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Targeted Disruption of *H2B-V* Encoding a Particular H2B Histone Variant Causes Changes in Protein Patterns on Two-dimensional Polyacrylamide Gel Electrophoresis in the DT40 Chicken B Cell Line*

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The chicken H2B gene family comprises eight members (*H2B-I* to *H2B-VIII*), which are all located in two major histone gene clusters. All of them have been shown to encode four different protein variants (classes I to IV). In the DT40 chicken B cell line, the *H2B-V* gene, encoding the class III H2B variant, constituted about 10% of the total intracellular mRNA from all the H2B genes. To study the nature of this particular variant *in vivo*, we generated heterozygous (*H2B-V*, +/−) and homozygous (*H2B-V*, −/−) DT40 mutants by targeted integration. The remaining H2B genes were shown to be expressed more in these mutants than in the wild-type cell lines. The growth rate of DT40 cells was unchanged in the absence of the *H2B-V* gene. Two-dimensional polyacrylamide gel electrophoresis showed that the protein patterns were, on the whole, similar between the wild-type and homozygous cell lines. However, within this constant background, some cellular proteins disappeared or decreased quantitatively in the homozygous mutants, and several other proteins increased or newly appeared. These results suggest that the class III H2B variant participates negatively or positively in regulation of the expression of particular genes that encode the proteins that vary in DT40 cells. This type of regulation is possibly mediated through alterations in nucleosome structure over the restricted regions involving the putative genes of the DT40 genome.

Chickens have fewer copies of the histone genes, ranging from six copies of the H1 gene to about ten copies of the core genes (H2A, H2B, H3, and H4) (1–3) than most higher eukaryotes, which possess large numbers of the genes of each histone subtype, ranging from several dozen to hundreds (4–7). Recently, furthermore, compensation for disruption of particular histone genes has been shown in yeast and chickens (8–15). Thus, in eukaryotes, this type of regulation, as well as the presence of multiple copies of the histone genes, should ensure that all the core histone subtypes remain in stoichiometric balance so that the chromatin structure is maintained precisely during cell proliferation.

On the other hand, there are several protein variants for each histone subtype in many higher organisms (16–20). Of the 44 chicken H1 and core genes, 30 have been sequenced, and available nucleotide sequence data indicate that the H1, H2A, H2B, and H3 families, respectively, comprise at least six, two, four, and three different protein variants (3, 21–29). All of the eight H2B genes belong to two major histone gene clusters with a total length of about 140 kb[1] (24). Five H2B genes (*H2B-I*, *H2B-II*, *H2B-III*, *H2B-IV*, and *H2B-VI*) encode the same amino acid sequence (class I), and that of *H2B-VII* differs from that of class I in three amino acid residues ($Lys^{31} \rightarrow Arg$, $Ser^{32} \rightarrow Ala$, $Gly^{60} \rightarrow Ser$; class II) (24). *H2B-V* contains a single amino acid alteration ($Lys^{30} \rightarrow Arg$; class III) (26), and the amino acid sequence of *H2B-VIII* is distinct from that of class I in two amino acid residues ($Lys^{30} \rightarrow Arg$, $Ser^{32} \rightarrow Thr$; class IV).[2] We have further demonstrated that the intracellular mRNA levels from *H2B-V* in the oviduct and lung of chickens were at most one-half those in the kidney, but the total mRNA level from all the H2B genes was roughly equal in these three tissues (30). In other organisms, several variants of each histone subtype have also been reported to be synthesized differentially throughout the cell cycle and development (16–20).

The influence of the core histone mutation on transcription regulation has been studied in yeast (12, 31). In a *Saccharomyces cerevisiae* mutant with disruption of one of two *H2A/H2B* gene pairs encoding two different variants H2A and H2B, the arrangement of nucleosomes over *CYH2* and *UBI4* and the centromere of chromosome III was dramatically disrupted, but nucleosomes over *HIS4* and *GAL1* and the telomeres appeared essentially normal. In this mutant, *HIS4* was constitutively expressed and *GAL1* repression was unaffected by the mutation. Interestingly, the intracellular levels of *CYH2* transcripts were unchanged, but those of *UBI4* transcripts increased about 2-fold. Thus, deletion of the particular histone gene pair influenced the expression of several genes differentially, through chromatin disruption localized in specific regions of the yeast genome. In addition, results obtained in *in vivo* and *in vitro* experiments showed that H1 histone acted as a general repressor of transcription in several higher eukaryotic systems (32–37), although not in yeast.

Together, these results led us to speculate that the variants of each of the chicken histone subtypes have specific individual functions in particular biological events including gene expression. To clarify the nature of the class III H2B variant *in vivo*, we constructed transfectants devoid of *H2B-V*, encoding it by targeted integration, since the gene distinctly produced about 10% of the total intracellular level of mRNAs from all the H2B

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[1] The abbreviations used are: kb, kilobase(s); bp, base pair(s); PAGE, polyacrylamide gel electrophoresis.

[2] Y. Takami, M. Higashi, T. Fukuoka, S. Takechi, and T. Nakayama, submitted for publication.

