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Organization of human histone genes

(hybridization/Southern blot technique/reverse Southern blot technique/cloning)

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ABSTRACT **We describe the isolation and initial characterization of seven independent λ Charon 4A recombinant phages which contain human histone genomic sequences (designated λHHG). Restriction maps of these clones and localization of the genes coding for histones H2A, H2B, H3, and H4 are presented. The presence of histone encoding regions in the λHHG clones was demonstrated by several independent criteria including hybridization with specific DNA probes, hybrid selection/*in vitro* translation, and hybridization of λHHG DNAs to reverse Southern blots containing cytoplasmic RNAs from G_1-, S-, and arabinofuranosylcytosine (cytosine arabinoside)-treated S-phase cells. In addition, the λHHG DNAs were shown to protect *in vivo* labeled H4 mRNAs from S1 nuclease digestion. Based on the analysis of the λHHG clones, human histone genes appear to be clustered in the genome. However, gene clusters do not seem to be present in identical tandem repeats. The λHHG clones described in this report fall into at least three distinct types of arrangement. One of these arrangements contains two coding regions for each of the histones H3 and H4. The arrangement of histone genes in the human genome, therefore, appears to be different from that in the sea urchin and *Drosophila* genomes in which each of the five histone-encoding regions (H1, H2A, H2B, H3, and H4) is present only once in each tandemly repeated cluster. At least one clone, λHHG 41, contains, in addition to the histone genes, a region that hybridizes with a cytoplasmic RNA approximately 330 nucleotides in length. This RNA is not similar in size to known histone-encoding RNAs and is present in the cytoplasm of HeLa cells predominantly in the G_1 phase of the cell cycle.**

Histone proteins complex with DNA to form structures, known as nucleosomes, that are fundamental components in the organization of the eukaryotic genome. The synthesis of histone proteins is tightly coupled to DNA replication in a number of higher eukaryotic cells (1–4). Although more definitive experiments are required, several lines of evidence point toward transcriptional level control of histone gene expression during the cell cycle in HeLa cells and in normal human diploid fibroblasts (5–10). To elucidate the levels and mechanisms of human histone gene regulation, homologous probes specific for individual histone genes, as well as knowledge about the structure and organization of the human histone genes, are required. Cloned human genomic histone sequences should provide such specific probes and are requisite for the identification of those DNA sequences involved in the regulation of these genes. Histone genes of several other species have been cloned and characterized. In sea urchins and *Drosophila melanogaster* these genes are clustered and tandemly repeated (reviewed in ref. 11), whereas in yeast (12), mouse (13, 14), and chicken (15, 16) the histone genes are also clustered but have no apparent repeat.

In this paper, we report the isolation and initial characterization of seven genomic clones containing human histone genes (designated λHHG). Hybridization studies with these clones show the histone genes to be clustered but not tandemly repeated. Six of the seven clones can be grouped into one of two types of arrangement. At least one of the clones studied contains, in addition to H3 and H4 histone genes, another gene which codes for an RNA present predominantly during the G_1 phase of the HeLa S_3 cell cycle.

MATERIALS AND METHODS

Bacteria and Bacteriophage. Clones were obtained by screening a human genomic DNA library containing human fetal liver DNA cloned in λ Ch4A (17). The library was generously provided by T. Maniatis. Bacteriophage were grown in *Escherichia coli* strain DP_{50}supF (obtained from F. Blattner) in NZCYM.DT broth as suggested by Blattner *et al.* (18). Screenings were done on 15-cm Petri plates, each containing approximately 10^4 pfu of recombinant bacteriophage. Selection of positive clones was according to the technique of Benton and Davis (19). Phage DNA was isolated by a modification of the method described by Blattner (18). All experiments involving viable bacteriophage and bacteria containing recombinant DNA were performed under conditions specified by the National Institutes of Health guidelines for research involving recombinant DNA.

