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Chromatin Structural Transitions and the Phenomenon of Vitellogenin Gene Memory in Chickens

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We have previously shown that the steroid hormone-mediated transcriptional activation of the chicken vitellogenin II gene (*VTGII*) in the liver is accompanied by a series of chromatin structural changes, including the formation of two sets of 5′-proximal nuclease-hypersensitive sites and the demethylation of a single 5′-flanking *Msp*I site which lies within a region of DNA that recently has been shown by Jost and co-workers to specifically bind the estrogen receptor complex in vitro. To assay the stability and possible functional significance of these induced structural changes, we transiently activated the *VTGII* gene during embryonic development and then allowed the chickens to hatch and grow for various periods of time before analyzing their livers. By 7 weeks posthatching all of the induced 5′-flanking hypersensitive sites had decayed. Moreover, the loss of these sites occurred without consequence to the "memory effect," that is, these structural features did not need to be present in hormone withdrawn birds to allow this gene to be activated more rapidly in response to a secondary presentation of estradiol. Although the demethylation was more stable, it also appeared not to be the basis of the memory phenomenon. The birds that still exhibited memory after 25 weeks of hormone withdrawal were not more extensively demethylated within the receptor-binding site than were the birds which failed to show memory at this age. A similar uncoupling of these two parameters was also observed when embryos were first injected with submaximal doses of estradiol and then assayed 1 week after hatching; the chickens which acquired memory were not demethylated to any greater extent than those which did not acquire memory. Other parameters that may be relevant to memory are discussed.

To explore the relationship between chromatin structure and the potential for gene expression, we have been studying the major chicken vitellogenin II gene (*VTGII*) under various physiological and developmental states. Two aspects of this system make it very attractive for studies of this sort. First, whereas the vitellogenin genes (which encode the major yolk proteins) are normally expressed only in the livers of mature oviparous females during periods of egg laying, males and immature females are equally capable of transcribing the gene in response to exogenous estradiol. That the liver is fully determined with respect to the potential for vitellogenin gene expression before the administration of hormone is evidenced by the fact that neither protein synthesis (17) nor DNA replication (14, 21, 29) is required to affect the transcription of this gene once estradiol is administered. Moreover, the gene appears to be transcriptionally activated in the majority of the hepatocytes, as demonstrated recently by in situ autoradiography (29). Thus this gene is experimentally accessible to structural analysis, since it resides within a fully differentiated tissue in its determined state in vivo before, during, and after transcriptional activation by estradiol. Second, this gene exhibits a very interesting "memory effect" (2), which is operationally defined by comparing the kinetics of *VTGII* expression after primary and secondary injections of estradiol (9, 18). In particular, the secondary response is typified by a more immediate accumulation of *VTGII* RNA after the injection of estradiol, whereas the primary response is characteristically biphasic with the rapid phase being preceded by an approximately 6-h window of relatively slow *VTGII* message accumulation. Thus, whereas the gene in the hormone-naive state has already acquired the potential to be directly activated, the system is clearly poised to respond more efficiently after having been matured during the primary response.

In the determined state before transcriptional activation by estradiol, the vitellogenin genes are extensively methylated in both frogs (12) and chickens (22, 30). In frogs it also appears that these genes reside within regions of chromatin which are generally insensitive to digestion by DNase I (10, 12, 13). In chickens the *VTGII* gene is punctuated by a class of preactivation-state, nuclease-hypersensitive sites that are tissue specific and occur both within and flanking the 3′ end of the gene but are not observed upstream of the transcription unit (5). To date, this is the extent of our knowledge of the permissive state of chromatin for this particular gene.

Other structural features, such as a general DNase I sensitivity over the entire gene region in frogs (10, 12, 13) and nuclease-hypersensitive sites located upstream of the chicken gene (5), are first observed hours after the initial addition of estradiol as the gene becomes transcriptionally activated. A slower event, which is detectable within a few days after hormone presentation and is observed for the chicken but not the frog vitellogenin genes, is the demethylation at one particular *Msp*I site within the *VTGII* gene region, located approximately 610 base pairs upstream from the start site of transcription (5, 22, 30). Curiously, this demethylation does not seem to require DNA synthesis, although the possibility that local repair-type synthesis may be involved cannot be ruled out (29). Although the mechanism of this particular demethylation is not understood, the initial suggestion that an activated steroid receptor complex might be directly involved (at least in triggering the event) received indirect support in the recent studies of Jost and

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co-workers, which demonstrated that this responsive *Msp*I site lies within an estrogen receptor DNA-binding site (19).

Since each of these types of induced structural features has been implicated in numerous other systems as a parameter that may bear on gene regulation (8), it has been appealing to consider that one or more of these might facilitate the secondary transcriptional responses to estradiol and thus be relevant to the phenomenon of hepatic memory. In previous studies from our laboratory we were able to rule out one of the induced features from consideration in this context. In particular, the most distal 5'-flanking, nuclease-hypersensitive site, which lies just upstream of the putative estrogen receptor-binding site (4), was observed only during periods of hormonal stimulation (5) and thus was clearly not correlated with hepatic memory. Whereas it appears that generalized DNase I sensitivity is acquired over the frog vitellogenin genes during the primary response to estradiol, the issue as to whether the decay of this DNase I sensitivity precedes (12) or is coincident with (31) the loss of memory has not been resolved.

