SV40 enhancer can dramatically stimulate transcription *in vivo* of the human β-globin gene (13). Similarly, SV40 sequences have recently been shown to influence transcription of a lower eukaryotic (sea-urchin) histone gene cluster after transfection into HeLa cells (14). Here we compare the SV40 enhanced versus nonenhanced transcription *in vivo* of a cloned human H4 histone gene in cultured cells.

MATERIALS AND METHODS

Plasmid Construction and Purification: All DNA constructs were made by standard recombinant DNA techniques (15) in accordance with the National Institutes of Health Guidelines for Recombinant DNA Research. Plasmid pF0108A was prepared by subcloning a 1.8 Kb EcoRI/Hind III genomic DNA fragment containing a human H4 histone gene into pBR322 as described previously (3,9). A 254 bp fragment of SV40 DNA containing the 72 bp tandem repeat enhancer region (but not the origin of replication) was removed from plasmid pDG014 (generously provided by Drs. Dan Greenspan and Sherman Weissman) by Kpn I digestion and then blunt end-ligated into the EcoRI site of pF0108A, generating the plasmid pSVE108A (Fig. 1). Circular supercoiled plasmid DNA was prepared by alkaline lysis (15) followed by cesium chloride/ethidium bromide equilibrium density gradient centrifugation (15) and Biogel A15-M gel filtration.

Cell Culture and Transfection: HeLa S3 cells and mouse LTK$^-$ cells were maintained at 37^{o}C with 5% CO_2, in Eagle's minimal essential medium (E-MEM) and Dulbecco's modified E-MEM respectively, supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Subconfluent (40-60%) cell monolayers (1-2x10^6 cells) in 10cm petri dishes were transfected with 25 µg of supercoiled DNA using the calcium phosphate co-precipitation (16) and glycerol-shock (17) procedures.

RNA Isolation: Total cellular RNA was isolated from cells harvested 36 to 48 hours after transfection, using proteinase K/DNase I digestion and phenol/chloroform extraction as described previously (5).

5'-end Labelling of DNA: pF0108A DNA was first digested with Sst II (BRL), dephosphorylated with bacterial alkaline phosphatase (Worthington Biochemicals) and labelled at the 5' terminus by T4 polynucleotide kinase (P.L. Biochemicals) using [^{32}P-γ] ATP (specific activity 4,500 Ci/mmole, New England Nuclear). Labelled DNA was then digested with EcoRI (BRL) and strand-separated (18) by denaturation in 30% DMSO followed by electrophoresis on a 5% polyacrylamide gel. The appropriate strand (indicated in Fig. 1) was recovered by electroelution and used as probe in S1 nuclease mapping (see below). A G+A nucleotide reaction (18) was performed on the labelled DNA and used to generate sequencing markers.

S1 Nuclease Mapping: RNA (25-250µg) was hybridized (19) to excess 5'-end labelled DNA (approximately 5ng) for three hours at 55^{o}C in 12.5-50µl of hybridization buffer (80% formamide, 0.4M NaCl, 0.04M Pipes (pH 6.4), 1mM EDTA). Hybridization mixtures were quenched with eight volumes of ice-cold S1 nuclease assay buffer (250mM NaCl, 30mM Na acetate (pH 4.4) 1mM $ZnSo_4$ and incubated with 2000 U/ml S1 nuclease (P.L. Biochemicals) at 37^{o}C for 30 minutes. S1 resistant hybrids were phenol/chloroform extracted, ethanol precipitated, then sized by electrophoresis on 6% polyacrylamide/8.3 M urea gels (18). Gels were soaked in 50% (v/v) ethanol for 30 minutes, dried and subjected to autoradiography at -70^{o}C for up to five days, using XAR-5 x-ray film (Eastman Kodak) and Dupont lightning plus, intensifying screens.

RESULTS AND DISCUSSION

The effect of an SV40 enhancer element on transcription of a cloned human H4 histone gene was examined using a transient expression system. We elected to link the transcriptional enhancer directly to the 5'-flanking sequences of this H4 gene (Fig. 1) as the activity of certain eukaryotic gene promoters has been reported to vary according to their relative positions with respect to enhancers (20). The "enhanced" or "nonenhanced" recombinant plasmids containing a human genomic H4 histone gene were transfected into cultured mouse LTK$^-$ cells and human HeLa S3 cells via a calcium phosphate coprecipitation technique.