Gene-Specific Histone Probes. Cloned genomic chicken sequences containing H3 and H4 encoding regions were used to screen the human recombinant library. Gene localization in the isolated human histone gene clones was determined by Southern blot analysis using chicken H3, H4, and H2B probes and an H2B probe from sea urchin DNA. These probes were characterized by DNA sequence analysis and by hybrid selection-translation (unpublished data). Plasmid DNA was isolated by the cleared lysate procedure (20) or by the alkaline method of McMaster *et al.* (21). Nick-translated DNA probes were prepared according to Maniatis *et al.* (22) after isolation of appropriate fragments from low-gelling-temperature agarose gels (Sigma) (23).

Preparation of cDNA. The TS–11S RNA from S phase HeLa S_3 cells was polyadenylylated by using ATP-polynucleotidylexotransferase from maize (24) and was then reverse-transcribed by avian myeloblastosis virus reverse transcriptase in the presence of [α-^{32}P]dCTP (25).

Hybridization. DNA fragments were fractionated on 0.8% agarose gels and transferred to nitrocellulose filters according to the technique described by Southern (26). DNA·DNA hybridizations were carried out for 20–30 hr at 68°C as directed by Lawn *et al.* (17). DNA hybridizations to reverse Southern blots were as described (10).

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Abbreviation: kb, kilobase(s).

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RNA Isolation. Total cytoplasmic RNA from G_1-and S-phase HeLa S3 cells was isolated, electrophoretically fractionated, and transferred to diazobenzyloxymethyl-paper as described by Rickles *et al.* (10). *In vivo* labeled H4 mRNAs from HeLa S3 cells were prepared as described by Lichtler *et al.* (27).

S1 Nuclease Digestion of RNA·DNA Hybrids. *In vivo* ^{32}P-labeled H4 mRNAs were separated in 8.3 M urea/6% polyacrylamide gels and RNA was extracted from individual bands as described (27). All H4 mRNAs were mixed, precipitated along with DNA from λHHG clones, and hybridized in 80% formamide. The products were digested with S1 nuclease according to Berk and Sharp (28), denatured, and electrophoresed on neutral "strand separation" gels (29).

RESULTS

Isolation of Clones Containing Human Histone Sequences. One complete equivalent of the human genomic DNA library (approximately 8×10^5 recombinant phage) (17) was screened by using a probe containing chicken H3 and H4 gene sequences. The first screening yielded 109 positives and, during further screenings, 15 recombinants were shown to contain sequences that cross-hybridized with the same probe. Seven of the 15 clones have been characterized in further detail and are described in this report.

Restriction Mapping. The seven clones suspected to contain human histone genes were mapped with respect to *Eco*RI, *Hin*dIII, and *Bam*HI sites (Fig. 1). λHHG DNAs were digested with each of the three enzymes singly as well as with all combinations of two enzymes. Confirmation of the maps was provided by similar analyses of the *Eco*RI fragments that were subcloned in pBR322. Maps constructed in this way were confirmed by hybridization of various probes (discussed below) to blots of the digested λHHG DNAs. Six of the seven clones fell into two distinct types of restriction patterns (Fig. 1). λHHG 6, λHHG 17, and λHHG 22 constitute one group. λHHG 6 and λHHG 17 have exactly the same restriction pattern. This result can be due to replication of a single recombinant during the amplification of the library (17) or to isolation of independent recombinants. The insert of λHHG 22 has a restriction pattern that is similar but not identical to that of λHHG 6 and 17, but it is 2.9 kilobases (kb) larger and has the opposite orientation with respect to λ. λHHG 5, λHHG 41, and λHHG 55 form a second group of clones with similar restriction maps. The inserts of these clones have a number of restriction sites in common and appear to form an overlapping set of sequences. The restriction pattern of λHHG 39 does not resemble that of any of the other six clones presented in this report.