To try to better characterize these induced structural features, with respect to their stabilities as well as their possible relevance to the memory effect, we have transiently activated the *VTGII* gene during embryonic development (11) and then allowed the chickens to hatch and grow for up to 25 weeks in the absence of exogenous estradiol. We present evidence that the proximal 5'-flanking hypersensitive sites, as well as the more generalized DNase I sensitivity, have decayed within 7 weeks posthatching, whereas the memory is still quantitatively retained at this time. Thus neither of these chromatin structural parameters appears to be required for the memory effect, and neither is maintained indefinitely in the absence of *VTGII* transcription.

By 25 weeks of age we show that hepatic memory has significantly decayed in two of the three hormone-withdrawn birds examined in this regard. This loss of memory occurs without a correlative remethylation within the estrogen receptor-binding site, indicating that the methylation status at this position does not dictate the type of response observed.

MATERIALS AND METHODS

Films were scanned with an E-C Apparatus Corp. densitometer in combination with a Hewlett-Packard Co. integrator. DNA sequencing was carried out by the dideoxy method (24) exactly as described previously (4). Experimental details for all of the other methods can also be found in an earlier publication (5); supplementary details are noted in the figure legends.

RESULTS

Propagation of hepatic memory in chickens. The phenomenon of hepatic memory has been defined based on the observation that the initial rate and maximal level of vitellogenin RNA accumulation are greater after secondary presentations of estradiol relative to those observed in response to the first exposures to this steroid hormone. The acquisition of memory is well documented in both avian (9, 18) and amphibian (1, 31) systems. On the other hand, the decay of memory has been clearly demonstrated only for the case of adult male frogs (31). Furthermore, the relationship (if any) between the instability of memory and cell division has not been addressed experimentally other than to note that the half-life for each is quite long (25).

To address this issue more directly, we set up an experiment with chickens in such a way as to allow a maximal number of cell divisions to occur after hormone withdrawal. This was accomplished by injecting estradiol at the earliest time in development that the hepatocytes are capable of inducing significant amounts of *VTGII*, namely, at day 12 of embryonic development (11). This regimen also enabled us to assay the various chromatin structural changes (induced during the primary responses) in their ability to be passively propagated to daughter cells in the absence of *VTGII* gene transcription (see below). Two points are relevant here. First, we know that for embryos injected with estradiol on day 12 the induced *VTGII* message levels decline sharply at the time of hatching (unpublished results). Second, within the first 4 weeks after hatching (which occurs on day 21) the hepatocytes undergo approximately four rounds of cell division, followed by two additional rounds by the time they are adults (16).

To examine the stability of memory in the chicken, we injected 12-day eggs with estradiol and let the birds hatch out and grow for various times before initiating the secondary responses. To characterize these responses with respect to memory, we examined the accumulation of *VTGII* RNA within individual livers as a function of time after the readdition of estradiol and compared these profiles with analogous data obtained with 4-week-old chickens which had been injected 3 weeks previously with either propylene glycol (the carrier solvent) or estradiol (Fig. 1). The latter is a traditional protocol (9) and serves as an internal reference for visualizing the memory phenomenon with this assay. Control experiments indicate that secondary response profiles identical to this standard reference for memory are also obtained (during the first 6 h after the injection of estradiol) for 1- and 2-week-old chickens that had first been injected with estradiol as 12-day embryos (unpublished results).

The results of such an analysis for birds that were first injected as 12-day-old embryos and then reinjected and sacrificed at either 8 or 25 weeks posthatching are presented in Fig. 1. All of the birds maintained hepatic memory for the first 8 weeks posthatching, and some of the birds even maintained a potential to respond more rapidly when reinjected after a period of 25 weeks without hormone. Thus it is clear that memory can be maintained for a long time in the chicken and, moreover, that this can occur in livers which are undergoing multiple rounds of cell divisions.

5'-Flanking DNA nuclease-hypersensitive sites and DNase I sensitivity. In considering parameters that might be relevant to the long-term stability of the hepatic memory, we decided to see whether there might be a correlation between memory and any of the chromatin structural features that were identified in previous studies as being induced over the *VTGII* gene region during the primary response to estradiol. In addition, we were also curious to examine generalized DNase I sensitivity in this context for two reasons: first, because this feature has classically been correlated with the potential for gene expression (8) and, second, because analogous experiments carried out with frogs have led to disparate conclusions regarding whether the decay of this structure may be correlated with the loss of memory (12, 31).