After approximately 48 hours total cellular RNA was extracted from transfected cells and analysed by an S1 nuclease assay for human histone gene transcripts with the same 5' termini as the authentic human H4

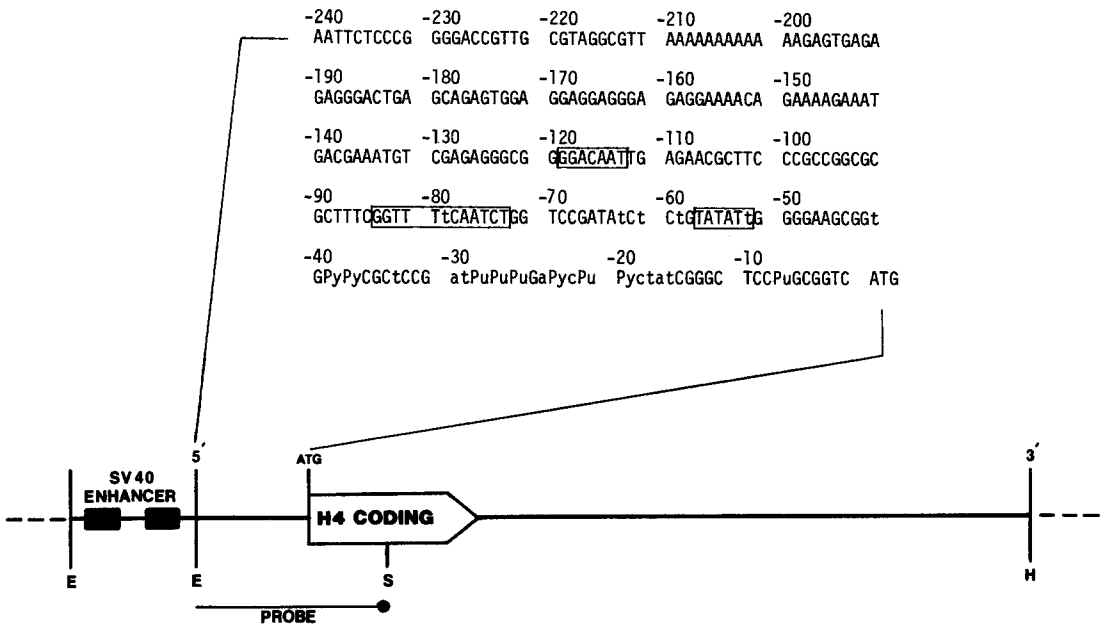

Fig. 1. Schematic representation of the human genomic H4 histone gene in plasmids pFO108A and pSVE108A which were utilized for transfection experiments. An SV40 transcriptional enhancer was inserted at the EcoRI site of plasmid pFO108A immediately upstream from the H4 5'-flanking region generating plasmid pSVE108A. Consensus 5' regulatory sequences of the H4 gene include two tandem 'CAAT' boxes (nucleotides -116 and -78) and a 'TATA' box (nucleotide -57). A histone related motif, GGTCC (nucleotide -72) and a series of G and A repeat sequences (nucleotides -120 to -190) are also present. The 408 bp Sst II/EcoRI fragment was 5' end-labelled, and the 'sense' strand was used as DNA probe in S1 nuclease mapping experiments. E-EcoRI, S-Sst II, H-Hind III, (——)pBR322 derived sequences.

histone mRNAs. A 5'-end labelled, single-stranded Sst II/Eco RI DNA fragment (408 nucleotides), spanning approximately half of the H4 coding and the entire 5'-flanking regions (Fig. 1), was used to map the 5' terminus(i). When correctly initiated mRNA hybridized to the labelled complementary strand, S1 nuclease then digested any unhybridized sequences, leaving a protected 198-nucleotide labelled fragment (arrows Figs. 2 and 3) resolved by denaturing electrophoresis and autoradiography.

The amount of H4 probe used (0.04 pmol) for each sample represents an excess of DNA, thus the assay quantitatively measures H4 mRNA levels. Control experiments with RNA from L-cells transfected with pBR322 showed no detectable protection of the labelled DNA probe fragment (Fig. 2, lane 1 and Fig. 3, lane 2). Thus mouse and human H4 mRNAs could be clearly distinguished in this assay.

