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Developmental Regulation of Hypomethylation of δ-Crystallin Genes in Chicken Embryo Lens Cells

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Sequences in the two δ-crystallin genes become hypomethylated when they are expressed in the chick lens. This system is particularly advantageous for studying temporal changes in hypomethylation, since lens tissue can be isolated at all developmental stages. In previous work we have shown that most *Hpa*II sites become hypomethylated within the δ1-crystallin gene long after δ-crystallin gene activation. One site is hypomethylated when crystallin mRNA begins to be synthesized at high levels at 50 h; we show here that this site maps to the 3′ end (intron 15) of the δ1-crystallin gene. In addition, we have examined the methylation status of *Hpa*II and *Hha*I sites found near the 5′ end of the δ1-crystallin gene. Two *Hha*I sites adjacent to a viral core enhancer sequence in intron 2 are also first hypomethylated at 50 h. These findings point to regions of the δ1 gene that should be investigated further for functional significance in regulating δ-crystallin transcription.

δ-Crystallin is the product of one or both of the two adjacent δ-crystallin genes found in the chicken genome and is the predominant protein synthesized by the embryonic chick lens (18). The δ1-crystallin gene has the typical transcriptional control elements found upstream of most RNA polymerase II genes. Sequence data have identified the locations of a CAAT box and TATA box 5′ to δ1 (14, 15) and the locations of two regions with homology to viral core enhancer sequences; one is located 300 base pairs (bp) 5′ to δ1, and the other is within the second intron (4, 13). The δ2-crystallin gene is located 4 kilobases (kb) downstream from δ1 in the same orientation (13). Both genes contain 17 exons and polyadenylation signals at the 3′ end, but gene 2 is missing enhancer-like sequences and CAAT sequences in the 5′ region. Also, the δ1 promoter has fivefold greater activity than does the δ2 promoter in driving transcription of the bacterial chloramphenicol acetyltransferase gene in transient transfection assays (4). These data support the current hypothesis that δ1 is the primary δ-crystallin gene expressed during lens differentiation, and recent experiments have determined that only 1 to 2% of embryonic lens mRNA is transcribed from δ2 (17).

One additional characteristic of a transcribed gene is that it often has reduced levels of cytosine methylation compared with DNA from cells in which the gene is not transcribed (1). Several restriction enzymes that can be used to identify hypomethylated DNA are available (3). *Hpa*II ($C^{me}CGG$) will cleave DNA if the cytosine residue in the second position is unmethylated on both strands of DNA, while its isoschizomer, *Msp*I, will cleave at this sequence (CCGG) regardless of the status of methylation of the internal cytosine. Several CCGG sequences within the δ-crystallin genes are hypomethylated (assayed by *Hpa*II digestion) when these genes are expressed in the embryonic chick lens (11). In most studies, hypomethylation of a gene has been determined only in a tissue where the gene is inactive and in a tissue where the gene is expressed. However, by following the kinetics of hypomethylation in carefully staged lenses, we have found at least one *Hpa*II site that is especially intriguing because it undergoes hypomethylation at 50 h of development (9), when δ-crystallin transcripts begin to accumulate at high levels in the lens (20).

In the present study we have expanded our analysis of hypomethylation of the two δ-crystallin genes by using several new 5′ probes and a second restriction enzyme, *Hha*I, which is used to examine methylation at $G^{me}CGC$ sequences since this enzyme will not cleave DNA when the latter sequence is methylated. In these experiments, lens and nonlens tissues were dissected from staged embryos (16) and processed as described previously (21). Initially, samples were collected from 13-day lenses, where δ-crystallin genes are transcribed at high levels, and from nonlens tissue (40-h headless embryos). When these initial experiments revealed an interesting difference in the pattern of hypomethylation in a particular region, DNA was isolated from lenses at intermediate times between 40 h and 13 days.

Methylation at *Hha*I sites in gene 1. Figure 1 is a map of all *Hha*I and *Hpa*II sites in the two δ-crystallin genes, assembled from published sequence information for the δ-crystallin locus (13–15). Four *Hha*I sites have been located at the 5′ end of δ1: site 1 (abbreviated Hha^1) is 2,411 bp upstream of the start of transcription; site 2 is 448 bp upstream; and sites 3 and 4 are in intron 2, 476 and 1,038 bp, respectively, downstream from the transcription start site.