In our previous studies we identified three chromatin structural features that were induced as a consequence of estradiol treatment, two of which persisted after the primary wave of *VTGII* RNA had decayed to undetectable levels. In particular, a pair of 5'-proximal, nuclease-hypersensitive sites (the so-called B sites) and the demethylation that is induced at the *Msp*I site which resides within the 5' flanking estrogen receptor binding site were each found to be stable

for as long as 6 weeks posthatching for birds which had been exposed to estradiol during embryonic development (5). Although this conclusion was based on the analysis of a single chicken, we have subsequently carried out a similar analysis for a set of four birds, in this case with each being sacrificed at 3 weeks posthatching. These data are shown in Fig. 2 to provide a reference for the studies presented below, which will demonstrate the disappearance of such sites later in development.

For each of the birds, sub-bands which derive from cuts at the B sites are present in good yield (Fig. 2), as seen previously for hormone-withdrawn birds (5). To assay the extent of 5'-flanking demethylation, we used the isozyme *Hpa*II since this conveniently cleaves the *Msp*I recognition site only if it is not methylated. In particular, the genomic DNA was digested with *Hpa*II in combination with *Bam*HI; the latter generates a 3.6-kilobase band, whereas if the *Msp*I site which lies within this fragment is not methylated a 2.4-kilobase band is apparent instead. These bands were resolved by electrophoresis and visualized after Southern blotting by indirect end labeling (Fig. 2). From these data we conclude that by 3 weeks posthatching the demethylation which was initiated by the primary injection of estradiol has progressed to completion, i.e., to the same extent as is observed in the livers of laying hens (5). This statement is most readily demonstrated for the two birds which are homozygous for the *Msp*I allele (Fig. 2, lanes 5 and 11); for the other two birds (lanes 7 and 9) the analysis is complicated somewhat by the fact that these birds are heterozygotic with respect to the critical *Msp*I site (lanes 2 and 3), and thus the yield of *Hpa*II cutting must be corrected to account for this. This polymorphism has been described previously (5), and its sequence is presented below.

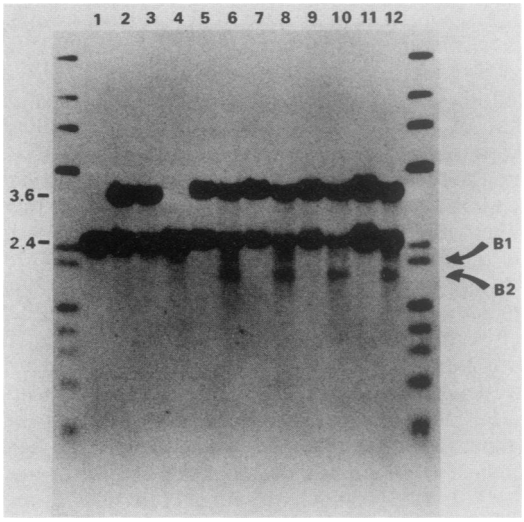

FIG. 2. For birds injected with a single dose of estradiol as 12-day embryos, the induced 5'-proximal, nuclease-hypersensitive sites persist for at least 3 weeks posthatching, as does the demethylated form of the *Msp*I site located 610 base pairs upstream of the transcriptional start site of the *VTGII* gene. In lanes 1 through 4, liver DNA isolated from four birds was digested with *Msp*I and *Bam*HI to determine the genetic composition of these chickens vis-a-vis the 5'-flanking *Msp*I polymorphism (see text). The degree of demethylation within the polymorphic allele containing the *Msp*I site was assayed by digesting the same DNA samples with *Hpa*II and *Bam*HI (see text), as shown in lanes 5, 7, 9, and 11, respectively. To assay for nuclease-hypersensitive sites within this 5'-flanking region, one sample of liver nuclei for each of the birds was incubated for 10 min at 37°C (conditions which are known to promote digestion at nuclease-hypersensitive sites by endogenous nucleases) before extraction of the DNA and digestion with *Hpa*II and *Bam*HI, as shown in lanes 6, 8, 10, and 12, respectively. The control and endogenous nuclease-digested samples for each bird are shown pairwise in adjacent lanes. After digestion with the restriction enzymes, the DNA samples were electrophoresed on a 1% agarose gel, Southern blotted, and probed with the nick-translated fragment 108BR1.4 (see reference 5 for a detailed schematic of this probing scheme) to examine the 5' end of the vitellogenin gene.