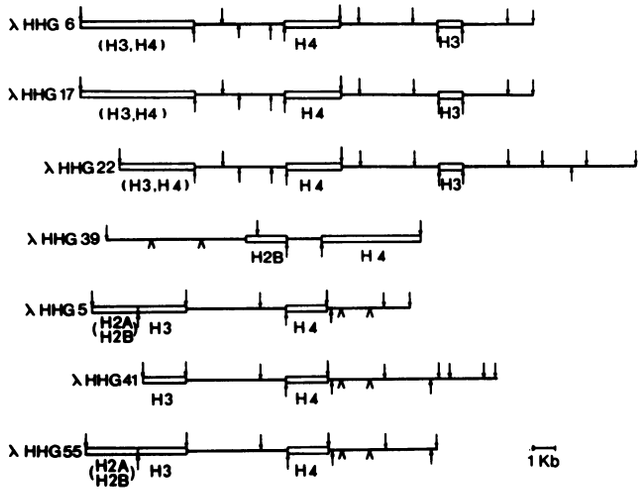

FIG. 1. Restriction maps of the seven λHHG recombinant phage. Restriction sites and histone-encoding regions were determined as described in the text. For purposes of comparison, the insert of λHHG 22 is displayed in an orientation (with respect to λ) opposite to that of the other clones. Boxes indicate restriction fragments to which histone-encoding sequences have been assigned. ↓, *Eco*RI; ↑, *Hin*dIII; ∧, *Bam*HI

Histone-Encoding Regions. Histone-encoding regions within each clone were initially mapped by hybridizing homologous or heterologous DNA probes to blots of λHHG restriction fragments. *Eco*RI-digested phage DNA was first hybridized to a ^{32}P-labeled homologous cDNA probe complementary to 7S–11S polysomal RNA from S-phase HeLa S3 cells. This RNA fraction has been shown to be enriched in histone mRNA sequences (7, 30–32). All seven λHHG clones contain *Eco*RI fragments that hybridize strongly with this cDNA probe (Fig. 2). The presence of multiple bands from each clone that hybridize to the cDNA suggests that each clone may contain more than one histone gene. From these results we could not determine whether these multiple genes coded for the same or different histone proteins. To distinguish between these alternatives, probes for individual histone genes were prepared from cloned sequences.

Probes for H3 and H4 were prepared from the same clone containing chicken histone genes used to screen the library. A 1.1-kb *Sac* II–*Sma* I fragment was used as an H4 specific probe. The presence of H4-encoding regions in this fragment was determined by hybridization-selection and cell-free translation as well as by partial sequence analysis (data not shown). A 570-bp *Hin*dIII–*Sac* II fragment was used as the H3 probe; the sequence of this fragment has been partially determined and shown to contain H3-encoding sequences. H2B-encoding regions were identified by using a sea urchin H2B-specific probe, the 1.45-kb *Hha* I fragment of pCO2 (pCO2 contains an entire histone gene repeat unit from *Strongylocentrotus purpuratus*) (33). DNA fragments were nick-translated and hybridized to blots containing DNA from λHHG clones that had been di-

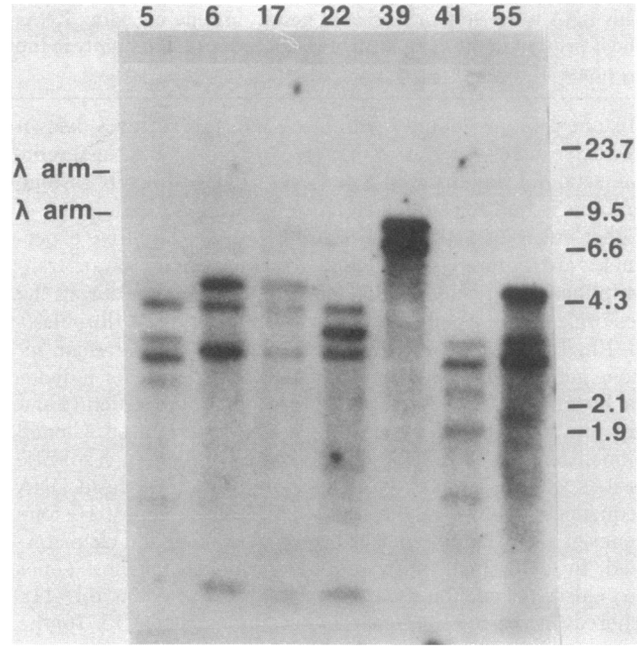

FIG. 2. Hybridization of λHHG clones to histone-enriched cDNA. ^{32}P-Labeled DNA complementary to 7S–11S polysomal RNA from S-phase HeLa cells was hybridized (20 hr) to a Southern blot containing *Eco*RI-digested λHHG DNA. Numbers on the right indicate the size (in kb) of λ *Hin*dIII markers electrophoresed in the same gel.