Our initial experiments with probe pδ1.3, located near the 5′ end of δ1 (Fig. 1), revealed a restriction fragment polymorphism at a *Bam*HI site at the 5′ end of δ1: Bam^2, located 1,629 bp 5′ to δ1, is cleaved about 50% of the time (data not shown). Therefore, mapping the location of hypomethylation events was made easier because each hypomethylation event generated two unique fragments, one from the 5.8-kb precursor (Bam^1-Bam^3) and one from the 3.6-kb precursor (Bam^2-Bam^3).

*Hha*I-*Bam*HI digests of DNA from 40-h headless embryos probed with pδ1.3 were identical to *Bam*HI digests alone, indicating that all *Hha*I sites were methylated (Fig. 2a).

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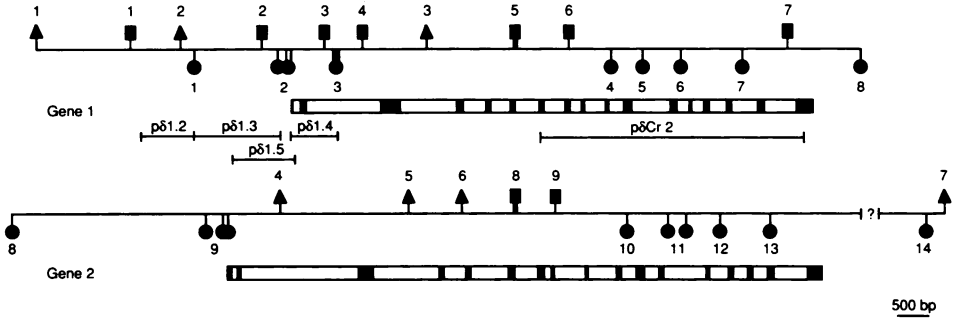

FIG. 1. Restriction maps of the two δ-crystallin genes of the chicken (from the work of Nickerson et al. [13, 14]). The map shows locations of exons (closed boxes), introns (open boxes), and restriction sites for *Bam*HI (▲), *Hpa*II (●), and *Hha*I (■). The probes used to analyze hypomethylation at the 5′ and 3′ ends of the δ-crystallin genes are shown.

However, *Hha*I-*Bam*HI digests of 13-day lens DNA revealed extensive hypomethylation at many sites (Fig. 2b). We examined DNA obtained from lenses of several stages between 40 and 96 h of development (Fig. 2c to f). At 50 h, two *Hha*I sites in intron 2 became hypomethylated (Fig. 2d) in a small fraction of lens cells. Hypomethylation at Hha^3 reduced the 5.8-kb fragment to 4.3 kb and reduced the 3.6-kb fragment to 2.1 kb. Hypomethylation of Hha^4 reduced the 5.8-kb fragment to 4.8 kb and reduced the 3.6-kb fragment to 2.7 kb. The extent of hypomethylation of these two *Hha*I sites was still increasing at 96 h (Fig. 2e and f) as the levels of δ-crystallin transcription in the lens increased as well (20).

Hypomethylation at site Hha^1 appeared to follow hypomethylation of sites 3 and 4. By 96 h, a 3.4-kb (Hha^1-Hha^4) restriction fragment was clear (and it may appear earlier) in DNA from lens cells in which only Hha^4 had been cleaved (Fig. 2f). Later, Hha^1 became hypomethylated in cells in which Hha^3 already had been cleaved, producing a 2.9-kb fragment (Hha^1-Hha^3) seen in 13-day lens samples (Fig. 2b). Not all lens cells undergo hypomethylation of Hha^3, because several restriction fragments (4.8, 3.4, and 2.7 kb) having Hha^4 as the 3′ boundary persisted in the 13-day lens.

Hypomethylation of Hha^2 was not detected (Table 1).

