An estrogen-dependent demethylation at the 5' end of the chicken vitellogenin gene is independent of DNA synthesis

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ABSTRACT

A HpaII site 611 base pairs upstream from the 5' end of the chicken vitellogenin II gene exhibits an estrogen-dependent demethylation in hormone-responsive cells. The complete demethylation of this site occurs considerably later than the transcriptional activation of the gene, and therefore appears to be an effect rather than a cause of this activation. In vivo studies using two inhibitors of DNA synthesis (cytosine arabinoside and hydroxyurea) demonstrate that activation of transcription of the gene is independent of DNA synthesis. Surprisingly, the estrogen-dependent demethylation of the HpaII site is also independent of DNA synthesis. The possible involvement of a site-specific demethylase is discussed.

INTRODUCTION

Eukaryotic DNA methylation occurs exclusively at the 5'position of cytosine, and predominantly in the dinucleotide CpG (1-3). The notion that DNA methylation plays a role in the control of vertebrate gene expression is an attractive one, and is supported by three lines of evidence. First, in most systems thus far examined, CpG methylation correlates inversely with gene activity [e.g. chicken α globin (4); chicken β globin (5); chicken ovalbumin, ovomucoid and conalbumin (6); X. laevis rDNA (7)]. Second, the transcriptional activity of DNA transferred into cells is highly (and specifically) ^{me}CpG sensitive (8). Third, inhibitors of DNA methylation, such as 5-azacytidine and ethionine, have been shown to have profound effects on the phenotype of cells (9) and at the level of the gene, their effects have been correlated with undermethylation at activated gene loci (10,11).

The issue is, however, clouded by the existence of several

situations where DNA methylation does not correlate with gene expression (12,13).

The chicken vitellogenin gene is transcribed in the liver of egg laying hens under the control of the steroid hormone estrogen. Precocious induction of high levels of transcription of this gene in immature male or female chickens can be brought about by the administration of a single large dose of estrogen (14,15). Since virtually all the hepatocytes in the estrogen-induced liver respond to produce vitellogenin (16), this system holds the advantage of both quantity and homogeneity of cell type, when compared with other systems, such as the chicken oviduct system.

This laboratory has recently demonstrated an estrogen-dependent undermethylation at the 5' end of the chicken vitellogenin gene (17,18). In the present study we have investigated the time course of induction of this undermethylation in the presence or absence of the DNA replication inhibitors cytosine arabinoside (AraC) and hydroxyurea, and conclude that the mechanism of undermethylation is independent of DNA synthesis.

EXPERIMENTAL PROCEDURES

Animal Treatment

Immature white Leghorn chickens (200 g), both male and female, were used in all experiments. Estradiol was dissolved in propylene glycol at 40 mg ml^{-1}, and injected intramuscularly into the leg (0.1 ml per 100 g body weight). Control chickens received propylene glycol only. Hydroxyurea and cytosine arabinoside were dissolved in 0.85% sodium chloride and injected intraperitoneally in the amounts indicated in the legend of figures.

Southern Hybridisation

Large size genomic DNA was prepared as previously described (17). Restriction digests of genomic DNA were performed at 8 units of restriction enzyme per µg DNA, for 4 hours at 37°C in the buffers recommended by the manufacturers (Boehringer Mannheim and New England Biolabs). Ten micrograms of DNA were loaded onto each slot of a horizontal 1% agarose gel (Sigma), and after electrophoresis transferred to nitrocellulose paper (Schleicher

and Schuell) by the method of Southern (19). Filters were hybridised with ^{32}P-labelled nick translated probes (20) and washed as previously described (17). Radioautography was performed at -70°C on Kodak X-5 film using two Illford intensifying screens.

Determination of Vitellogenin mRNA in Total Liver RNA

Total liver RNA was prepared and hybridised with an excess of ^{3}H-cDNA as previously described (14). RNA dot blot hybridisation was carried out according to Thomas (21). A titration curve was established with purified vitellogenin mRNA. Quantitation of vitellogenin mRNA was done only within the linear portion of the titration curve.