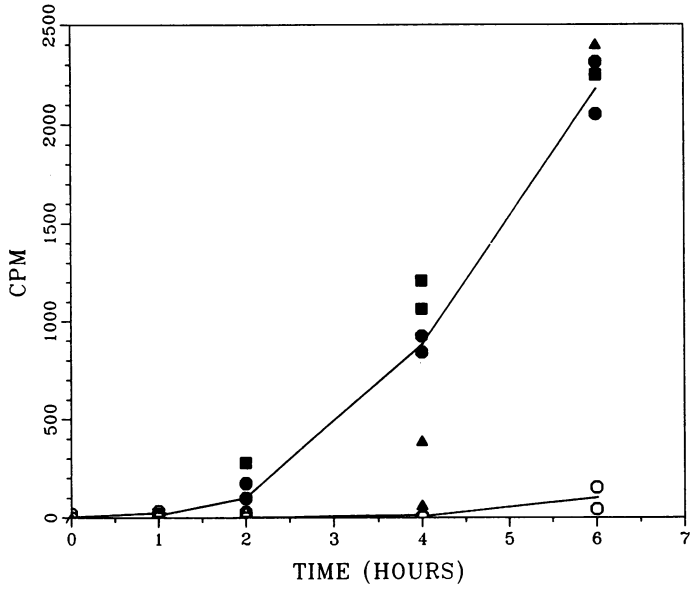

FIG. 1. Acquisition and decay of hepatic memory in chickens. Chickens that had been injected with estradiol (1 mg per egg) on day 12 of embryonic development were hatched and allowed to grow for either 8 or 25 weeks before receiving a second injection of estradiol (20 mg/kg). In each case, chickens were sacrificed at the indicated times after injecting estradiol, and liver RNA was isolated by the guanidinium isothiocyanate method. RNA was similarly obtained from the livers of 4-week-old birds that had been injected with either propylene glycol (the carrier solvent) or estradiol in propylene glycol (20 mg/kg) as 1-week-old hatchlings to provide an illustration of the memory effect for the sake of reference. RNA samples (0.5 μg) were dotted in duplicate onto a nitrocellulose filter with a 96-well manifold, and the filter was hybridized to an SP6 antisense probe for *VTGII*. After washing, the filter was previewed by exposure to preflashed film (21). The dots were then cut out, immersed in vials containing Aquasol-2, and counted in a scintillation counter. Each symbol represents an individual bird, showing the primary response to estradiol (○) and the responses of birds sacrificed at 4 (●), 8 (■), and 25 (▲) weeks posthatching. Curves have been drawn for classic primary and secondary responses.

In contrast to these results, by 7 weeks posthatching the picture was quite different. Although the *Msp*I site was stably maintained in its demethylated state, the set of 5'-proximal hypersensitive sites was evident in only one of the three birds that we examined. The data for one of the birds that showed virtually no evidence of the B sites are presented in Fig. 3 (in Fig. 3B the autoradiogram has been intentionally overexposed to emphasize this point). Given the very rapid *VTGII* secondary responses when birds that had been withdrawn from hormone for this length of time were reinjected with estradiol (Fig. 1), it appears that these promoter-proximal hypersensitive sites are not required for the hepatic memory.

