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Cell cycle-dependent expression of a stable episomal human histone gene in a mouse cell

L. GREEN*, I. SCHLAFFER†, K. WRIGHT*, M. L. MORENO*, D. BERAND†, G. HAGER†, J. STEIN*, AND G. STEIN*

*University of Florida, College of Medicine, Gainesville, FL 32610; and †National Institutes of Health, National Cancer Institute, Bethesda, MD 20205

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ABSTRACT **We have constructed a recombinant plasmid that includes a cell cycle-dependent human H4 histone gene with 650 base pairs of 5′ and 900 base pairs of 3′ flanking sequences and the 69% transforming fragment of bovine papilloma virus. When transfected into C127 mouse cells, this plasmid is maintained as a stable episome with approximately 20 copies per cell. Micrococcal nuclease digestion indicates that the episomal human histone gene is packaged as chromatin. The human H4 histone transcript is initiated at the correct 5′ start site and undergoes selective destabilization when DNA synthesis is inhibited. When C127 cells containing the episomal H4 histone gene are synchronized, the human H4 histone mRNA levels are regulated coordinately with DNA replication and parallel those of transcripts from the murine chromosomal H4 histone genes. Our results suggest that the regulatory sequences and/or regulatory molecules associated with murine and human histone genes are compatible. The human histone gene–bovine papillomavirus episome is therefore a viable system for studying cell cycle-regulated histone gene expression under conditions where control is not influenced at the site of chromosomal integration by *cis*-acting elements of genes normally not contiguous.**

Human histone genes are represented as a family of moderately repeated sequences with differences in the structure, organization, and regulation of the various copies (1–8). The genes are organized in clusters of core (1–8) or core together with H1 histone genes (7, 9) and are located on at least three human chromosomes—1, 6, and 12 (10, 11). Sequence analysis of several human histone genes reveals 5′ consensus sequences that indicate that the individual genes in a cluster might function as independent transcription units (4, 5, 8, 12). This suggestion is supported by UV inactivation studies (13) and also by experiments in which expression was assayed from individual copies of intact human histone genes (14, 15), from histone genes with deletions in 5′ flanking sequences (14, 15), and from fusion genes in which histone 5′ flanking sequences were used to drive prokaryotic and other eukaryotic genes (16).

Functionally, there are three classes of human histone genes. Most are expressed in a cell cycle-dependent manner and temporally as well as functionally coupled with DNA replication (17–28); several are expressed constitutively throughout the cell cycle and after inhibition of DNA replication (8, 29–32) and some are nonfunctional pseudogenes (6). The organization and expression of murine histone genes are similar (33, 34).

To address mechanisms operative in the regulation of a cell cycle-dependent human histone gene, it is necessary to establish the structural and functional limits of the gene and to identify sequences both within the mRNA coding region and in 3′ and 5′ flanking regions that are involved with the principal elements of histone gene expression. This requires localizing sequences associated with: (*i*) the preferential expression during the S phase of the cell cycle and (*ii*) the rapid and selective turnover of histone mRNAs at the termination of S phase or after DNA synthesis inhibition.

By systematically modifying human histone gene structure, we can assess the influence of such structural perturbations on the various parameters of histone gene expression. This approach is dependent on the ability to: (*i*) synchronize the cells containing the modified cell cycle-dependent human histone gene, (*ii*) maintain the modified gene in a physical state in which it is not influenced by *cis*-acting elements of genes normally not contiguous, and (*iii*) distinguish transcripts of the modified gene from those of the host cell. Short-term transient assays, in which transcripts from modified histone genes are expressed during the initial 48 hr after transfection into eukaryotic cells, can provide insight into sequences that influence the extent of expression and histone mRNA stability. However, the limited expression in transfected cells and the inability to synchronize the cell population limit the parameters of histone gene expression that can be examined. Although stable cell lines can be obtained by cotransfecting with a gene encoding a selectable marker, limitations are imposed by the inability to control the sites of chromosomal integration.