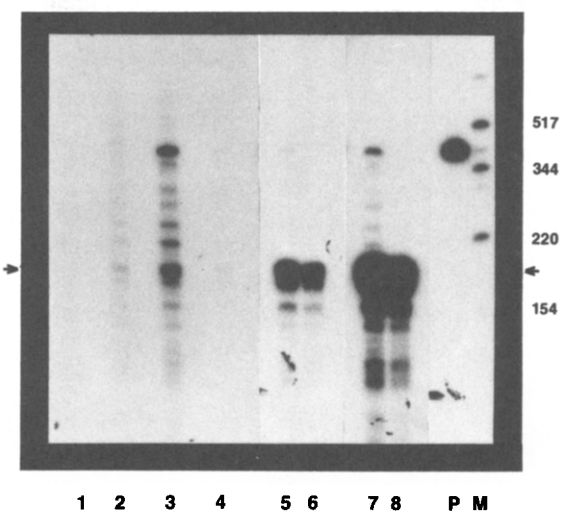

Fig. 2. S1 nuclease protection analysis of human H4 histone gene mRNAs from transfected cells. Lane 1--250μg of RNA from pBR322 transfected L-cells. Lane 2--250μg of RNA from pF0108A (nonenhanced H4) transfected L-cells. Lane 3--100μg of RNA from pSVE108A (enhanced H4) transfected L-cells. Lane 4--100μg of RNA from pSVE108A (enhanced H4) transfected L-cells treated with 1mM Hydroxyurea for two hours prior to harvest. Lane 5--25μg of RNA from pSVE108A (enhanced H4) transfected HeLa cells. Lane 6--25μg of RNA from pBR322 transfected HeLa cells showing expected size (arrows) of endogenous H4 mRNAs. Lanes 7 and 8--Prolonged (1 month) exposure of lanes 5 and 6 respectively. Lane P--Full length 5'-end labelled, strand-separated H4 DNA probe (408 nucleotides). Lane M--HinfI digested pBR322, 3'-labelled markers.

Transfection of L-cells with pF0108A (nonenhanced H4) produced low levels of correctly initiated H4 mRNAs (Fig. 2, lane 2). In addition, a number of transcripts, both longer and shorter than the anticipated 198-nucleotide fragment, were protected (Fig. 2, lane 2). When L-cells were transfected with pSVE108A (enhanced H4), a similar 5' protection pattern was resolved with approximately 50-fold greater signal intensity from correctly initiated H4 mRNA (arrows Fig. 2, cf. lanes 2 and 3) as determined by densitometric scanning of the autoradiograms. An analogous 5' pattern of fragments larger than the endogenous 198 nucleotide fragment was protected when pSVE108A (enhanced H4) was transfected into homologous HeLa cells. Although this pattern was very weak compared to the endogenous signal after short exposure (Fig. 2, lane 5) it was pronounced after prolonged autoradiography (Fig. 2, lane 7). No such additional 5' pattern was protected however, with pBR322-transfected