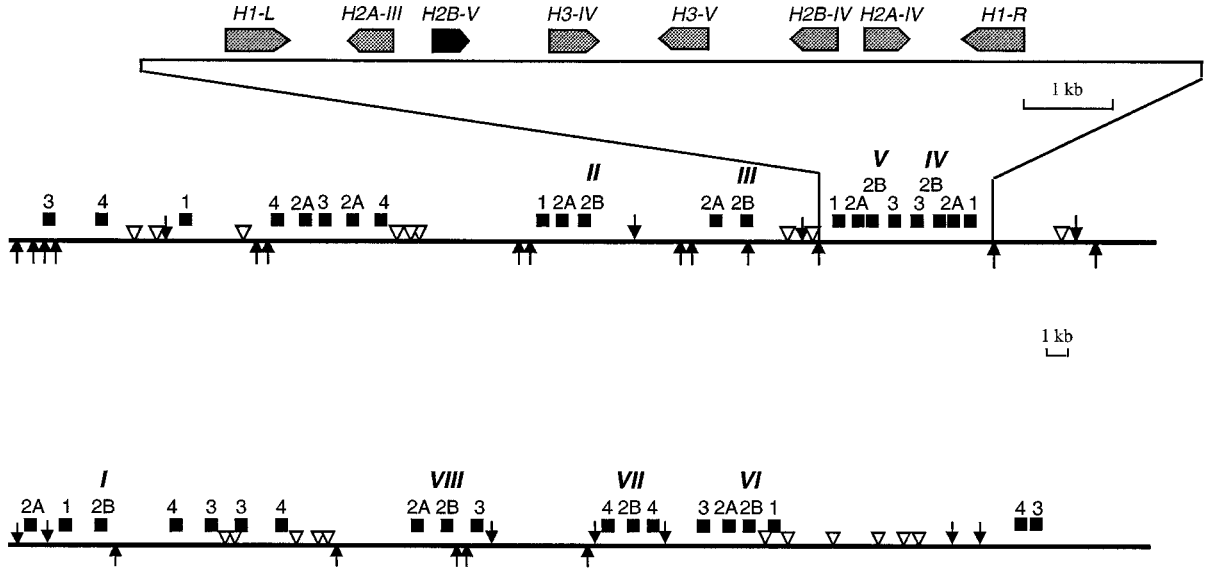


FIG. 1. **Organization of the chicken histone genes.** The two major histone gene clusters reported (24) are shown with slight modifications. *1*, *2A*, *2B*, *3*, and *4* indicate H1, H2A, H2B, H3, and H4, respectively. The eight H2B genes are designated as *I* to *VIII* (26). A subcluster, carrying two H1-H2A-H2B-H3 gene sets, is extended, and the ORF with their orientations are shown by *arrows*. Cleavage sites: *upward arrows*, *Eco*RI; *downward arrows*, *Bam*HI; *open downward arrowheads*, *Hin*dIII.

genes in DT40 cells. Analyses by two-dimensional PAGE revealed that the protein patterns of the homozygous mutants were slightly, but obviously, distinct from those of the wild-type cell lines, indicating the involvement of the class III H2B variant in regulation of the expression of putative genes encoding the proteins that varied.

MATERIALS AND METHODS

Cell Cultures—DT40 cells and all subclones were cultured essentially as described (8, 38–40) in Dulbecco's modified medium containing 10% (v/v) fetal calf serum, 1% (v/v) chicken serum, 2 mM L-glutamine, 0.01 mM β-mercaptoethanol, and penicillin/streptomycin at 37 °C under 5% CO_2 in air in a humidified incubator unless stated otherwise. At the indicated times, the cell numbers were counted to determine the growth rate.

Gene Constructs—As a source of chicken genomic DNA of *H2B-V*, we used the 11.5-kb *Eco*RI fragment carrying two H1-H2A-H2B-H3 gene sets and the 3.8-kb *Eco*RI/*Kpn*I fragment carrying an *H1L-H2A-III-H2B-V* gene set (26). To obtain the H2B-V/hisD targeting construct, we inserted the fragment carrying *hisD* under the chicken β-actin promoter (41) into the *Bst*XI site of the *H2B-V* coding region of the 11.5-kb *Eco*RI fragment, and the chimeric DNA was inserted into the Bluescript II plasmid. To obtain the H2B-V/Eco-gpt targeting construct, the fragment carrying *Eco-gpt* under the chicken β-actin promoter (42, 43) was inserted similarly into the *Bst*XI site of the *H2B-V* coding region of the 3.8-kb *Eco*RI/*Kpn*I fragment, and the resultant fragment was inserted into the Bluescript II plasmid. Before transfection into DT40 cells, we linearized the H2B-V/hisD or H2B-V/Eco-gpt construct by *Sph*I or *Bam*HI digestion. Probe 1 was the *Hin*dIII/*Eco*RI fragment of about 400 bp originating from the 5′ outside of the *Eco*RI fragment of 11.5 kb. Probe 2 was the *Eco*RI/*Sca*I fragment of *hisD* of about 750 bp, and probe 3 was the *Eco*RI/*Eco*RV fragment of *Eco-gpt* of about 400 bp.

Transfection and Isolation of Transfectants—The transfection conditions were essentially as described previously (8, 38–40). Transfectants with the H2B-V/hisD construct were selected in medium containing 600 μg of histidinol/ml. We transfected the H2B-V/Eco-gpt construct into clones in which one of two *H2B-V* genes had already been disrupted, and selected transfectants in medium containing 600 μg of histidinol/ml and 25 μg of mycophenolic acid/ml, respectively.

Southern Blot Analysis—Samples of 10 μg of genomic DNAs were digested with the indicated enzymes, separated in a 0.8% (w/v) agarose gel, transferred to a Hybond N^+ membrane according to the supplier's protocol (Amersham), and then hybridized by the method of Southern (44) with probe 1, 2, or 3. The DNA probes were labeled with ^{32}P by random-priming as recommended by the supplier (Amersham).