We have constructed an episome containing a cell cycle-dependent human H4 histone gene with 650 nucleotides of 5′ and 900 nucleotides of 3′ flanking sequences. The inclusion of the 69% transforming segment of bovine papillomavirus (BPV) provides an origin of replication and genetic sequences necessary for maintenance of the transformed phenotype (35, 36). When introduced into C127 mouse cells, this human histone gene is packaged as chromatin and maintained as a stable episome. Expression occurs in a cell cycle-dependent manner, with human H4 histone mRNAs distinguishable from mouse H4 mRNAs by S1 nuclease analysis. The human H4 histone mRNA exhibits correct *in vivo* 5′ site-specific initiation and is selectively destabilized when DNA synthesis is inhibited. When cells containing the H4 histone–BPV episome are synchronized, expression of the episomal human H4 histone gene varies coordinately with DNA replication and parallels that of chromosomal murine histone genes.

MATERIALS AND METHODS

Cell Culture and Cell Synchronization. C127 mouse cells and I-8 cells were grown in monolayer culture in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. Cells were synchronized by two cycles of 2 mM thymidine blockage.

Isolation of Extrachromosomal DNA. Extrachromosomal DNA was isolated by a modification of the Birnboim and Doly procedure (37). Semiconfluent cell monolayers (2–5 × 10^7 cells) were harvested and collected by centrifugation at

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Abbreviations: BPV, bovine papillomavirus; kb, kilobase(s).

500 × *g* for 5 min. The cell pellets were resuspended in 1 ml of 25 mM Tris·HCl/50 mM glucose/10 mM EDTA, pH 8.0, and then were incubated on ice for 5 min; 1% $NaDodSO_4$/0.2 M NaOH (2 ml) was added, and the resulting lysate was incubated at 4°C for 5 min. One-half volume of 5 M sodium acetate (pH 4.8) was added, and incubation at 4°C was continued for 20 min. The denatured DNA precipitate was pelleted by centrifugation at 180,000 × *g* for 10 min at 4°C and discarded. Diethyl pyrocarbonate was added to the supernatant to 0.2%, and after vigorous agitation the mixture was incubated at room temperature for 5 min. Two volumes of 95% ethanol were added, and DNA was precipitated overnight at −20°C. The precipitate was resuspended in TE medium (10 mM Tris·HCl/1 mM EDTA, pH 8.0) and was extracted twice with phenol/chloroform/isoamyl alcohol, 25:24:1 (vol/vol), and twice with chloroform/isoamyl alcohol, 24:1. Sodium acetate (5 M; pH 5.0) was added to 0.25 M, and the DNA was precipitated with 2 volumes of 95% ethanol at −20°C.

Isolation and Quantitation of Total Cellular Nucleic Acid. For DNA isolation, cell pellets were resuspended in 0.015 M NaCl/0.0015 M sodium citrate, pH 7/0.5% $NaDodSO_4$/200 μg of proteinase K per ml and gently shaken overnight at room temperature. The lysates were extracted with 1 volume of phenol, then twice with 0.5 volume of phenol/chloroform/isoamyl alcohol, and finally with 1 volume of chloroform/isoamyl alcohol. Sodium acetate was added to a final concentration of 0.25 M, and total nucleic acid was precipitated with ethanol at −20°C. The pellets were resuspended in 50 mM Tris·HCl/10 mM EDTA, pH 8.0; RNase A was added to 20 μg/ml, and incubation was carried out at 37°C for 90 min. After extraction with phenol/chloroform/isoamyl alcohol and with chloroform/isoamyl alcohol, sodium acetate was added to 0.3 M, and DNA was precipitated with 95% ethanol at −20°C. The pellet was resuspended in TE medium. Total cellular RNA was isolated as described (23), and histone mRNA levels (human and mouse) were determined by S1 nuclease-protection analysis.