The other point addressed in Fig. 3 regards the generalized

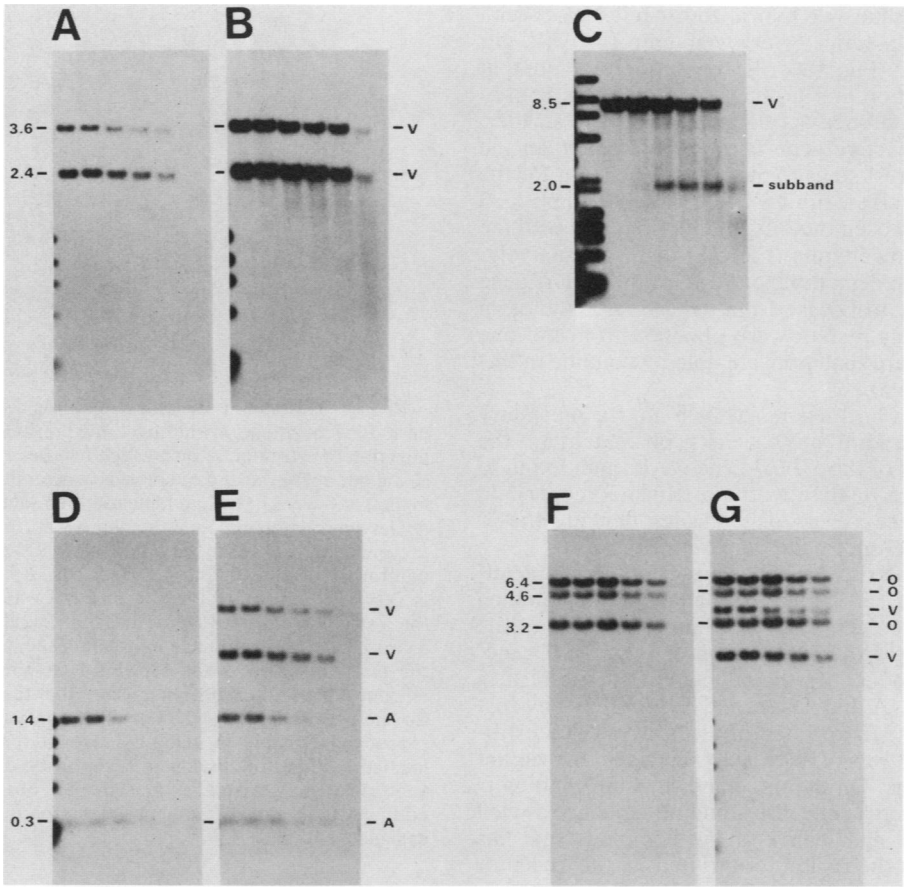

FIG. 3. The 5′-flanking, nuclease-hypersensitive sites and the general DNase I sensitivity over the *VTGII* gene decayed within 7 weeks from the time of estradiol presentation, but the 5′-flanking estradiol-induced demethylation persisted. Liver nuclei isolated from a 7-week-old chicken that had been exposed to estradiol on day 12 of embryonic development were incubated with various amounts of DNase I (left to right: 4°C without DNase I; 15°C without DNase I; and 25, 27.5, 30, and 32.5 μg of DNase I per ml, respectively, all at 15°C). Then the DNA was isolated, digested with the restriction enzymes *Bam*HI and *Hpa*II, electrophoresed on 1% agarose gels, blotted onto nitrocellulose filters, and probed with one of the following: (A) 108BR1.4, a fragment from a genomic vitellogenin clone (5); (D), pA2, a genomic beta-actin clone (6); or (F) p80v, a genomic ovalbumin clone. Panel B shows a longer exposure of the blot from panel A to emphasize the complete absence of subbands, and hence hypersensitive sites, in this region of the *VTGII* gene. As a positive control, a parallel blot was hybridized with the nick-translated *VTGII* probe 101HK1.3 to demonstrate the presence of the so-called A1 preactivation state hypersensitive site (5), which is located within the *VTGII* gene (panel C). Panel E shows the superposition of the films from panels A and D to directly compare the rate of digestion of the *VTGII* gene (fragments denoted V for *VTGII*) with an active gene (fragments denoted A for actin). Similarly panel G, which superimposes films A and F, allows a comparison of the rate of digestion of the *VTGII* gene with a gene that is inactive (fragments denoted O for ovalbumin) in this tissue.

DNase I sensitivity. As noted in the introduction, in hepatocytes of adult male frogs the vitellogenin genes reside within DNase I-insensitive structures before hormone presentation and become nuclease sensitive during the primary response, and this sensitivity decays between 2 and 6 months after hormone withdrawal (12, 31). Regarding the last point there is some disagreement as to whether this may be correlated with the loss of memory. Since these equivocal conclusions were arrived at on the basis of two different assays of DNase I sensitivity, we decided to use yet a third assay to address this issue in chickens. In particular, we have taken advantage of the fact that the hepatocytes which respond to the primary injection of estradiol become marked, in that the resident *VTGII* genes become demethylated at the 5′-flanking *Msp*I site (5). Thus, by digesting nuclei with various concentrations of DNase I and then restricting the resultant purified genomic DNA with *Hpa*II, it is possible to specifically examine the nuclease sensitivity of the genes which by definition must have responded to the primary injection of estradiol. Moreover, by examining the nuclease sensitivity of the demethylated copies after multiple rounds of cell division have occurred in the absence of *VTGII* transcription, it is possible to critically address whether the loss of nuclease sensitivity is a true decay or might represent replacement at the cellular level by a population of hepatocytes which have failed to respond to the primary administration of estradiol (25).

Data from this kind of experiment are presented in Fig. 3. In contrast to a comparable analysis carried out with livers which were actively transcribing the *VTGII* gene where the 2.4-kilobase demethylated (i.e., responsive) DNA target was digested more readily than the 3.6-kilobase methylated (i.e., nonresponsive) DNA target (Fig. 4), for the 7-week-old hormone-withdrawn bird examined in Fig. 3 this was not observed, indicating that the previously responsive *VTGII* gene is no longer as DNase I sensitive as it had been during the preceding episode of overt expression. To relate this observation to reference genes which are known to be active

or inactive in this tissue, we hybridized parallel blots with DNA probes for beta-actin (6) or ovalbumin (p80V; a gift from Stan McKnight) (Fig. 3D or F, respectively). Note that the restriction digestion and probing schemes were chosen so as to have the reference active gene fragments smaller, and the reference inactive gene fragments larger, than the *VTGII* bands of interest so as to more easily account for the issue of DNA target sizes. For ease of comparison, in Fig. 3 E and G we superimposed the *VTGII* data (Fig. 3A) with the actin (Fig. 3D) and ovalbumin (Fig. 3F) data, respectively. We conclude that the demethylated form of the *VTGII* gene displays a sensitivity to DNase I that is characteristic of an inactive gene. Similar results were obtained from the one other homozygous bird that we were able to examine in this detail (data not shown).

As expected, for a bird examined 24 h after a secondary presentation of estradiol at 7 weeks posthatching, the demethylated copies of the *VTGII* gene were again found to be DNase I sensitive relative to the methylated copies. In addition, the nuclease-hypersensitive sites flanking the 5′ end of the gene were once again apparent (Fig. 4).