RNase Protection Method—An antisense RNA probe for measurement of the amounts of H2B mRNAs was prepared as follows. A blunt-ended *Bgl*I fragment of 352 bp, containing the 128-bp 5′-flanking and 224-bp 5′-coding regions of *H2B-V*, was inserted into the *Eco*RV site of Bluescript II. ^{32}P-Labeled antisense RNAs were synthesized with phage T7 RNA polymerase after cutting with *Bam*HI and used as probe H2B-V. This probe was 430 nucleotides long (see Fig. 2*A*), since it consisted of the 352-nucleotide fragment from *H2B-V* flanked by the 25- and 53-nucleotide fragments derived from the vector.

Total RNAs were isolated from exponentially growing DT40 subclones as described (45). The intracellular levels of H2B mRNAs were determined by the RNase protection method with a [^{32}P]CMP-labeled RNA probe and an Ambion RPAII kit according to the manufacturer's protocol. This probe revealed no bands for yeast RNA fractions (see Figs. 2*A* and 5), since the chicken H2B gene exhibits no homology with that of yeast. After electrophoresis in a denaturating polyacrylamide gel, autoradiography was carried out. The intensities of the radioactivity of protected fragments for *H2B-V* and for the remaining H2B genes were then determined with a Fuji BAS 1000 Image Analyzer. In the former case, the value was corrected as to the ratio of the number (38) of nucleotide C between positions +93 and +226 to that (79) between positions −57 and +226 of the antisense RNA probe, since the putative initiation site of *H2B-V* is position −57.

Two-dimensional Gel Electrophoresis—Total cellular proteins were prepared from exponentially growing DT40 subclones as described (46) and then separated with an automated apparatus for two-dimensional electrophoresis, TEP-1 (Shimadzu, Japan) (47). The conditions for the first-dimensional isoelectrofocusing gel were 5% (w/v) acrylamide, 6% (v/v) ampholines (pH 3–10), 8 M urea, and 2% (v/v) Nonidet P-40. After electrophoresis for 16 h at 800 V and 20 °C, the isoelectrofocusing gel was transferred automatically to equilibration buffer (5% (v/v) β-mercaptoethanol, 2.5% (w/v) SDS, and 8 M urea) on the second dimensional SDS-PAGE gel for 5 min. Then the second run was carried out under the conditions of 0.1% (w/v) SDS and 12.5% (w/v) acrylamide for 30 min at 150 V and then for 3.5 h at 300 V at 15 °C. Then, the SDS-PAGE gel was stained with Coomassie Blue.

RESULTS

H2B-V Is Responsible in Part for the Total Intracellular Level of H2B mRNAs in the DT40 Cell Line—In chickens, eight H2B genes (*H2B-I* to *H2B-VIII*) are all located in two major histone gene clusters (24), which are shown with extension of a subcluster carrying two H1-H2A-H2B-H3 gene sets in Fig. 1. These eight H2B genes encode four different protein variants (classes I to IV), each of which is composed of 125 amino acid residues including a putative initiation Met (24, 26).[2] They exhibit extensive sequence homology (92–99%) in their ORF, but are considerably different in their 5′-flanking regions, ex-

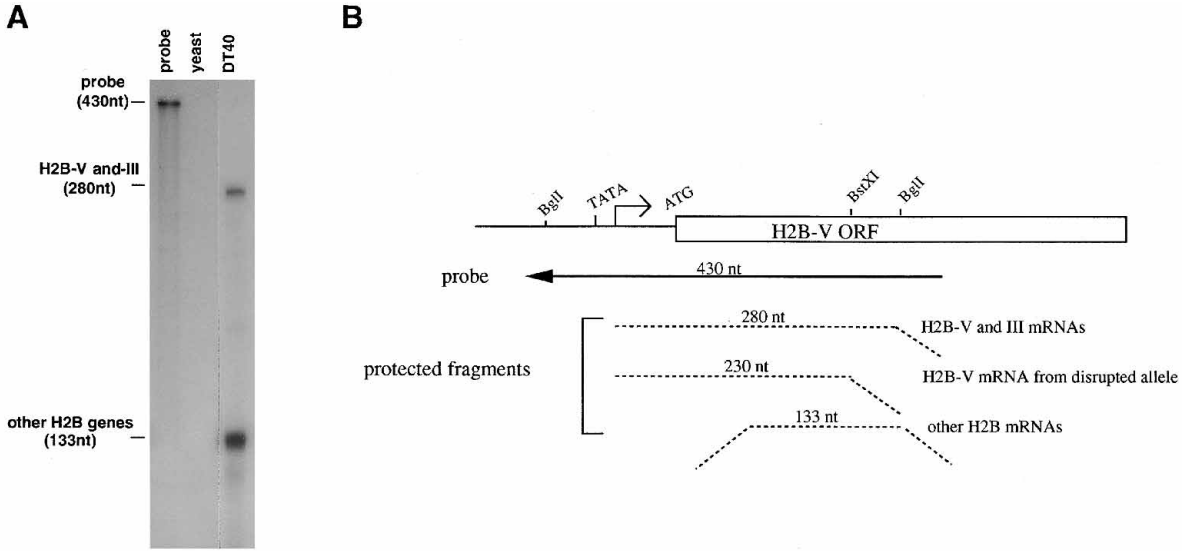


FIG. 2. **Expression of H2B genes in DT40 cells.** *A*, intracellular mRNA levels from *H2B-V* and from the remaining H2B genes. DT40 cells were grown in Dulbecco's modified medium to the logarithmic phase (38), and then total RNAs were extracted (45). Total RNAs (15 μg), together with 15 μg of total RNAs of yeast, were analyzed using ^{32}P-labeled antisense RNA probe H2B-V. The labeled probe was also run. After electrophoresis in a denaturating polyacrylamide gel, autoradiography was carried out. The radioactive intensities of the protected fragments of *H2B-V* and of the residual H2B genes were measured with a Fuji BAS 1000 Image Analyzer. *B*, schematic illustration of the RNase protection method using probe H2B-V.

cept that *H2B-V* and *H2B-III* are extremely similar in both the 5′-flanking and 5′-coding regions. Moreover, between positions +80 and +90, these two genes have continuously distinct sequences of 4 or 5 nucleotides from other H2B genes.