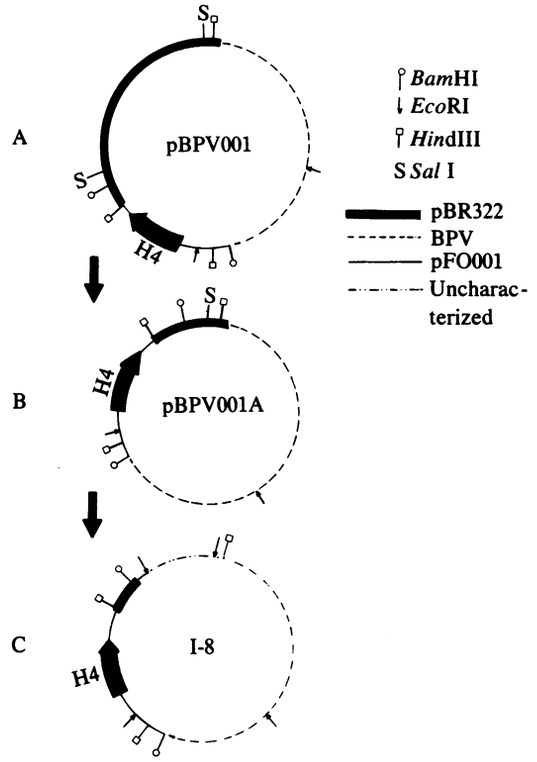

FIG. 1. Schematic diagrams showing the construction of the BPV-human H4 histone chimeric plasmid present in the I-8 cell line. (*A*) Original pBPV001 construct used to transfect C127 mouse cells. (*B*) Subsequent construct after 85% of the pBR322 sequences had been removed by *Sal* I digestion and religated to circularize the larger fragment (pBPV001A). (*C*) Final map of the plasmid present in I-8 cells. This map was constructed from data obtained by Southern blot analysis of restriction endonuclease-digested I-8 episome-enriched DNA hybridized with ^{32}P-labeled human H4 or BPV probes.

RESULTS AND DISCUSSION

Selection and Characterization of Cell Lines Containing a Stable Episomal H4 Human Histone Gene. A recombinant plasmid pBPV001 was constructed containing a cell cycle-dependent human H4 histone gene from pFO001 (14) with 650 nucleotides of 5′ and 900 nucleotides of 3′ flanking sequences. Initially, the 2.65-kilobase (kb) *Bam*HI fragment of pFO001 was inserted into the *Bam*HI site of p69P-S, which contains the *Hin*dIII-*Bam*HI 69% transforming fragment from BPV inserted into the *Hin*dIII and *Bam*HI sites of a pBR322 derivative in which the *Eco*RI site was converted into a *Sal* I site (Fig. 1*A*). pBPV001 was used to transform C127 mouse cells. Thirty-five cell lines were grown from selected foci and were screened for the presence of the pBPV001 episome. Extrachromosomal DNA was isolated and was digested with restriction endonuclease, blotted to nitrocellulose, and hybridized to a ^{32}P-labeled (nick-translated) human H4 histone gene or BPV probe. None of the cell lines from this transformation nor from cells transformed with the H4 histone gene in the reverse orientation gave the expected restriction pattern. Sixty-six percent of the cell lines had both human H4 histone gene and BPV sequences present, but they had all undergone some rearrangement or integration into the genome.

Since pBR322 sequences have been reported to reduce transformation efficiency as well as to promote integration of transfected DNA into the genome (35), we used *Sal* I restriction endonuclease to remove most of the pBR322 sequences present in the original construct. The larger *Sal* I fragment was recircularized (pBPV001A) and used to transfect C127 mouse cells (Fig. 1*B*). Cell lines were established and screened for the presence and organization of the pBPV001A episome. Of the 15 cell lines analyzed by Southern blot analysis of episomal DNA with a ^{32}P-labeled human H4 histone gene probe, 4 yielded a 1.95-kb *Hin*dIII fragment, indicating the presence of an unrearranged pFO001 human H4 histone gene (Fig. 2). A cell line designated I-8 contained an episome with both an unrearranged pFO001 H4 histone gene (Fig. 2 *Upper*) and an intact BPV 69% transforming fragment (Fig. 2 *Lower*) and was selected for further studies. The I-8 episome contains an additional 1.0-kb DNA fragment in the pBR322 segment. This extra fragment was presumably acquired during plasmid construction (Fig. 1*B*) or during the transformation process. The functional significance of this DNA segment with respect to the stability of the episome remains to be established; however, a similar phenomenon was observed by Di Maio *et al.* (38). A restriction map of the I-8 episome is shown in Fig. 1*C*.