In Situ Hybridisation

Cryostat sections (8 µM) of unfixed liver were cut at -15°C, and taken up onto a prefixed slide, subbed in Denhardt's solution (22). Each slide contained a control and an estrogen-induced section. The sections were air-dried at 25°C and then fixed in Bouin's fixative (BDH) for 15 min at room temperature. The fixed slides were washed extensively in 50% ethanol (4-8 changes 30 min each) until the characteristic yellow colour had completely disappeared. Slides were prehybridised for 3 hr in 50% formamide, 50 mM sodium phosphate, 600 mM sodium chloride, 5.0 mM EDTA, 1 x Denhardt's solution at 37°C and subsequently hybridised to ^{32}P-labelled nick translated pVT3 and pVT329 (23) for 72 hr at 37°C (24).

Hybridised slides were washed once in 1 x SSC at room temperature and twice in 1 x SSC at 40°C (30 min each time). To reduce background, slides were dipped in 2% gelatine before being dipped in Kodak NTB2 emulsion diluted 1:1 in 0.6 M ammonium acetate. Slides were exposed in a dry air tight container at 4°C for several days and developed as described elsewhere (25). Sections were stained in Giemsa stain and examined by light microscopy.

RESULTS

Time Course of the Estrogen-Dependent Demethylation of a HpaII Site Close to the 5' End of the Vitellogenin Gene

As it has already been shown, the HpaII site (M_2) situated at -6II by upstream from the 5' end of vitellogenin II gene is

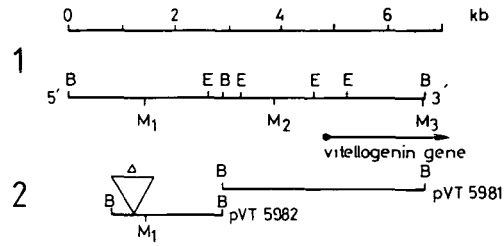

Fig. 1
Probes used in the analysis of the methylation state of the M_2 and M_1 HpaII sites.
1. A restriction map of 5' end of the gene is shown for the enzymes BamHI, (B); EcoRI, (E); and HpaII (M).
2. Site M_2 was probed using pVT598.1, and site M_1 using pVT598.2. pVT598.2 contained a 0.8 kb deletion, 0.2 kb from the M_1 HpaII.

the only site which undergoes hypomethylation upon estrogen treatment (17,18,26).

As an obvious first step towards an understanding of the possible role played by the demethylation of the M_2 site in the activation of the vitellogenin gene, we compared the time course of transcriptional induction of the gene with the time course of demethylation of the M_2 site. Vitellognin mRNA levels were quantitated by hybridisation analysis of total RNA with labelled tritiated cDNA, as described by Jost et al. (16). Methylation of the M_2 locus was investigated by Southern blot analysis of high molecular weight DNA digested with the restriction enzymes HpaII and BamHI. The blots were hybridised to ^{32}P-labelled nick translated probe pVT598.1, which corresponds to the entire large BamHI fragment shown in Figure 1.

From Figure 2 it can be seen that vitellogenin mRNA first appears 6 hr after estrogen treatment, and, thereafter, accumulates steadily; by 24 hr it represents approximately 0.06% of total cellular RNA. The vitellogenin mRNA content reaches a maximum between 48 hrs and 72 hrs and subsequently decreases until by day 6 no vitellogenin mRNA sequences can be detected. The demethylation of the M_2 site displays very different kinetics, however. The highly methylated pattern characteristic of non-expressing tissues is retained for many hours after the gene has become transcriptionally active (Fig. 2, panel B). Indeed, the