DNA sequence analysis of the allelic polymorphism identified within the *VTGII* 5′-flanking estradiol receptor-binding site. At this point we decided to determine the basis of the polymorphism in the 5′-flanking region of the *VTGII* gene. As noted in the introduction, the affected *Msp*I site resides within a region of DNA that has recently been shown by Jost and co-workers to be footprinted in vitro by purified estrogen receptors (19). It was of interest to sequence this variant allele for two reasons. On the one hand, this information is important in arriving at a consensus binding sequence as well as in evaluating the significance of the homology that has been noted between the region footprinted by this receptor and the core enhancer element, a point we shall return to in the Discussion. Second, we were curious to know whether the allele which lacks an *Msp*I site nonetheless maintains a CpG sequence at this position which could serve as a substrate for DNA methylation (15).

To address these issues, we cloned and sequenced the region of interest by using a genomic library constructed and kindly provided by Kathleen Conklin. The difference between this allele and the previously sequenced allele (4) lies in a guanine-to-cytosine transversion within the third nucleotide of the *Msp*I restriction site (data not shown). Interestingly, each of the two polymorphic forms therefore contains a CpG sequence at roughly the same position within the receptor-binding site which may, at least in principle, be methylated. For the Seattle variant this is merely displaced by a single base pair relative to the previously characterized allele.

DNA demethylation. We next tested the relationship between memory and the estradiol-mediated demethylation that occurs within the receptor-binding site. First, the notion that this demethylation might be adequate to account for memory was directly tested by analyzing the methylation status of the hormone-withdrawn birds at 25 weeks posthatching. In particular, since some of the birds showed appreciably more memory than others (Fig. 1), a simple prediction of the hypothesis is that the former set should be more extensively demethylated than the latter set. Based on densitometer scans of Southern blots which resolve the methylated and demethylated copies from one another, it is clear that this was not the case (Fig. 5). Despite the large variation in *VTGII* responsiveness (i.e., 7 to 111%, relative to a typical secondary response), the extent of demethylation at this *Msp*I site fell within a surprisingly narrow range,

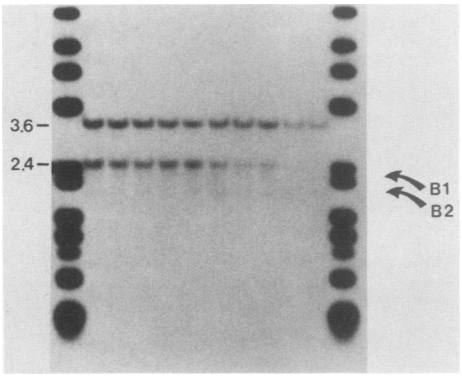

FIG. 4. Chromatin structural features which decay during the period of hormone withdrawal are regained after a secondary injection of estradiol. A bird which had been injected with estradiol (1 mg per egg) as a 12-day embryo was reinjected with estradiol (20 mg/kg) at 8 weeks posthatching and then sacrificed 24 h later. Liver nuclei were prepared and incubated at 15°C for 10 min in the presence of increasing amounts of DNAse I (left to right: 4°C control, 15°C control, and 1, 2.5, 5, 10, 12.5, 15, 17.5, and 20 μg of DNase I per ml, respectively). The DNA was isolated, digested with the restriction enzymes *Hpa*II and *Bam*HI, electrophoresed on a 1% agarose gel, transferred to a nitrocellulose filter, and hybridized with the nick-translated probe 108BR1.4. A similar analysis with the enzymes *Msp*I and *Bam*HI indicated that the bird was homozygous for the *Msp*I-containing *VTGII* allele (data not shown). The lower parent band is due to demethylation at the 5′-flanking *Msp*I site located within the estrogen receptor-binding site. The smaller subbands which arise as a consequence of the DNase I are due to cutting within the pair of promoter-proximal hypersensitive sites designated B1 and B2 (5).

with no bird deviating more than 6% from the average of 39%. From this analysis we conclude that demethylation is apparently not sufficient to confer memory.

The second approach that we took to address this issue was to treat 12-day embryos with submaximal doses of estradiol in the hope that we might be able to uncouple the demethylation event from the conferral of memory. Indeed, this approach proved useful (Fig. 5). In particular, birds that received 1/10 the normal primary dose (i.e., 100 μg per egg) were given a maximal secondary dose (20 mg/kg) at 1 week posthatching and then sacrificed after 4 h to assay *VTGII* RNA levels and DNA methylation. Whereas the submaximal primary injections provoked only partial demethylation (less than or equal to 26%) in the two cases examined, one of these birds nonetheless showed a maximal secondary response indicative of having quantitatively gained memory. As in the experiment described above, the extent of demethylation did not correlate with memory. The bird which acquired memory did so despite the fact that it was demethylated even slightly less than the bird which failed to acquire memory (20 versus 26%, respectively). These results demonstrate that the correlation normally observed between demethylation and the acquisition of memory is not mandatory.