Characterization of the Episomal H4 Histone Gene. The episomal nature of the pFO001 H4 histone gene–BPV construct in the I-8 cell line was confirmed by Southern blot analysis. Total cellular and episome-enriched DNAs were isolated from I-8 cells, and the intact (undigested) DNAs were examined by hybridization with a radiolabeled human H4 histone gene probe. Fig. 3 *Upper* shows that the H4 histone–BPV construct migrates as both supercoiled (form I) and nicked-circular (form II) molecules, indicating that it is extrachromosomal. There was no evidence for integrated copies of the human H4 histone gene.

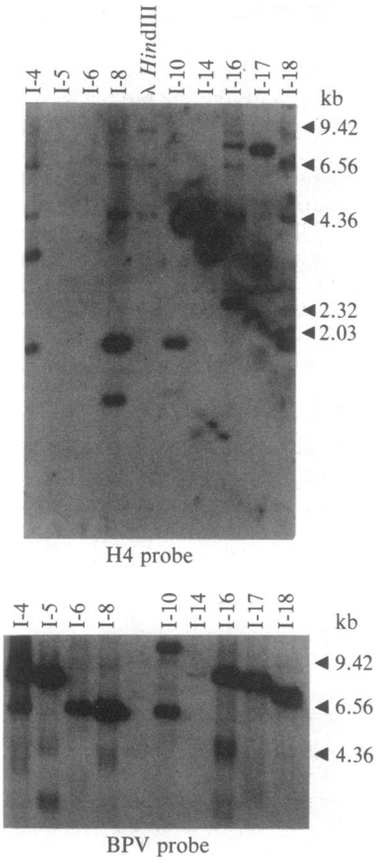

FIG. 2. Southern blot analysis of DNAs enriched for episomal material isolated from C127 cell lines transfected with the human H4–BPV construct shown in Fig. 1*B*. Each sample was digested with *Hin*dIII restriction endonuclease and fractionated electrophoretically. The filter-immobilized DNAs were hybridized with pFO108A, a pBR322-based plasmid that contains a truncated version of the human H4 histone gene used in the construct (*Upper*) or the 8.0-kb BPV fragment from pBPV-1 (*Lower*). The blots were then analyzed by autoradiography.