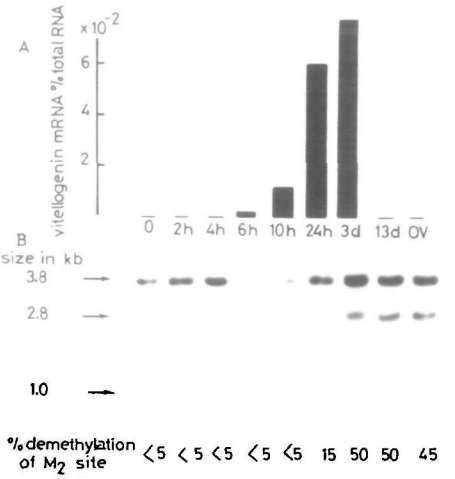

Fig. 2
Comparison of the time course of vitellogenin mRNA induction with the estrogen-dependent demethylation of site M_2.
Panel A shows the kinetics of vitellogenin mRNA accumulation at various times after estrogen administration as determined by solution hybridisation of total cellular RNA with ^{3}H-labelled vitellogenin cDNA. Panel B shows the kinetics of estrogen-induced demethylation of the M_2 HpaII site. DNA (10 µg) from each time point was digested sequentially with restriction enzymes HpaII and BamHI. The digested DNA was run on a 1% agarose gel, transferred to nitrocellulose paper and hybridised to ^{32}P-labelled pVT598.1. The presence of bands at 2.8 kb and 1.0 kb indicates hypomethylation at the M_2 HpaII site. A shorter exposure of the same blot was measured on a densitometer and the extent of demethylation was calculated; % demethylation = $\frac{\text{intensity of bands 2.8 + 1 kb}}{\text{intensity of bands 3.8 + 2.8 + 1 kb}} \times 100$.

24 hr time point, which contained high levels of vitellogenin mRNA, is only 15% undermethylated with respect to the control. At day 3, however, there is evidence of profound undermethylation at the M_2 site, which is retained quantitatively thereafter, even after transcription of the gene has ceased (e.g. Fig. 2, panel B, lane 13 days). We have previously noted the anomalous methylation status of the M_2 site in oviduct DNA, which despite being transcriptionally inactive at the vitellogenin gene locus possesses an "active" methylation pattern (17).

Effect of DNA Synthesis Inhibitors on DNA and RNA Metabolism in the Chicken Liver

Since it is widely held that DNA demethylation is a replica-

tion-dependent phenomenon (2) and that previous data from this laboratory suggested that the estrogen-dependent induction of phosvitin synthesis was DNA-replication independent (27), we initiated the following series of DNA inhibitor studies as an attempt to separate the two phenomena. In order to minimize potential artifacts we chose two inhibitors with different sites of action, namely cytosine arabinoside (Ara C) and hydroxyurea, for these experiments. Hydroxyurea depletes the pools of deoxyribonucleotide triphosphates by inhibiting the enzyme ribonucleotide reductase (28,29) while Ara C is modified *in vivo* to Ara CTP and is in this form a potent inhibitor of certain DNA polymerases (30-34).

We have previously published data concerning dosage requirements for the inhibitor hydroxyurea (27), however, similar data for the inhibitor Ara C were not available, and we therefore first performed a series of concentration dependence studies and time courses (Fig. 3). Figure 3 shows that a maximal inhibition of DNA synthesis is obtained with 300 mg of cytosine arabinoside/kg body weight. For a single injection of 250 mg cytosine arabinoside/kg the inhibition of DNA synthesis is of about 90% during the first 12 hours and then gradually decreased (inset of Fig. 3), therefore in the experiments described below we injected 250 mg of cytosine arabinoside/kg body weight every 12 hours for 4-5 days. Since an increased detoxification or degradation of cytosine arabinoside could possibly take place in the liver following repeated injections of the drug, it was of importance to test whether the inhibition of DNA synthesis in the liver was affected after prolonged injections of cytosine arabinoside. Table I shows the repeated injections of cytosine arabinoside every 12 hours for 1-5 days inhibited liver DNA synthesis by over 90%. Moreover, during the prolonged treatment with cytosine arabinoside the specific activity of thymidine triphosphate pool increased by about 5-15% (data not shown).

In order to determine if DNA synthesis is not a prerequisite for the activation of the vitellogenin gene, we have used the dot-blot hybridisation technique to estimate the extent of transcription of the gene in the presence or absence of the two inhibitors. Table II shows the results of these experiments.