DISCUSSION

We have shown that neither the set of 5′-flanking hypersenstive sites nor the general DNase I sensitivity in the *VTGII* gene region is maintained indefinitely in chicken livers that have become transcriptionally silent at this locus. Since these experiments were carried out with chickens that had been injected with estradiol during embryonic develop-

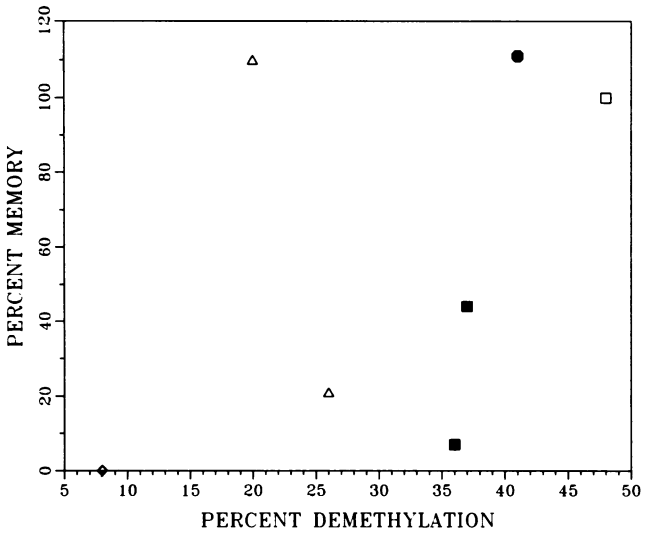

FIG. 5. Neither the acquisition nor the stability of hepatic memory is correlated with the extent of demethylation at the 5'-flanking *Msp*I site located within the estrogen receptor-binding site. The extent of demethylation within the 5'-flanking *Msp*I site was determined by probing Southern blots (containing *Hpa*II- and *Bam*HI-digested liver DNA) with the nick-translated probe 108BR1.4, exposing them to preflashed film (21), and then scanning the two bands in each lane with a densitometer, working within the linear range of film density. A similar analysis with the enzymes *Msp*I and *Bam*HI allowed the genotype of each bird to be determined with respect to this *Msp*I site; for heterozygotes, the *Hpa*II data were adjusted by a factor of 2 to correct for the fact that only one allele is being assayed. The memory index (plotted as the ordinate on this graph) is used to express *VTGII* RNA as a percentage of what is observed for a classic secondary response; for each time point analyzed, *VTGII* levels typical of a primary response were subtracted to conveniently define the scale from 0 (no memory; i.e., primary response) to 100 (complete memory). Two sets of experiments are summarized in this figure. Shown are data for reinjected (20 mg/kg) 25-week-old birds that were initially injected with estradiol (1 mg/egg) as 12-day embryos and sacrificed at 4 (■) and 6 (●) h after the secondary injections of estradiol (Fig. 1). Also shown (△) are data obtained 4 h after reinjecting (20 mg/kg) 1-week-old birds that were initially injected with submaximal (0.1 mg/egg) doses of estradiol as 12-day embryos. Data from a 1-week-old hormone-naive bird (◇) and a 1-week-old bird that had been injected previously with a maximal (1-mg/egg) primary dose of estradiol as a 12-day embryo (□) are also represented for reference purposes. See the text for further details.

ment, the protocol allows for a number of cell divisions to take place after hormone withdrawal. We have not yet addressed the significance of this parameter vis-a-vis the decay of these chromatin structural features, but it is absolutely clear that cell division poses no obstacle to the maintenance of hepatic memory. Thus, whereas other (as yet unidentified) chromatin structural changes may be induced as a consequence of the primary injection of estradiol and as such facilitate subsequent responses of the *VTGII* gene, it would appear that neither 5'-flanking hypersensitive sites nor general DNase I sensitivity is deterministic in this regard. In contrast to the loss of these structures, the change in methylation (that is induced in the 5'-flanking region of the *VTGII* gene during the primary response to estradiol) is relatively stable. This is not surprising, since this feature is presumably passively maintained during rounds of DNA synthesis and would only be changed in the event of de novo methylation. Despite its stability and the fact that it occurs within an estrogen receptor-binding site (19), we have also shown that this demethylation is neither necessary nor sufficient for memory. The fact that a mechanism nonetheless clearly exists which is capable of promoting precise demethylation events in response to estradiol suggests that other methylation-sensitive loci may be involved in mediating the memory effect.

It is striking that by 7 weeks posthatching, the state of the *VTGII* gene in the livers of birds that were transiently stimulated during embryonic development has reverted to a structure that appears almost identical to the structure observed in both the hormone-naive liver and estradiol-stimulated oviduct. Aside from the estradiol-dependent hypersensitive sites upstream of the critical *Msp*I site, which we presume would decay upon the removal of hormone in the oviduct as happens in the liver, there is only a single feature that we have found thus far (i.e., an additional hypersensitive site located within the gene in the oviduct) which serves to distinguish the chromatin structure of the silent *VTGII* gene in these two tissues (5).

