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

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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 S-phase-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 1–8 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 Birnstiel and Doly procedure (37). Semiconfluent cell monolayers (2–5 × 10⁶ cells) were harvested and collected by centrifugation at

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Abbreviations: BPV, bovine papillomavirus; kb, kilobase(s).
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- and doxorubicin-dependent human H4 histone gene from pFO001(14) with 650 nucleotides of 5’ and 500 nucleotides of 3’ flanking sequences. Initially, the 2.65-kilobase (kb) BamHI fragment of pFO001 was inserted into the BamHI site of pE9P-S, which contains the HindIII-BamHI 69% transforming fragment from BPV inserted into the HindIII and BamHI sites of a pBR322 derivative in which the EcoRI site was converted into a SalI site (Fig. 1A). 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 episomal plasmid. Extrachromosomal DNA was isolated and was digested with restriction endonucleases, blotted to nitrocellulose, and hybridized to a 32P-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. Thirty-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 SalI restriction endonuclease to remove most of the pBR322 sequences present in the original construct. The larger SalI fragment was recircularized (pBPV001A) and used to transfect C127 mouse cells (Fig. 1B). 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 restriction endonuclease-digested I-8 episome-enriched DNA hybridized with 32P-labeled human H4 or BPV probes.

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.
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-labeled 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.

**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. 1B. Each sample was digested with HindIII 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 (pFP435C and pFP435) 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 HindIII restriction endonuclease and fractionated electrophoretically. Also included are known amounts of an H4 histone plasmid digested with EcoRI and HindIII (10–250 pg) to serve as standards for determining copy number. Phage λ DNA digested with HindIII or HindIII/EcoRI 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.
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 ^32P^-end-labeled human (Fig. 5A) or mouse (Fig. 5B) 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. 5C) suggests that the stability of human H4 histone gene transcripts from the chromosomal gene in HeLa cells and the episomal gene in the I-27 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 CI27 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 G2, in parallel with determinations of the rate of DNA synthesis. Fig. 6A shows an autoradiogram of an S1 nuclease protection analysis of I-8 cell RNAs using a ^32P^-end-labeled probe derived from the human H4 histone gene contained in the I-8 episome. Fig. 6B shows the rate of DNA synthesis in I-8 cells as monitored by [3H]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
S1 nuclease analysis of I-8 cell RNAs using a 32P-end-labeled fragment from a mouse histone gene designated pBR-mushhi-I-H4-HinfI (44). mRNAs transcribed from the mouse H4 histone gene were found in I-8 cells predominantly during S phase (Fig. 5C), as observed for RNAs from the episcopal human H4 histone gene. Therefore, it appears that expression of the episcopal 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 episcopal 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 episcopal nature of the pFOO01 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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