Methylation at *Hpa*II sites at the 5′ end of gene 1. Restriction enzyme analysis of *Hpa*II sites near the 5′ end of δ1 did not reveal any tissue-specific pattern of methylation. When DNAs from both 40-h headless embryos and 13-day lenses were examined with pδ1.2 (Fig. 1), site Hpa^1 was cleaved by *Msp*I but was resistant to *Hpa*II (data not shown). Therefore, Hpa^1 remained methylated in all tissues. Conversely, Hpa^2 (which is a cluster of three *Hpa*II sites separated by only 145 bp of DNA) and Hpa^3 (which is a cluster of three *Hpa*II sites separated by only 48 bp of DNA) sites in DNAs from all tissues were cleaved to the same extent by *Msp*I or *Hpa*II (data not shown). The latter result, obtained in experiments with probes pδ1.4 and pδ1.5 (Fig. 1), revealed that at least one of the three sites in Hpa^2 and Hpa^3 is hypomethylated at all times (Table 2). The closeness of the three *Hpa*II sites within each cluster makes it impossible to rule out tissue or age-specific regulation of hypomethylation at the other sites that would produce very small DNA fragments. However, genomic sequencing (7) could be used to examine the methylation status of these *Hpa*II sites.

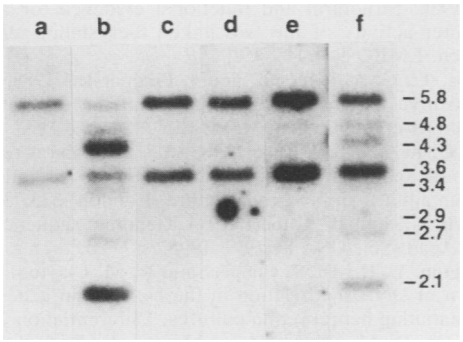

FIG. 2. DNA methylation patterns of δ1-crystallin gene in chicken embryos. DNA samples (2 μg) were digested with *Hha*I and *Bam*HI and probed with nick-translated pδ1.3. Samples were from 40-h headless embryos (a); 13-day lens (b); 40-h lens (c); 50-h lens (d); 65-h lens (e); and 96-h lens (f). The sizes of restriction fragments (in kilobases) are indicated.

Methylation at other HpaII sites in genes 1 and 2. DNA sequence data for the δ1-crystallin gene (14, 15) and the δ2-crystallin gene (13) made it possible to locate the *Hpa*II sites throughout this region whose methylation status had been studied previously by us (with probe pδCr2 [9, 21]) and by others (8). We reviewed the results of *Hpa*II digests of DNA from lens and nonlens tissues to locate regions of constitutive hypomethylation, while comparisons of *Msp*I and *Hpa*II digests revealed age or lens-specific hypomethylation and regions that remained methylated (Table 2). From this analysis we predict the locations of two additional restriction sites (Hpa^{14} and Bam^7 in Fig. 1) in the unsequenced region beyond the 3′ end of δ2.

We can now identify Hpa^7 as the site that undergoes hypomethylation beginning at 50 h, resulting in the appearance of a transient 5.0-kb *Hpa*II restriction fragment from *Hpa*II-*Bam*HI digests of lens DNA (9). Its location is confirmed by the kinetics of its disappearance. This fragment contains an internal site, Hpa^8, that becomes hypomethylated at 75 h, leading to the observed decline in the 5.0-kb fragment (9) and the appearance of the 1.8-kb product (reported earlier [21] as a 2.0-kb fragment). The remaining 3.2 kb from the 3′ end of the 5.0-kb fragment extends beyond the probe used here (pδCr2), so it is not detected.

TABLE 1. Methylation of *Hha*I sites in δ-crystallin gene 1[a]

*Hha*I site	Location	Status
1	5′ to gene 1	Hypomethylated by 96 h
2	5′ to gene 1	Always methylated
3	Intron 2	Hypomethylated by 50 h
4	Intron 2	Hypomethylated by 50 h

[a] All sites were located in this study.