Interestingly, whereas in liver the demethylation of the *Msp*I site occurs after the locus has been made DNase I sensitive, this is almost certainly not the case in the oviduct since the *VTGII* gene remains DNase I insensitive in this tissue even during periods of hormonal stimulation (unpublished obervations). The assumption that this particular demethylation is induced in the oviduct in response to estradiol (as happens in the liver) is consistent with the fact that we did not detect demethylation at this site in any (of seven) tissues examined which are not estrogen responsive (unpublished results). Thus, we are led to suggest that at least a subset of the chromatin that is DNase I insensitive may be functionally more dynamic than previously supposed. In addition, whereas in hepatocytes the interaction of estrogen receptors with the *VTGII* gene is apparently necessary to promote the conversion of the chromatin structure to a DNase I-sensitive state, the oviduct data suggest that such an interaction is not in and of itself sufficient.

As a final comment regarding this estrogen receptor-binding site, we note that the variant form which was initially identified as a Seattle polymorphism (by virtue of the fact that it lacks the critical *Msp*I restriction site) has recently been found (unpublished observation) to be well represented in at least two east coast hatcheries (Truslow Farms of Chestertown, Md., and Spafas of Norwich, Conn.). The fact that this allele is so widespread in the population, along with the fact that the two alleles appear to be indistinguishable with respect to their response profiles, suggests that this receptor-binding site is probably functionally unaltered by the base change. It may be somewhat surprising therefore to note that the Seattle substitution further weakens the previously noted sequence homology (from a 7-out-of-11 match to a 6-out-of-11 match) between this element and the core enhancer element (19), leaving only the 3' GAAAG portion of the homology appreciably intact.

The failure of the induced 5'-proximal hypersensitive sites to be propagated indefinitely in the absence of *VTGII* gene transcription would appear to be analogous to the observation by Birnsteil and co-workers (3) that hypersensitive sites located in the promoter regions of the embryonic sea urchin histone genes disappear after their transcriptional inactivation at the hatching blastula stage of embryogenesis. In both cases the hypersensitive sites persist for a finite time after transcription of the adjacent genes has ceased and then gradually decay, indicating that there is not an efficient

mechanism for maintaining this class of hypersensitive sites once the respective genes are no longer being used as transcriptional templates.

In contrast to these similarities, the functional contexts in which these changes take place are diametrically opposed. In particular, whereas the embryonic histone genes are destined to remain transcriptionally silent throughout later development, the *VTGII* gene is not only still determined in the hormonal withdrawn hepatocytes, but is actually poised in such a way as to be virtually immediately responsive to the readdition of estradiol. The absence of these hypersensitive sites in hormone-withdrawn chickens is especially intriguing considering their location (when present) flanking the 5′ end of the *VTGII* gene. In particular, the data provide no evidence to suggest that stable transcription complexes might be present under these conditions. Nuclease-hypersensitive sites analogous to this class of *VTGII* gene 5′-proximal sites have also recently been described for the *apoVLDLII* gene (20), another estrogen-inducible, liver-specific avian gene (26, 32). In the latter case the induced sites are even less stable, becoming undectable within 3 weeks after the removal of hormone. Thus, 5′-proximal hypersensitive sites are apparently not required for the memory at the *apoVLDLII* locus either (7).

It would appear that the vitellogenin genes in frogs reside within DNase I-insensitive regions of chromatin before hormone presentation (12, 31). Moreover, although induced to assume nuclease-sensitive structures as a consequence of exposure to estradiol, the genes revert to insensitive states within a period of months after hormone withdrawal and transcriptional inactivation. Similar results have recently been obtained for the chicken *VTGII* gene (this communication) as well as the chicken *apoVLDLII* gene (20). Whereas we previously suggested that the slow rate of accumulation of *VTGII* RNA during the first 6 h of the primary response might reflect a rate-limiting transition to a DNase I-sensitive state, this appears not to be the case. The reassembly of the *VTGII* gene into a nuclease-resistant structural domain, which occurs within 7 weeks after the withdrawal of hormone, proceeds without impairing in any discernible way the ability of the gene to respond virually immediately to the readdition of estradiol. It may be prudent to temper this conclusion, however, considering that in none of these studies has it been possible to discern more than a threefold difference in DNase I sensitivity between active and inactive genes. In particular, it is formally possible that in isolating liver nuclei some structural information is lost which otherwise might serve to distinguish the silent (but potentially active) *VTGII* gene from other inactive genes in this context.

Our analysis still leaves open the possibility that hepatic memory may be due to an as yet unidentified chromatin structural change which might serve to promote a faster secondary transcriptional activation of the *VTGII* gene. Alternatively, a *trans* mechanism, perhaps involving qualitative or quantitative differences in estrogen receptor levels (23, 27, 28), may underlie the differences between primary and secondary responses to estradiol. In either case, specific models must now take into account the fact that memory is both long lived and can be propagated to dividing cells after the withdrawal of estradiol.

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