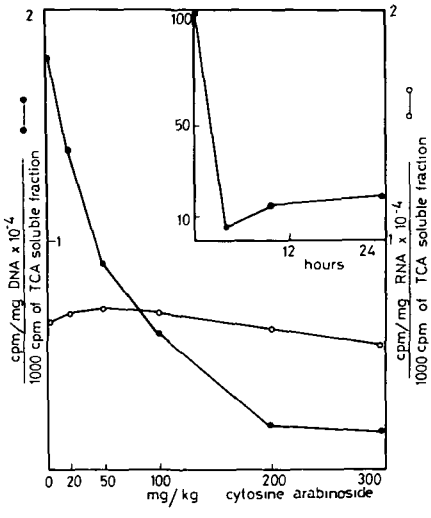

Fig. 3
Dose-response curve of the inhibition of DNA synthesis by cytosine arabinoside.
Chickens, 200 g body weight, were first injected with a single dose of 40 mg estrogen per kg body weight, and then received 20, 50, 100, 200 or 300 mg/kg body weight of Ara C, respectively. Four hours later groups of four chickens were labelled for 50 min with 100 µCi of ^{3}H-thymidine per kg body weight (a parallel group received 100 µCi of ^{3}H-uridine per kh body weight). The thymidine or uridine incorporation was determined in the 10% trichloroacetic acid precipitate of the liverhomogenate. The inset shows the time course of the inhibition of DNA synthesis in the liver of chicken receiving a single dose of 250 mg of Ara C per kg body weight. At the times indicated four chickens were injected with ^{3}H-thymidine as described above.

Neither inhibitor alone induces the accumulation of vitellogenin mRNA sequences. Estrogen, as predicted, induces the transcription of the gene to the expected level (0.06% total cellular RNA) and this level is retained during estrogen treatment in the presence of both Ara C and hydroxyurea. Whether cytosine arabinoside was injected 2 hours after or before estrogen treatment did not change the results presented in Table II. We conclude that the transcription of vitellogenin gene is independent of DNA synthesis.

Quantitation of Cells Producing Vitellogenin mRNA

In order to be able to interpret fully the data presented in this paper it was necessary to know the number of hepatocytes

Table I
Effects of repeated injections of cytosine arabinoside on liver DNA synthesis. Immature chicks (150-200 g) were injected first with 40 mg estradiol/kg and then with 250 mg cytosine arabinoside/kg body weight every 12 hours during a period of up to 5 days. Six hours after the last injection of cytosine arabinoside groups of 3 chicks were pulse-labelled with ^{3}H-thymidine as described in the legend of Fig. 3.

No. of days post treatment	cpm/mg DNA / 1000 cpm TCA sol.	% Inhibition
Control	24,560 ± 122	0
1	1,425 ± 35	94
2	1,467 ± 23	94
3	1,467 ± 43	94
4	1,758 ± 24	93
5	2,200 ± 30	91

Table II
Effect of cytosine arabinoside and hydroxyurea on the estrogen-dependent induction of vitellogenin mRNA synthesis in immature chickens.
Estrogen was injected at a dose of 40 mg/kg body weight. Ara C was injected 2 hr before estrogen and every 12 hr thereafter at a dosage of 250 mg/kg body weight. Hydroxyurea was injected 2 hr before estrogen and every 24 hr thereafter at a dosage of 3 g/kg body weight. Total RNA was prepared from groups of 5 chickens each as described in the Experimental Procedures after 48 hr estrogen treatment, and quantitated by dot-blot hybridisation.

Treatment	Vitellogenin mRNA hybridised to cloned cDNA probe	
	cpm/10 µg RNA	% of total RNA
Control	14	0.0009
Cytosine Arabinoside	< 10	< 0.0009
Hydroxyurea	< 10	< 0.0009
Estradiol	939 ± 63	0.062
Estradiol + Cytosine Arabinoside	976 ± 99	0.060
Estradiol + Hydroxyurea	949 ± 45	0.063