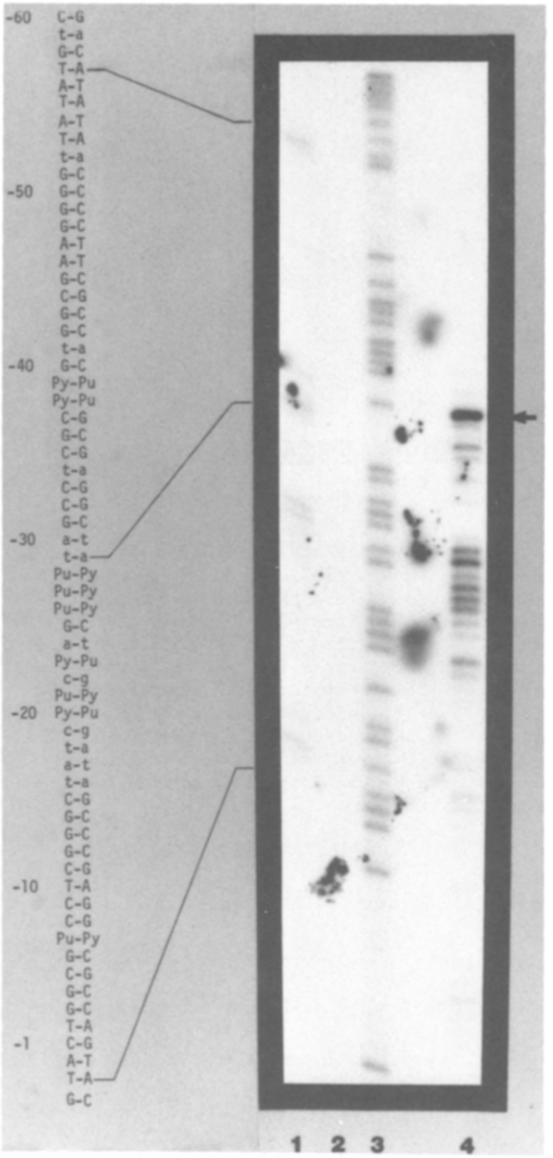

Fig. 3. S1 nuclease mapping of 5′ terminal sequences of human H4 histone gene mRNAs from HeLa cells and transfected L-cells. Lane 1--100 µg of RNA from pSVE108A (enhanced H4) transfected L-cells. Lane 2--100 µg of RNA from pBR322 transfected L-cells. Lane 3--G+A 'sequencing ladder' derived from the 5′-end labelled H4 DNA probe. Lane 4--25 µg of RNA from pBR322 transfected HeLa cells showing the sizes of authentic H4 histone mRNAs. The position of the band corresponding to the expected size of the correctly initiated transcripts is indicated by the arrow.

HeLa cells (Fig. 2, lane 6) even after prolonged autoradiography (Fig. 2, lane 8). It is also noticeable that with the enhanced H4 transfected cells a substantial proportion of H4 transcripts appeared to originate in

the pBR322/SV40 vector, thereby protecting the H4 DNA probe over its entire 408 nucleotides (Fig. 2, cf. lanes 3,4,5,7 and P).

More precise mapping of H4 mRNA 5′ termini was achieved on a sequencing gel using G+A sequencing markers generated from the 5′-labelled H4 DNA probe (Fig. 3, lane 3). The majority of authentic H4 mRNAs from HeLa cells transfected with pBR322 map to a cap site approximately 30 nucleotides upstream from the ATG translation start codon (arrow Fig. 3, lane 4). Also, a series of HeLa H4 5′ termini map to sites that are downstream from the transcription cap start site (Fig. 3, lane 4). The corresponding 5′ termini pattern obtained from pSVE108A (enhanced H4) transfected L-cells (Fig. 3, lane 1) is different from that protected in pBR322 transfected HeLa cells (Fig. 3, lane 4). For example, in addition to the correctly initiated human H4 mRNA transcripts in transfected L-cells, one of the other protected 5′-termini maps to the TATA box region (Fig. 3, lane 1). It is not clear from the present data if the additional H4 5′-termini from transfected cells, which map upstream and downstream from the cap site, are the result of incorrect transcriptional initiation, possibly by RNA polymerases I or III. In the case of HeLa cell RNA, 5′-termini mapping downstream from the cap site (Fig. 3, lane 4) may represent hybridization to a microheterogeneous population of endogenous H4 mRNAs (19). Alternative sites for initiation of transcription have been reported for human globin genes, whether expressed endogenously (21) or as a result of transfer into heterologous cells (22). The presence of additional H4 5′ and/or 3′ flanking sequences may be necessary to generate a population of transcripts, all arising from a single initiation site.

It is significant that inhibition of DNA synthesis, by 1mM hydroxyurea, during "enhanced" transient expression of a human H4 histone gene in mouse L-cells brought about a rapid destabilization of the human H4 mRNA (Fig. 2, lane 4). This suggests that the post-transcriptional mechanism(s) that couples histone gene expression with DNA replication in human cells (5,6) is also operative in murine cells.

ACKNOWLEDGEMENTS

We are indebted to Drs. Dan Greenspan and Sherman Weissman for providing plasmid pDG014 which contained the SV40 enhancer region. This work was funded by grants awarded by the National Institutes of Health (GM32010), the National Science Foundation (PCM 83 18075) and the March of Dimes Birth Defects Foundation (1-813).

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