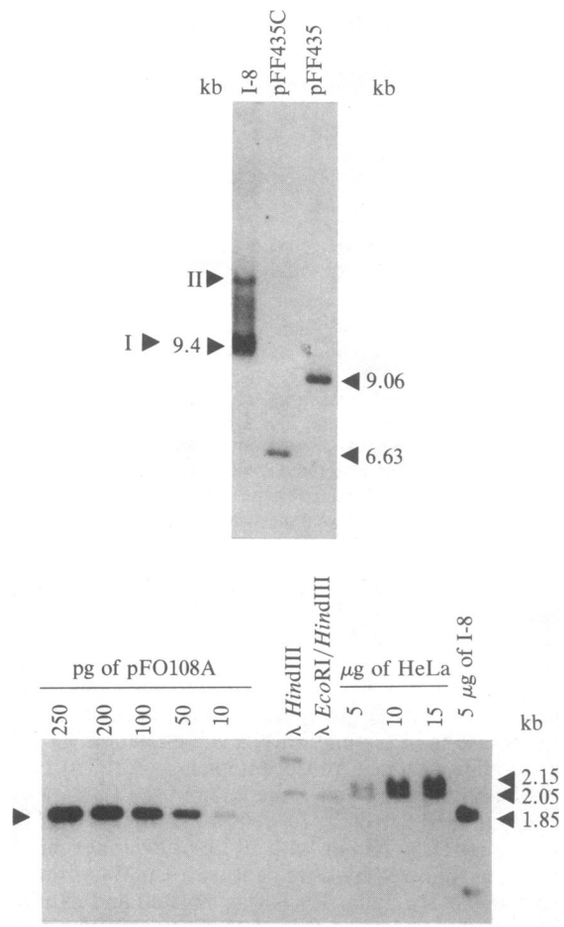

FIG. 3. (*Upper*) Southern blot analysis of DNAs enriched for episomal material from I-8 cells. The I-8 episomal DNA and two undigested plasmid DNAs (pFF435C and pFF435) were fractionated electrophoretically. The filter-immobilized DNAs were hybridized to a ^{32}P-labeled plasmid containing the human H4 histone gene and analyzed by autoradiography. Arrowheads: I, form I; II, form II. (*Lower*) Southern blot analysis of I-8 and HeLa total cellular DNA. Various amounts of the DNAs were digested to completion with *Hin*dIII restriction endonuclease and fractionated electrophoretically. Also included are known amounts of an H4 histone plasmid digested with *Eco*RI and *Hin*dIII (10–250 pg) to serve as standards for determining copy number. Phage λ DNA digested with *Hin*dIII or *Hin*dIII/*Eco*RI were 3′-end-labeled by using [α-^{32}P]dCTP and the Klenow fragment of *Escherichia coli* DNA polymerase I and were used as size markers. The filter-immobilized DNAs were hybridized to a ^{32}P-labeled human H4 histone gene and analyzed by autoradiography.

The copy number for the episomal human H4 histone gene was determined by comparing I-8 total cellular DNA with various known amounts of both human H4 histone plasmid DNA and HeLa cell DNA by Southern blot analysis using a radiolabeled [^{32}P]H4 histone gene probe. Densitometry of such Southern blots from several independent experiments (Fig. 3 *Lower*) indicated the presence of approximately 20 copies of the episome per I-8 cell. After several months of culture and after reconstitution of frozen cell stock, the copy number of the episomal I-8 H4 histone gene remained stable. Nuclease digestion studies indicated that the H4 histone-BPV episome is packaged as chromatin (data not shown).

Expression of I-8 Episomal DNA. To determine if the human H4 gene contained in the I-8 episome was expressed in the C127 mouse host cell, total cellular RNA was isolated from exponentially growing cells and hybridized with a ^{32}P-end-labeled probe derived from the human H4 histone gene. Hybridization was followed by S1 nuclease analysis. The protected DNA·RNA hybrids were analyzed in a 5.6% polyacrylamide gel along with ^{32}P-end-labeled size markers. Fig. 4 shows an autoradiogram from one such gel that indicates that the human H4 gene is indeed expressed in the I-8 mouse cell line and that there is 5′ site-specific initiation of transcription. HeLa total cellular RNA was also included in the S1 nuclease analysis. The signal from probe protected with I-8 RNA was 23% of that protected by HeLa cell RNA.

The decreased representation of human H4 histone gene transcripts in I-8 cells (where there are 20 copies of the episomal gene/genome) compared with HeLa cells (where this H4 histone gene is a single-copy gene) may indicate that the level of transcription of this human H4 histone gene is decreased in I-8 cells. Alternatively, the lower representation of human H4 histone mRNAs in I-8 cells may result from normal posttranscriptional regulation of cellular histone mRNA levels, where both human and mouse H4 histone mRNAs contribute to a common pool. Compensatory, posttranscriptional regulation of histone mRNA levels has been observed in other systems after introduction of homologous (39) or heterologous (40) histone genes.

Destabilization of Human H4 Histone Gene Transcripts in the I-8 Cell Line. A key parameter of histone gene expression is the posttranscriptionally mediated, selective destabilization of histone mRNA that occurs after inhibition of DNA replication and the requirement of protein synthesis for this degradation (24, 41–47). It has been well documented that the treatment of cells with hydroxyurea, a DNA synthesis inhibitor, results in a rapid loss of cellular histone mRNAs

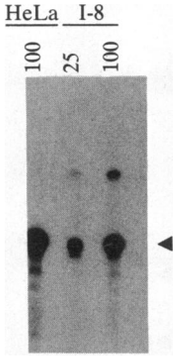

FIG. 4. S1 nuclease protection analysis of total cellular RNA from logarithmic-phase I-8 and HeLa cells in microgram amounts indicated. Hybridization was carried out between total cellular RNA and a ^{32}P-end-labeled probe derived from an *Sst* II restriction endonuclease digest of a human H4 histone gene (pFO108A). The hybrids were subjected to S1 nuclease digestion, and protected fragments were fractionated by gel electrophoresis and analyzed by autoradiography of the dried gel. The arrow indicates the 196-nucleotide band that is the major fragment protected by HeLa cell RNA. The minor upper band represents an alternative start site in HeLa and mouse cells.

and that treatment of cells with a protein synthesis inhibitor such as cycloheximide prevents histone mRNA breakdown and even results in histone mRNA accumulation.