To determine the intracellular levels of H2B mRNAs in DT40 cells, we applied the RNase protection method using probe H2B-V, which consisted of the antisense RNA fragment derived from the 128-bp 5′-flanking region involving the 5′-untranslated sequence plus the 224-bp 5′-coding region of *H2B-V*, in addition to the 78-bp flanking sequence of the plasmid vector (Fig. 2*B*). Therefore, this antisense RNA probe should protect the 5′-untranslated and 5′-coding sequences of mRNAs of about 280 nucleotides from *H2B-V* plus *H2B-III* completely, but only about 133 nucleotide internal portions of mRNAs from other H2B genes. As expected, the 280 nucleotide band of mRNAs from *H2B-V* plus *H2B-III* was distinguishable from some bands corresponding to about 133 nucleotides of mRNAs from the remaining H2B genes (Fig. 2*A*). Most of the intensity of the 280-nucleotide band disappeared in the homozygous DT40 mutants in which two *H2B-V* genes were deleted by targeted integration, indicating that the band was derived exclusively from *H2B-V* but slightly from *H2B-III* (see Fig. 5). Thus, the class III H2B variant encoded by *H2B-V* is definitely present in DT40 cells, although at low levels (its quantitative levels will be shown later).

High Frequency of Targeted Integration into the H2B-V Locus—To clarify the nature of *H2B-V*, we first transfected DT40 cells with targeting vectors of the gene. Genomic DNAs were prepared from the stable transfectants and then analyzed by Southern blotting after *Hin*dIII digestion or *Bam*HI plus *Sal*I digestion. The filter was first hybridized with probe 1 originating from the 5′ outside of the *Eco*RI fragment of 11.5 kb, containing two H1-H2A-H2B-H3 gene sets (Fig. 3*c*), followed by hybridization with probes 2 and 3 derived from *hisD* and *Eco-gpt*, respectively (Fig. 3, *d* and *e*).

In a control DT40 cell line (hisD-ecogpt) carrying both *hisD* and *Eco-gpt* integrated randomly, probes 1, 2, and 3, respectively, hybridized to several different fragments (Fig. 4, *A*, *B*, and *C*). The sizes of these fragments were distinct from those expected from targeted integration events as mentioned later.

As an initial step to obtain mutant cells deprived of *H2B-V*, we introduced the H2B-V/hisD construct into DT40 cells. In this targeting vector, *hisD* under the chicken β-actin promoter was inserted into the *H2B-V* coding region and flanked upstream and downstream by sequences surrounding the gene (Fig. 3*a*). As expected after integration of the H2B-V/hisD construct into the *H2B-V* locus (see Fig. 3*d*) in two of the seven stable transfectants selected with histidinol (cl-6 and cl-7), probe 1 newly hybridized to a *Hin*dIII fragment of 4.8 kb and a *Bam*HI/*Sal*I fragment of 7.5 kb (Fig. 4*A*). Probe 2 hybridized to a *Hin*dIII fragment of 9.8 kb and a *Bam*HI/*Sal*I fragment of 7.5 kb (Fig. 4*B*); probe 3 showed no bands (Fig. 4*C*). Similar results were obtained with three other clones (data not shown). In these five clones, thus, one of two *H2B-V* genes had been modified.

Two of the five histidinol-resistant clones (cl-6 and cl-7; *H2B-V*, +/−) were then chosen for transfection of the H2B-V/Eco-gpt construct. In this targeting construct, *Eco-gpt* transcribed by the chicken β-actin promoter was inserted into the *H2B-V* coding region (Fig. 3*b*). As expected after integration of the H2B-V/Eco-gpt construct into the remaining *H2B-V* gene (see Fig. 3*e*) in three of the 44 clones analyzed (cl-6-1, cl-6-11, and cl-7-4), probe 1 hybridized to a *Hin*dIII fragment of 4.8 kb and two *Bam*HI/*Sal*I fragments of 7.5 kb and 6.5 kb (Fig. 4*A*). Probe 2 hybridized to a *Hin*dIII fragment of 9.8 kb and a *Bam*HI/*Sal*I fragment of 7.5 kb (Fig. 4*B*), and probe 3 hybridized to a *Hin*dIII fragment of 8.8 kb and a *Bam*HI/*Sal*I fragment of 6.5 kb (Fig. 4*C*). Similar results were obtained with 12 other clones (data not shown). These results, thus, together with those for the first allele, indicate that targeted integration into the *H2B-V* locus occurred at a high frequency, as expected from results for the different loci tested, the rearranged and unrearranged immunoglobulin light chains, β-actin, ovalbumin, RAG-2, RAD51, Csk, and the H3 and H1 genes (8, 39, 40, 48, 49).[3]

Decrease and Disappearance of Intracellular H2B-V mRNAs in the Heterozygous and Homozygous Mutants—We analyzed the intracellular levels of mRNAs from *H2B-V* and from the remaining H2B genes in several DT40 subclones by the RNase

[3] Seguchi, K., Takami, Y., and Nakayama, T. (1995) *J. Mol. Biol.*, in press.