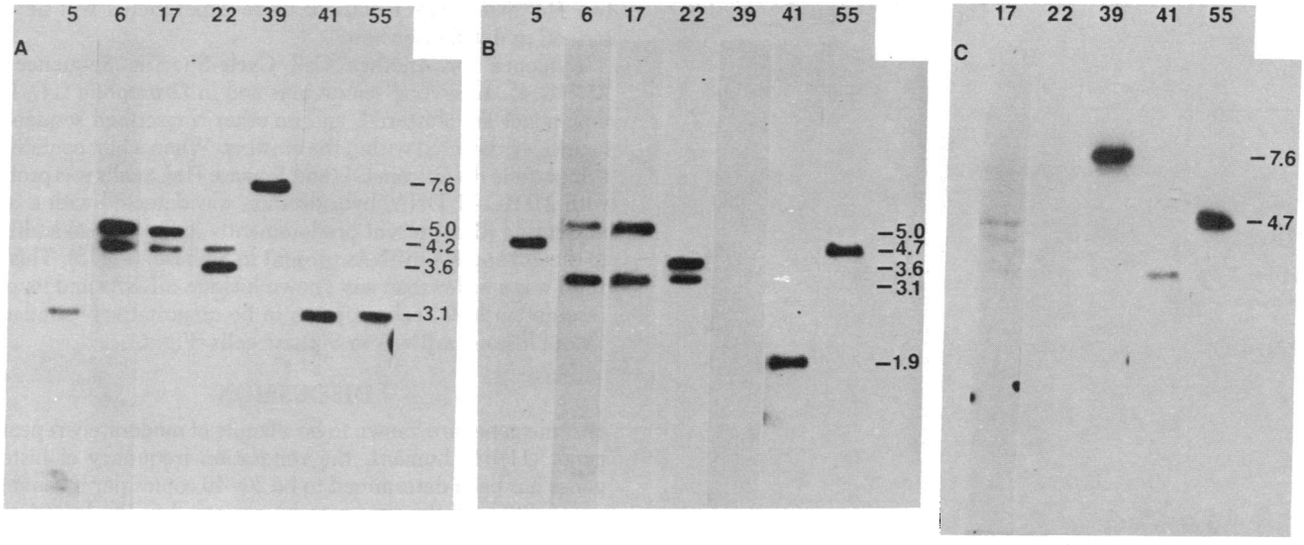

FIG. 3. Hybridization of λHHG clones to probes for specific histone genes. (*A*) Hybridization of a Southern blot containing *Eco*RI-digested λHHG DNA with chicken H4 probe. (*B*) Hybridization of a similar blot with chicken H3 histone sequences. (*C*) Hybridization to the 1.45-kilobase-pair *Hha* I fragment from pCO2 which contains sea urchin H2B sequences. Numbers at the right of each panel refer to the size (in kilobase pairs) of hybridizing bands present in each λHHG clone as determined by the migration of λ*Hin*dIII markers electrophoresed in parallel lanes of the same gels. Numbers at the top of each panel refer to the DNA present on the nitrocellulose filter.

gested with *Eco*RI, *Hin*dIII, and *Bam*HI (both single and double digestions). The results for the *Eco*RI digestions are shown in Fig. 3. H4-encoding regions are present in all seven clones. H3-encoding regions are present in all clones except λHHG 39. H2B-encoding regions are present only in clones λHHG 39 and λHHG 55. Clone λHHG 5 was not tested with this probe. Hybridization with a probe containing chicken H2B sequences independently confirmed these results and also indicated the presence of H2B-encoding regions in clone λHHG 5 (not shown).