To examine this posttranscriptionally controlled component of human histone gene regulation in the I-8 mouse cell line, exponentially growing I-8 cells were treated with 1 mM hydroxyurea for 60 min or with 10 μg of cycloheximide per ml for 5 min followed by treatment with 1 mM hydroxyurea for 60 min. Total cellular RNA was isolated and hybridized to ^{32}P-end-labeled human (Fig. 5*A*) or mouse (Fig. 5*B*) H4 histone gene probes. S1 nuclease analysis indicates that treatment with cycloheximide followed by hydroxyurea results in a 4-fold increase in the human H4 histone transcript and a 3- to 4-fold increase in the endogenous mouse H4 transcript. Similar treatment of I-8 cells with hydroxyurea resulted in an 80% decrease in the human H4 transcript and an 80% decrease in the endogenous mouse H4 transcript. These figures are expressed relative to untreated I-8 control cells. A comparison with the same inhibitor treatments of HeLa cells (Fig. 5*C*) suggests that the stability of human H4 histone gene transcripts from the chromosomal gene in HeLa cells and the episomal gene in the C127 mouse cells is similarly regulated at a posttranscriptional level.

Cell Cycle-Dependent Expression of the I-8 Episome. S1 nuclease analysis of RNAs isolated from synchronized I-8 cells indicates that the episomal human H4 histone gene in C127 mouse cells is expressed in a cell cycle-dependent manner. Exponentially growing I-8 cells were synchronized by two cycles of 2 mM thymidine blockage, and total cellular levels of mouse and human H4 histone mRNAs were assayed every 60 min as cells progressed through S phase and into G_2, in parallel with determinations of the rate of DNA synthesis. Fig. 6*A* shows an autoradiogram of an S1 nuclease protection analysis of I-8 cell RNAs using a ^{32}P-end-labeled probe derived from the human H4 histone gene contained in the I-8 episome. Fig. 6*B* shows the rate of DNA synthesis in I-8 cells as monitored by [^{3}H]thymidine incorporation. Levels of human H4 histone mRNAs in the I-8 cells closely followed cellular DNA synthesis, exhibiting an increase of >20-fold at the peak of S phase (4 hr) compared with late G2 (8 hr). This relationship was confirmed by similar results when human H4 histone mRNA levels were determined in these cells as they progressed through mitosis into G1 and then into the second round of DNA synthesis, and additionally by several independent experiments (data not shown).

Cellular levels of mRNAs from a murine chromosomal H4 histone gene were similarly assayed during the cell cycle by

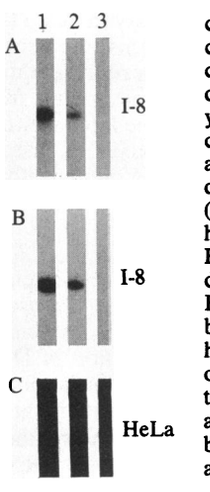

FIG. 5. S1 nuclease analysis of total cellular RNAs isolated from I-8 or HeLa cells untreated (lane 3) or treated with: cycloheximide for 5 min, followed by hydroxyurea for 90 min (lane 1) or hydroxyurea for 60 min (lane 2). (*A*) I-8 total cellular RNA (25 μg) was hybridized with a ^{32}P-end-labeled human H4 histone probe derived from an *Nco* I digest of pFO108A. (*B*) I-8 total cellular RNA (25 μg) was hybridized with a ^{32}P-end-labeled mouse H4 histone probe derived from a *Bst*NI digest of pBR-mus-hi-1-H4-Hinf I. (*C*) HeLa total cellular RNA (25 μg) was hybridized with a ^{32}P-end-labeled human H4 histone probe derived from an *Nco* I digest of pFO108A. The hybridization mix was then subjected to S1 nuclease digestion, and protected fragments were fractionated by gel electrophoresis and analyzed by autoradiography of the dried gel.