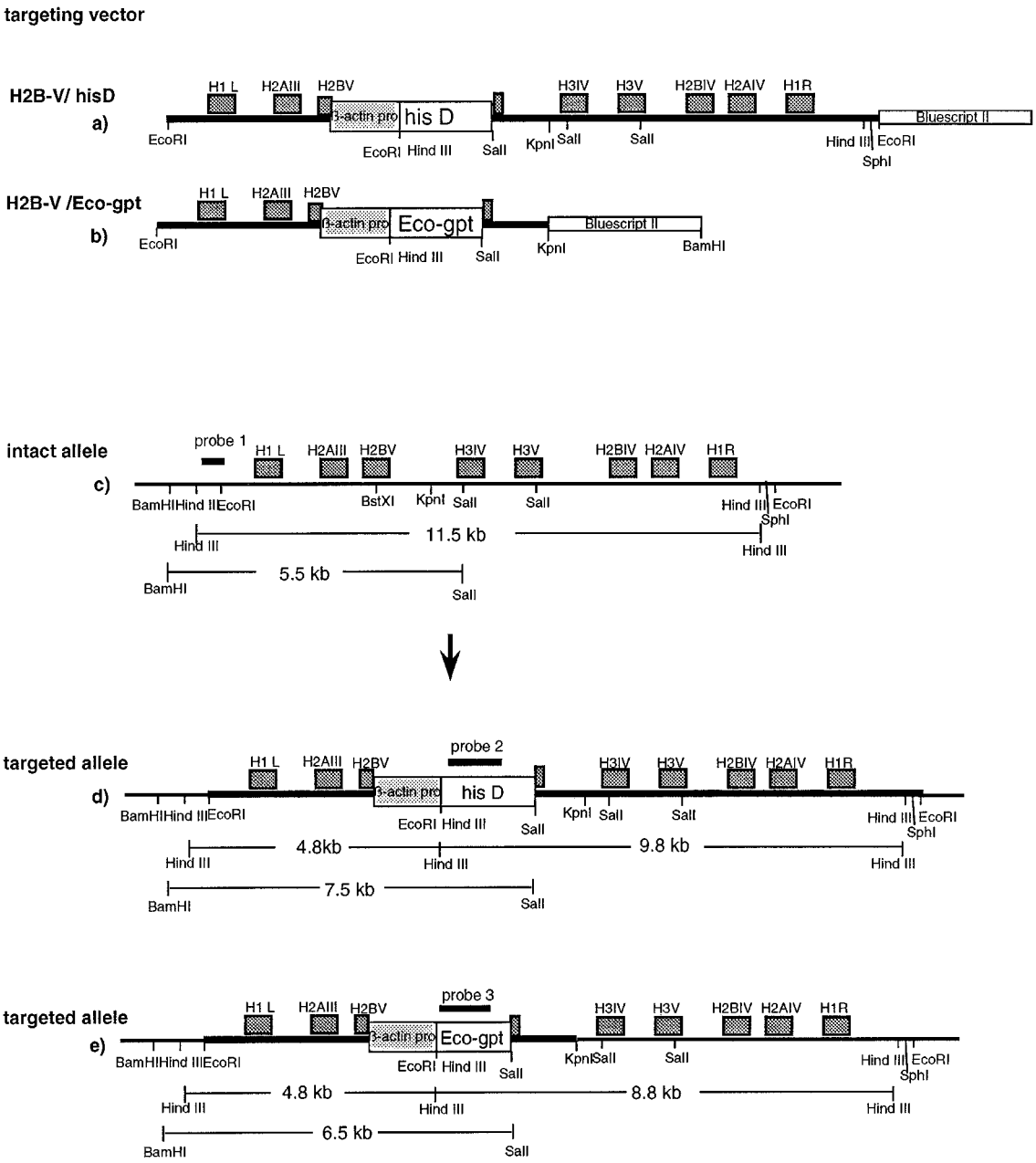


FIG. 3. **Schematic diagram of the homologous recombination resulting in deletions of the first and second *H2B-V* genes.** *a*, targeting H2B-V/hisD construct. *b*, targeting H2B-V/Eco-gpt construct. The *open boxes* (*hisD* and *Eco-gpt*) indicate *hisD* and *Eco-gpt*, respectively, under the control of the chicken β-actin promoter represented by the *lightly shaded area* (*β-actin pro*). The plasmids were linearized at the *Bcl*I or *Cla*I site. *c*, *H2B-V* locus in the genome of DT40 cells. *d*, *H2B-V* locus in DT40 clones after targeted integration of the H2B-V/hisD construct. *e*, *H2B-V* locus in DT40 clones after targeted integration of the H2B-V/Eco-gpt construct. *H1L*, *H2A-III*, *H2B-V*, *H3-IV*, *H3-V*, *H2B-IV*, *H2A-IV*, and *H1R*, respectively, indicate their ORF. The locations of probes 1, 2, and 3 are indicated by *bars*. Only relevant restriction sites are indicated. Possible relevant fragments obtained on *Hin*dIII digestion and *Bam*HI plus *Sal*I digestion are shown with their lengths in kilobases.

protection method using probe H2B-V, as described above. The amounts and patterns of H2B mRNAs of a drug-resistant control cell line (hisD-ecogpt; *H2B-V*, +/+) were essentially identical with those of DT40 cells (Fig. 5), showing that the two exogenous genes, *hisD* and *Eco-gpt*, were scarcely related to the expression of any of the H2B genes.