TABLE 2. Methylation of *Hpa*II sites in the two δ-crystallin genes

*Hpa*II site[a]	Location	Status
1	5′ end of gene 1	Remains methylated[b]
2 (3)	5′ end of gene 1	Always hypomethylated[b]
3 (3)	Intron 2	Always hypomethylated[b]
4	Intron 10	Hypomethylated by 96 h
5	Intron 11	Always hypomethylated
6	Intron 12	Remains methylated
7	Intron 15	Hypomethylated by 50 h
8	Spacer region	Hypomethylated by 75 h
9 (3)	5′ end of gene 2	Always hypomethylated
10	Intron 10	Hypomethylated by 96 h
11 (2)	Intron 11	Always hypomethylated
12	Intron 13	Always hypomethylated
13	Exon 16	Remains methylated
14	3′ end of gene 2	Always hypomethylated

[a] Single site except where indicated by a number in parentheses.
[b] Sites determined in this study. The other *Hpa*II sites were located by using previously published data (8, 9, 21) as described in the text.

The close arrangement and similar organization of the two δ-crystallin genes has led to the suggestion that the δ2 crystallin gene probably arose from δ1 by a duplication event (18). From the data in Table 2, it is interesting that the pattern of hypomethylation of many *Hpa*II sites in δ1 and δ2 appears to have been conserved too. Each gene has a cluster of three *Hpa*II sites at its 5′ end, which contain at least one site that is always hypomethylated, and each contains a site in intron 11 that is constitutively hypomethylated in lens and nonlens tissues. Also, both genes contain *Hpa*II sites that remain methylated in lens tissue. The latter observations highlight the difficulty in assigning a role to hypomethylation in gene activation because the correlation between gene activity and hypomethylation is not always precise. Yet by examining the entire δ-crystallin region, we found that hypomethylation of a few key sites correlates well with δ-crystallin gene expression in the lens. We have located one *Hpa*II site (9) and two *Hha*I sites (this study) in δ1 that become hypomethylated at the time when δ-crystallin mRNA levels begin to increase dramatically during development (at 50 h), observations that may be important for understanding the high levels of transcription of δ1 in the lens (23).

The complexity in the pattern of hypomethylation may be explained by the existence of different levels of control of δ-crystallin expression. Constitutive hypomethylation of some sites within a gene might be expected if the gene product is present in all tissues. Constitutive sites detected in δ1 and δ2 genes may correlate with low levels of expression of one or both of these genes in many embryonic tissues other than the lens (2). A possible explanation for the appearance of constitutive hypomethylation may be the high degree of similarity between δ-crystallin and a ubiquitous metabolic enzyme, argininosuccinate lyase (25). One of the chicken δ-crystallin genes is clearly related to the human gene for argininosuccinate lyase, while in ducks, the same gene encodes both proteins and purified duck δ-crystallin protein has high argininosuccinate lyase activity (19). Therefore, constitutively hypomethylated regions of the δ-crystallin genes could be explained by constant expression of the gene to produce one or both of its products.

Tissue-specific expression of the δ1-crystallin gene appears to be controlled mainly by sequences within the gene (5, 10). The one *Hpa*II site and two *Hha*I sites that became hypomethylated at 50 h of development and that are possibly associated with changes in levels of transcription were located within introns found in δ1. The latter three hypomethylation events may be associated with enhancer sequences, which can exert a strong quantitative effect on tissue-specific transcription even when located beyond the 3′ end of a gene (6, 22). A region with homology to a viral core enhancer sequence has been detected within intron 2 of δ1 (4), close to two of the hypomethylation events first detected at 50 h of development (*Hha*[3] is 114 bp downstream and *Hha*[4] is 676 bp downstream). Recently, a functional assay has revealed an enhancer element in intron 3 of δ1 (10). Although this enhancer does not contain a consensus sequence common to most enhancers (24), it does stimulate transcription of a transfected chloramphenicol acetyltransferase gene 20- to 40-fold. It remains to be seen whether other assays for functional control elements in regulating δ-crystallin transcription will reveal whether sites where we see methylation changes can modulate gene activity. In this regard, there may be a distinction between enhancer elements, which are needed to activate transcription, and modification of control sequences by hypomethylation, which may serve to maintain transcription, as appears to be the case with κ immunoglobulin genes (12).

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