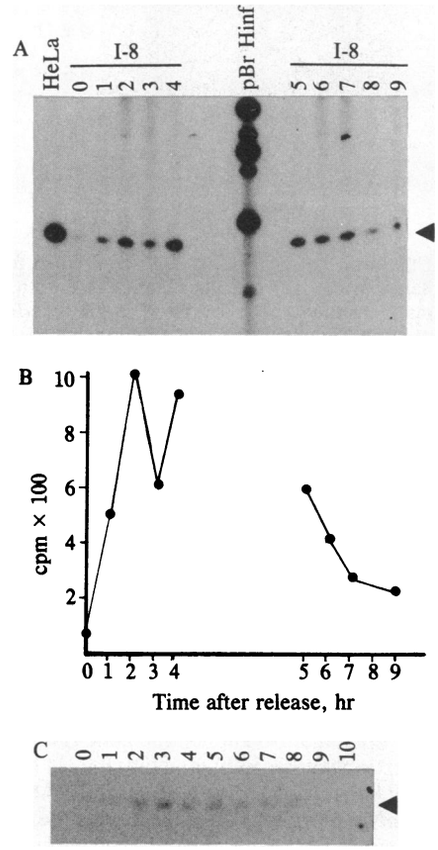

FIG. 6. Cell cycle regulation of the human H4 histone gene in I-8 cells. Total cellular RNA was isolated from I-8 cells harvested at various times (shown in hours) after release from a double thymidine blockage. RNAs were hybridized to appropriate ^{32}P-end-labeled probes and then subjected to S1 nuclease digestion. (*A*) RNAs (25 μg) were hybridized with a probe derived from an *Sst* II digest of pFO108A (human H4 histone gene). The arrowhead indicates the 196-nucleotide protected fragment. pBr Hinf, *Hinf*-digested pBR322. (*B*) DNA synthesis during the I-8 cell cycle. Every hour after release from thymidine block, cells were pulse-labeled for 30 min with [^{3}H]thymidine (2 μCi/ml; 1 Ci = 37 GBq), and trichloroacetic acid-precipitable radioactivity was determined. (*C*) RNAs (25 μg) were hybridized with a probe derived from a *Bst*NI digest of pBR-mus-hi-1-H4-Hinf I (mouse H4 histone gene). The arrowhead indicates the 110-nucleotide protected fragment.

S1 nuclease analysis of I-8 cell RNAs using a ^{32}P-end-labeled fragment from a mouse histone gene designated pBR-mushi-1-H4-Hinf I (44). mRNAs transcribed from the mouse H4 histone gene were found in I-8 cells predominantly during S phase (Fig. 6*C*), as observed for RNAs from the episomal human H4 histone gene. Therefore, it appears that expression of the episomal human H4 gene is regulated in a cell cycle-dependent manner in the I-8 mouse cells. The relative importance of transcriptional and posttranscriptional components in this regulation remains to be established.

These results indicate the usefulness of this episomal human H4 minichromosome for examining molecular parameters of histone gene expression. It appears that sequences required for the cell cycle-dependent expression of the human H4 histone gene reside within the structural gene or in the immediate 650-nucleotide-upstream and 900-nucleotide-downstream flanking sequences that are present in the I-8 episome. Additionally, our results suggest that the regulatory molecules present in the I-8 mouse cells are compatible with expression of the human H4 histone gene predominantly during S phase. Compatibility of transcription and mRNA turnover factors, which are required for the transcriptionally and posttranscriptionally regulated components of histone gene expression, appears to cross the mouse–human species barrier. The episomal nature of the pFO001 H4 histone–BPV construct, coupled with its packaging as chromatin, permits the direct isolation of the minichromosome from I-8 cells. Hence, the isolation and characterization of factors that influence the cell cycle-dependent expression and modifications in chromatin structure of this human H4 histone gene can be realistically approached by using the I-8 minichromosome.

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