The intracellular levels of mRNAs from *H2B-V*, appearing as a 280-nucleotide band, in two heterozygous mutants deprived of one of two *H2B-V* genes (cl-6 and cl-7; *H2B-V*, +/−) were essentially the same (Fig. 5) and were about 70%, instead of 50%, of those in the control cell lines (DT40 and hisD-ecogpt; *H2B-V*, +/+). On the other hand, the 230-nucleotide band, which was derived from the chimeric *H2B-V* gene after the targeting event (see Fig. 2*B*), newly appeared. In four homozygous mutants deprived of two *H2B-V* genes (cl-6-1, cl-6-2, cl-6-11, and cl-7-4; *H2B-V*, −/−), the intensity of the 280-nucleotide band decreased dramatically, and that of the chimeric H2B mRNAs of 230 nucleotides increased inversely (Fig. 5). Therefore, the residual intensity of the 280-nucleotide band in these homozygous mutants should be due to transcripts from *H2B-III*. Thus, the normal levels of *H2B-V* mRNAs in DT40 could be estimated by subtraction of the intensity of the 280-nucleotide band (0.7%) in the homozygous mutants from that (10.5%) in the wild-type cell lines and were about 10% of the total mRNAs from all the H2B genes. In the heterozygous mutants (cl-6 and cl-7), other H2B genes were transcribed at higher levels than in the wild-type cell lines (DT40 and hisD-ecogpt), the increase being about 10% (table in Fig. 5). In the homozygous mutants

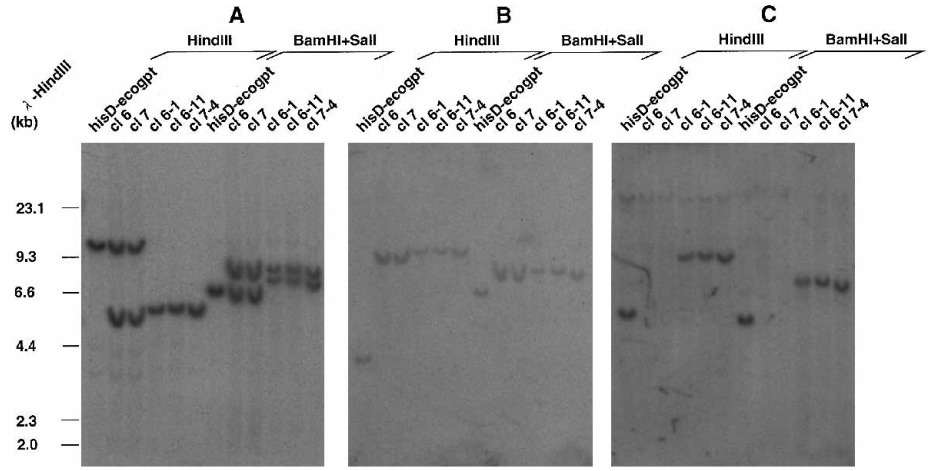
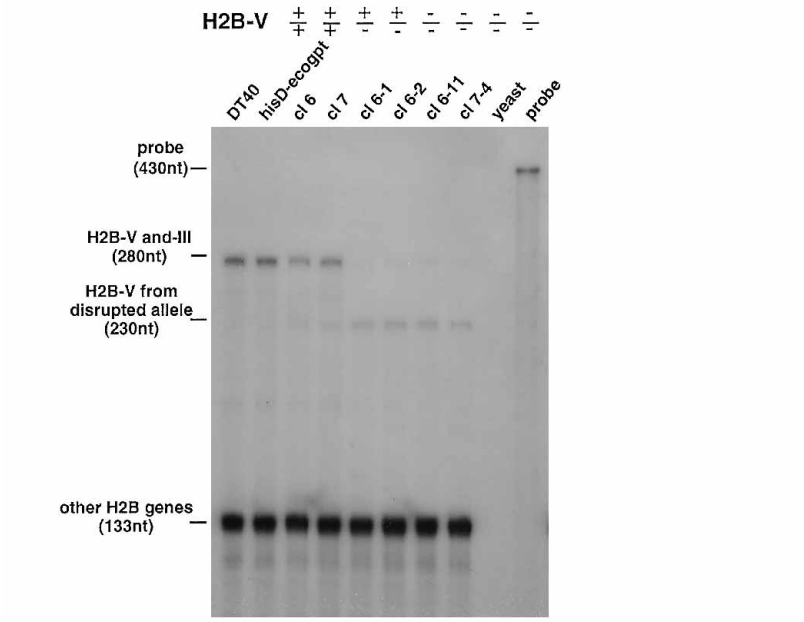


FIG. 4. **Southern blot analyses of homologous recombination events.** Genomic DNAs were prepared from one wild-type cell line carrying both *hisD* and *Eco-gpt* integrated randomly (hisD-ecogpt), two histidinol-resistant mutants after integration of the H2B-V/hisD construct (cl-6 and cl-7), and three histidinol and mycophenolic acid-resistant mutants after integration of the H2B-V/Eco-gpt construct (cl-6-1, cl-6-11, and cl-7-4). The *Hin*dIII fragments and *Bam*HI/*Sal*I fragments were hybridized with probe 1 (*A*), 2 (*B*), or 3 (*C*).



Relative radioactive intensity and ratio

	DT40	hisD-ecogpt	cl6	cl7	cl6-1	cl6-2	cl6-11	cl7-4
other H2B genes	100.0	106.7	103.8	123.1	118.3	134.6	124.0	107.7
H2B-V and-III	11.9	12.2	7.1	9.8	1.1	1.2	0.7	0.5
% of H2B-V and -III	10.7%	10.2%	6.4%	7.3%	0.9%	0.9%	0.6%	0.4%

FIG. 5. **Effects of *H2B-V* disruption on the intracellular levels of H2B mRNAs.** The experimental procedures were essentially as in Fig. 2. Two wild-type (DT40 and hisD-ecogpt; *H2B-V*, +/+), two heterozygous (cl-6 and cl-7; *H2B-V*, +/−), and four homozygous (cl-6-1, cl-6-2, cl-6-11, and cl-7-4; *H2B-V*, −/−) cell lines were grown to the midexponential phase, and then total RNAs were prepared and analyzed with probe H2B-V. Yeast RNAs were also analyzed. The labeled probe was also run. After electrophoresis in a denaturating polyacrylamide gel, autoradiography was carried out. The radioactive intensities of the protected fragments of *H2B-V* plus *H2B-III* and of other H2B genes were quantified, and, in the former case, the value was corrected as to the ratio of the number (38) of nucleotide C between positions +93 and +226 to that (79) between −57 and +226 of the antisense RNA probe. The radioactive intensities of mRNAs from other H2B genes in DT40 were assigned values of 100, and the relative values obtained are shown in the table. The ratios of the intensities of mRNAs from *H2B-V* plus *H2B-III* to those of mRNAs from all the H2B genes are also shown.