Assignment of H2A encoding regions and confirmation of the locations of H2B-, H3-, and H4-encoding regions were made by hybrid selection and *in vitro* translation. For these experiments 5S–18S polysomal RNA (enriched in histone mRNAs) from S-phase HeLa cells was hybridized with nitrocellulose filter-immobilized DNA from each of the λHHG phages or with plasmid DNAs containing *Eco*RI fragments of the λHHG DNAs subcloned into pBR322. The hybridized RNAs were eluted and translated in a wheat germ cell-free translation system. The results obtained for the λHHG phage DNAs are shown in Fig. 4. Only λHHG 5 and λHHG 55 contain H2A-encoding regions.

Other independent approaches involving hybridization of λHHG DNAs with histone RNAs were used to confirm that the clones contain histone sequences. λHHG clones were hybridized to blots containing cytoplasmic RNA from G_1-, S-phase, and arabinofuranosylcytosine (cytosine arabinoside)-treated S-phase HeLa S3 cells. Histone mRNAs are present on polysomes preferentially during the S phase of the HeLa cell cycle, and inhibition of DNA replication by drugs such as arabinofuranosylcytosine or hydroxyurea results in a drastic depletion of histone mRNA from the polysomes (1–4, 10, 31, 32, 35–37). λHHG 41 hybridized with H3 and H4 mRNAs and λHHG 55 with H4 mRNA and the H3-H2A-H2B cluster of histone mRNAs from S-phase cells (Fig. 5). Significant hybridization with histone mRNAs was not observed for RNA from G_1 cells. Furthermore, when DNA synthesis was inhibited with arabinofuranosylcytosine, λHHG 55 failed to hybridize to any appreciable extent with cytoplasmic RNA extracted from these cells.

H4 from HeLa cells has been reported to be coded for by several different forms of H4 mRNA, distinguishable by their different migration in several nondenaturing and denaturing gel systems (27). The presence of H4-encoding regions in four of the λHHG clones (λHHG 6, 17, 39, and 41) was confirmed by hybridization of λHHG DNAs in 80% formamide with a mixture of purified ^{32}P-labeled H4 mRNAs and subsequent digestion with S1 nuclease (28). RNA·DNA hybrids that are perfectly base-paired are resistant to S1 nuclease digestion, whereas single-stranded RNA and mismatched regions of RNA·DNA hybrids are sensitive to S1 nuclease. The products of S1 nuclease

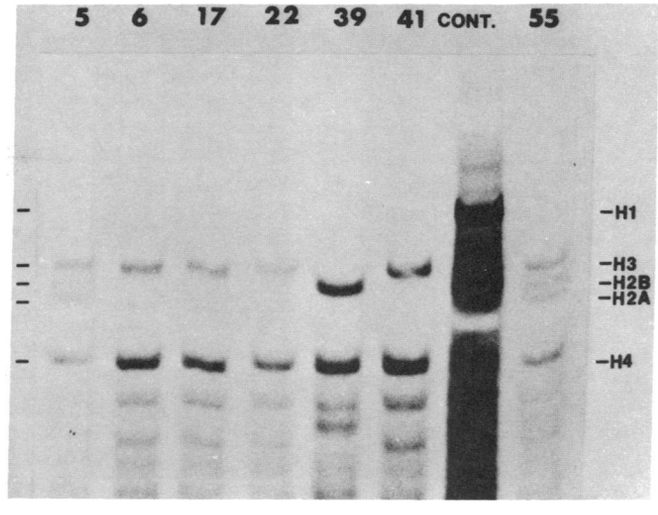

FIG. 4. *In vitro* translation of hybrid-selected 5S–18S polysomal RNA. Each λHHG phage DNA (30–120 μg) was immobilized on nitrocellulose filters. Four filters were hybridized on the same vial at 43°C for 6 hr in 50% (vol/vol) formamide/20 mM Hepes, pH 6.8/0.5 M NaCl/10 mM EDTA/ 0.5% $NaDodSO_4$. Filters were washed with buffers of decreasing ionic strength in the absence of formamide, followed by several washes in 10 mM Hepes (pH 6.8) to remove $NaDodSO_4$. Hybridized RNAs were eluted in 90% formamide/20 mM Hepes, pH 6.8/0.5 M NaCl/10 mM EDTA. RNAs were precipitated by ethanol and translated. *In vitro* translated proteins were analyzed on an acid/urea slab gel (34).