(cl-6-1, cl-6-2, cl-6-11, and cl-7-4), the expression of other H2B genes increased, and the mRNA levels were about 17% higher than those in the wild-type cell lines. In addition, in the heterozygous (*H2B-V*, +/−) and homozygous (*H2B-V*, −/−) mutants, the average sums of mRNAs from *H2B-V* and those from the other H2B genes including *H2B-III* were 105.6% and 105.7% of the normal levels, respectively. Thus, the H2B gene family, like the H3 gene family (8), has the ability to maintain steady-state levels of transcripts.

Changes in Protein Patterns after Disruption of Two H2B-V Genes—The influences of *H2B-V* disruption on the growth rate and chromatin structure were examined. Analyses of the heterozygous and homozygous mutants showed that the growth rate of DT40 cells was unchanged in the absence of *H2B-V*, and the doubling time of all the DT40 subclones examined was about 12 h. Furthermore, the chromatin structure in the mutants was, on the whole, identical with that in the wild-type cell lines (data not shown). However, because H2B-V mRNAs were definitely present in DT40 cells, the class III H2B variant encoded by *H2B-V* was expected to participate in transcription regulation, probably through local changes in the chromatin structure. To clarify the nature of this variant, we compared total cellular proteins of the homozygous mutant deprived of two *H2B-V* genes with those of the wild-type DT40 cell line (Fig. 6). As a control, we used the hisD-ecogpt cell line (*H2B-V*, +/+) in which *hisD* and *Eco-gpt* were inserted independently by random integration.

Total cellular proteins were prepared from exponentially growing DT40 subclones and analyzed by two-dimensional PAGE. Under the conditions used, we separated the proteins

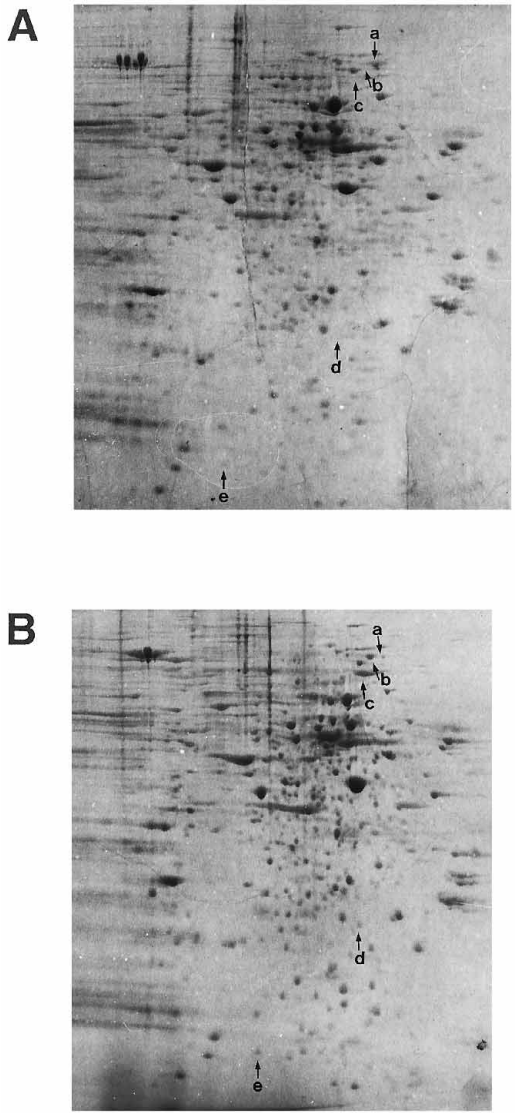


FIG. 6. **Comparison of the total cellular proteins of the wild-type and homozygous cell lines by two-dimensional PAGE.** Total cellular proteins were prepared as described in the text from a drug-resistant control cell line (hisD-ecogpt; *H2B-V*, +/+) (*A*) and a homozygous mutant (cl-6-11; *H2B-V*, −/−) (*B*). Isoelectrofocusing in the first dimension was performed in a gel using wide range ampholines (pH 3–10). The effective range was pH 4 (right) to 8 (left). SDS-PAGE in the second dimension was performed in 12.5% (w/v) acrylamide. The *downward arrow* indicates the protein that decreased in the homozygous mutant. *a*, 120 kDa. The *upward arrows* indicate the proteins that newly appeared or increased in the homozygous mutant. *b*, 120 kDa; *c*, 98 kDa; *d*, 30 kDa; *e*, 21 kDa.

based on the differences in pI, in ranges of about 4 to 8, and in molecular mass, in ranges of about 10 to 200 kDa, respectively. Therefore, all the histone subtypes with high pI values of about 12 could not be detected in our two-dimensional PAGE gels. No difference was observed in the electrophoretic patterns of DT40 and the drug-resistant control cell line, and the xanthine-guanine phosphoribosyltransferase of 17 kDa derived from *Eco-gpt* and the product of 50 kDa derived from *hisD* were undetectable even in the drug-resistant control cell line, probably because their amounts were very low (data not shown). These findings indicate that the expression of both *hisD* and *Eco-gpt* did not cause any change in the expression of endogenous genes.