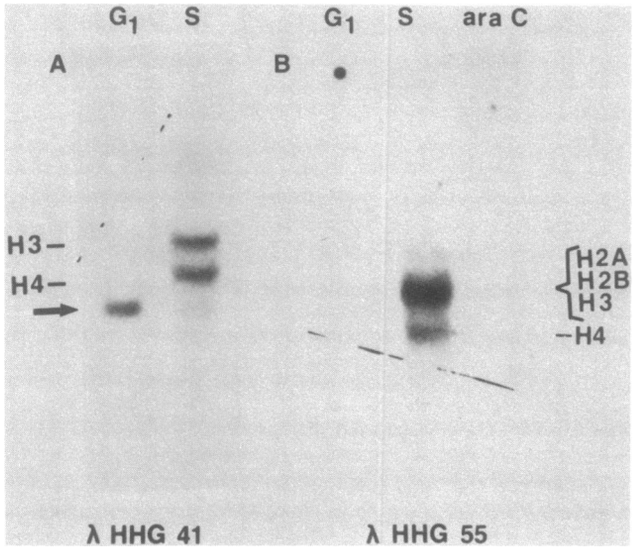

FIG. 5. Hybridization of λHHG DNAs to RNAs from different phases of the HeLa S3 cell cycle. (*A*) Hybridization of λHHG 41 DNA to blots containing total cytoplasmic RNA from G_1- and S-phase HeLa S3 cells. Arrow, 330-nucleotide RNA species. (*B*) Hybridization of λHHG 55 DNA to a blot containing total cytoplasmic RNA from G_1-, S-, or arabinofuranosylcytosine-treated (ara C) S-phase HeLa S3 cells.

digestion were denatured and electrophoresed in polyacrylamide gels. The results obtained with λHHG 6, λHHG 17, λHHG 39 and λHHG 41 are presented in Fig. 6. Each of the clones protected H4 mRNA from S1 nuclease digestion. λHHG 39 and 41 appeared to protect the same H4 mRNA species. Two different H4 mRNA species were protected by λHHG 6 and 17. This result is consistent with the observation that both clones have identical restriction maps and each contains at least

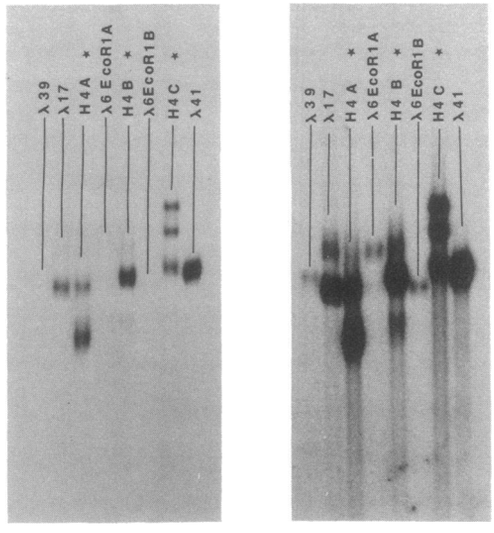

FIG. 6. S1 nuclease resistance of H4 mRNAs after hybridization to λHHG DNAs. *In vivo* ^{32}P-labeled H4 mRNAs from HeLa S3 cells were hybridized with λHHG DNAs and then digested with S1 nuclease. The products were denatured and separated electrophoretically in a nondenaturing gel. (*Left*) Two-day autoradiographic exposure. (*Right*) Eight-day autoradiographic exposure. *, Purified H4 mRNAs coelectrophoresed as markers: H4 mRNA A (lane 3), H4 mRNA B (lane 5), and H4 mRNA C (lane 7). Lanes 1, 2, 4, 6 and 8 contained H4 mRNAs that were protected from S1 nuclease digestion by hybridization with λHHG DNAs. Lanes: 1, λHHG 39; 2, λHHG 17; 4, 5.0-kb *Eco*RI fragment from λHHG 6 (Fig. 1); 6, 1.7-kb *Eco*RI/*Hin*dIII fragment from λHHG 6 (Fig. 1); 8, λHHG 41.

two H4 genes. The results of these experiments will be described in detail elsewhere.

Evidence for Another Cell Cycle-Specific Sequence in λHHG 41. In several sea urchins and in *Drosophila* (11), histone genes are clustered, and no other transcribed sequences have been detected within the clusters. When a blot containing cytoplasmic RNAs from G1- and S-phase HeLa cells was probed with λHHG 41 DNA, hybridization was detected with a 330-nucleotide RNA present predominantly in G_1 cells (in addition to the H3 and H4 mRNAs present in S phase) (Fig. 5). This G_1 RNA was smaller than any known histone mRNAs and its representation in G_1 cells appears to be quantitatively similar to that of histone mRNAs in S-phase cells (Fig. 5).

DISCUSSION

Histone genes are known to be a family of moderately repeated genes (11). In humans, the reiteration frequency of histone genes has been determined to be 20–40 copies per haploid genome (38), and they seem to be clustered in the long arm of chromosome 7 (ref. 39; unpublished data). We have described the isolation and characterization of seven genomic clones containing human histone genes. The identity of the clones as histone gene-containing sequences has been shown by hybridization with specific histone DNA probes, by hybrid selection-translation, and by hybridization with human histone mRNAs. The seven clones fall into three different arrangements with respect to restriction sites and location of histone-encoding regions. One of the arrangements contains multiple copies of H3 and H4 genes, and therefore the human histone genes, as those of other higher eukaryotes (13–16), do not fit the simple pattern of organization observed in sea urchin and *Drosophila* in which tandemly repeated clusters contain one each of the five histone genes. We cannot eliminate the possibility that there are more complicated repeats of more than one type of pattern. For example, each of the three clones λHHG 5, 41, and 55 (which constitute one arrangement) may represent repeats within the human genome. Alternatively, these clones may be independent isolates from the same genomic sequence. More detailed mapping of these clones may distinguish between these two possibilities. These arrangements may also be related to one another in the form of a large repeated gene cluster.

The seven clones we have characterized are probably not fully representative of the organization of the human histone genes. In the original screening, 15 positive clones were selected on the basis of strong hybridization with a probe containing both H3 and H4 sequences. Seven of these 15 were selected for further characterization because in additional screenings they showed the most intense hybridization with the same H3 and H4 probe. We therefore have probably selected for histone gene clusters containing multiple copies of H3 or H4 genes. Additionally, although at least seven H4 histone mRNA species have been identified in HeLa cells, (unpublished data) only three form perfect hybrids with the clones we have described. This result suggests further that we have not yet isolated clones representing the full complement of human histone genes.

Some *Eco*RI restriction fragments of the λHHG clones hybridize with a histone-enriched cDNA probe (Fig. 2) although no histone-encoding region has been assigned to these fragments (Fig. 1). These DNA fragments may contain sequences that code for RNAs other than histone mRNAs. For example, clone λHHG 41 contains a sequence that hybridizes with a 330-nucleotide RNA that is present predominantly in the cytoplasm of G_1 cells and at a much lower level in the cytoplasm of S-phase cells. The presence, within a histone gene cluster, of a transcribed sequence that is apparently cell cycle-regulated raises the possibility that this sequence might be involved in the reg-

ulation of histone gene expression.

While this manuscript was in preparation, a paper by Heintz *et al.* (40) similarly reported that human histone genes are clustered but not tandemly repeated.

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