The electrophoretic patterns of the proteins from the drug-resistant control cell line (hisD-ecogpt; *H2B-V*, +/+) were very similar to those in the case of the homozygous mutant (cl-6-11; *H2B-V*, −/−) (Fig. 6, *A* and *B*). However, on detailed comparison, several notable variations were observed within this constant background. The 120-kDa protein (indicated by *a*) and possibly some other proteins were present in the wild-type cell line, but were absent or present at lower amounts in the homozygous mutant. On the other hand, the 120-kDa, 98-kDa, 30-kDa, and 21-kDa proteins (indicated by *b*, *c*, *d*, and *e*), possibly with some other proteins, significantly increased in amount or newly appeared in the mutant. Judging from their molecular mass and pI, these proteins that varied did not correspond to either the chicken H2B histone (molecular mass, 14 kDa; pI, about 12), or the two exogenous 50-kDa and 17-kDa proteins from *hisD* and *Eco-gpt*, respectively. Similar results were obtained on comparison of total cellular proteins between DT40 and another homozygous mutant (cl-7−4; *H2B-V*, −/−) (data not shown). Therefore, these alterations in the protein patterns were not due simply to clonal deviation. Thus, our results clearly demonstrate that disruption of *H2B-V* encoding the class III H2B variant caused not only decreases in the amounts of the 120-kDa protein and some other proteins, but also increases in the amounts of the 120-kDa, 98-kDa, 30-kDa, and 21-kDa proteins and some others.

DISCUSSION

Many experiments have been performed on the roles of eukaryotic histones in both the maintenance of chromatin structure and transcription regulation (50). Recent studies involving gene disruption techniques have provided critical information concerning the nature of histones. For instance, histone H4 is required for the maintenance of the genome integrity in *S. cerevisiae* (51). Analysis of a *S. cerevisiae* mutant with deletion of one of two *H2A*/*H2B* gene pairs encoding a member of two H2A variants and a member of two H2B variants showed that chromatin disruption due to this mutation was localized in specific regions of the yeast genome and affected the expression of various genes differentially (31). Recently, Hirschhorn *et al.* (52) showed that a new class of histone H2A mutations in *S. cerevisiae* causes specific defects in transcription. Moreover, it has been shown that a histone H2A variant is important for chromosomal structure and function in *Schizosaccharomyces pombe* (53). Interestingly, in *D. melanogaster*, a histone variant, *H2AvD*, is essential either to provide an alternative capability for nucleosome assembly or to generate an alternative nucleosome structure (54).

Of the four chicken H2B variants (24, 26),[2] the class III variant would be a minor form in DT40 cells, since the intracellular levels of mRNAs from *H2B-V* encoding it were only about 10% (Figs. 2*A* and 5). To clarify the nature of this class III H2B variant *in vivo*, we transfected the H2B-V/hisD and H2B-V/Eco-gpt constructs into DT40 cells and obtained heterozygous and homozygous mutants, respectively, with disruption of one and two *H2B-V* genes. The intracellular levels of mRNAs from *H2B-V* in two heterozygous mutants (cl-6 and cl-7; *H2B-V*, +/−) were about 70% of the normal levels in wild-type cell lines (DT40 and hisD-ecogpt; *H2B-V*, +/+), and no H2B-V mRNAs were detected in four homozygous mutants (cl-6-1, cl-6-2, cl-6-11, and cl-7-4; *H2B-V*, −/−) (Fig. 5). As on the disruption of *H3-IV*/*H3-V*, which normally produce 24% of total H3 mRNAs in DT40 cells (8), we observed an alteration in the intracellular mRNA levels from the remaining H2B genes in all the *H2B-V*-disrupted mutants analyzed; nevertheless, the total H2B mRNA levels decreased at most 10%. Moreover, no variations were detected in either the growth rate or the overall chromatin structure, even after two *H2B-V* genes had been disrupted (data not shown).

Analyses by two-dimensional-PAGE showed that the proteins in the homozygous mutants were virtually identical with those in the wild-type cell lines. However, interestingly, the

protein patterns were slightly, but distinctly, different (Fig. 6). In the homozygous mutants, the 120-kDa protein and some other proteins had disappeared or decreased substantially, whereas the 120-kDa, 98-kDa, 30-kDa, and 21-kDa proteins, and some other proteins, had increased or newly appeared. These proteins that varied did not correspond to either the chicken H2B histone or the two products from *hisD* and *Eco-gpt*, judging from their molecular mass and pI. In addition, none of these proteins corresponded to any of the proteins that varied in the mutants devoid of the 01H1 variant, one of the six chicken H1 variants.[3] Together, these observations demonstrate that the class III H2B variant, like the 01H1 one, plays a specific role in transcription regulation in DT40 cells, in addition to a vital role in chromatin organization.

To explain the variations in the protein patterns in the DT40 mutants with the *H2B-V* disruption, we propose that *H2B-V* mutation alters the nucleosome structure over genes encoding proteins that varied, resulting in negative control of the expression of those encoding the 120-kDa protein and some other proteins, and in positive control of the expression of those encoding the 120-kDa, 98-kDa, 30-kDa, and 21-kDa proteins, and some others. In this model, nucleosomes over these putative genes in DT40 cells would normally include at least one molecule of the class III H2B variant as an H2B subtype, and in the *H2B-V*-disrupted mutants the variant should be replaced by any class I, II, or IV H2B variant. However, this model is hypothetical as there is no definitive biological evidence that the nucleosome structure really varies in the DT40 mutants, as in the yeast mutant (12, 31). Additional studies on disruption of each of the residual H2B genes are essential to determine whether or not each variant of the H2B family has a specific function in transcription regulation.

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