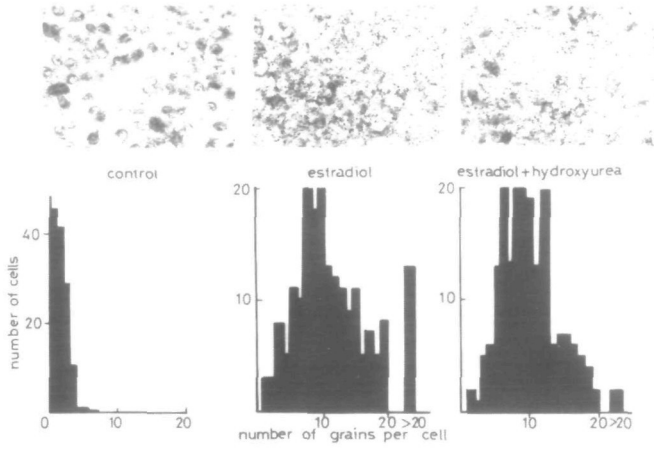

Fig. 4
Quantitation of hepatocytes producing vitellogenin mRNA in the presence or absence of hydroxyurea.
In situ hybridisation on 8 µm section of liver performed as described in the Experimental Procedures. Sections were stained with Giesma stain and examined by light microscopy at a 750 x magnification. Photomicrographs of section from control, estrogen-induced and estrogen-induced plus hydroxyurea livers are shown in panel A. Silver grains per cell were counted by eye for control (140 cells), estrogen-induced (189 cells) and estrogen-induced plus hydroxyurea (199 cells) and plotted against the number of cells (panel B).

responding to estrogen to produce vitellogenin. We would in this way be able to distinguish between the two extreme possibilities; a small number of hepatocytes producing very high levels of vitellogenin mRNA, or the majority of hepatocytes producing lower levels of mRNA. This distinction is especially important in this series of experiments, since the levels of vitellogenin mRNA we detect after inhibition of DNA synthesis by Ara C or hydroxyurea, may not derive from the total population of hepatocytes, as in the normal induction of the gene by estrogen (16), but may be the product of clones of cells resistant to the effects of the inhibitor. The quantitation of responsive cells has been performed by in situ hybridisation of ^{32}P-labelled cDNA probes to chicken liver sections.

Figure 4 shows a comparison of 8 µm sections from control estrogen-induced (2 days) and hydroxyurea-treated estrogen-induced (2 days) liver, probed in situ with ^{32}P-labelled vitello-

genin cDNA clones pVT3 and pVT329. An alteration of the morphology of the liver cells due to the estrogen treatment of the chicken is apparent in the photomicrographs in Figure 4. Particularly clear is the presence of large vacuoles in the induced samples. The high degree of cellular radioautographic labelling over the sections of estrogen-induced liver demonstrates the presence of vitellogenin mRNA in these cells. Control cells exhibited few, randomly scattered grains.

Quantitation of the number of grains per cell (Fig. 4B) permits us to conclude that the estrogenic response of the vitellogenin gene is essentially identical in the presence or absence of the DNA synthesis inhibitor hydroxyurea. In both cases the gene becomes transcriptionally active in the majority of hepatocytes.

Effect of Inhibition of DNA Synthesis on the Estrogen-Dependent Demethylation of the Chicken Vitellogenin Gene

It has long been suspected that changes in DNA methylation patterns are the effects of inhibition of DNA "repair" methylation activity, which reproduces the existent methylation pattern during DNA replication. We, therefore, tested this hypothesis by looking at the methylation state of the M_2 site during estrogen induction of the gene, in the presence of Ara C or hydroxyurea. Using the conditions we had determined for the mRNA quantitation experiments (i.e. 250 mg Ara C/kg body weight; 3 g hydroxyurea/kg body weight) we prepared DNA from control, estrogen-induced (5 days), Ara C alone (5 days), hydroxyurea alone (5 days), Ara C plus estrogen (5 days) and hydroxyurea plus estrogen (5 days) chicken livers. High molecular weight DNA was prepared and then cleaved with the restriction enzymes BamII and HpaII and Southern blots were prepared and hybridized with ^{32}P-labelled nick translated pVT598.1. The data shown in Figure 5 show that despite the inhibition of DNA synthesis by 90%, the estrogen-dependent demethylation of the M_2 site is quantitatively (50% demethylated) identical to that observed in the absence of DNA synthesis inhibitor. Using other probes (e.g. pVT598.2 which includes the HpaII M_1 site), we have demonstrated that there is no alteration of the specificity of the demethylation phenomenon in the absence DNA synthesis (data not shown).

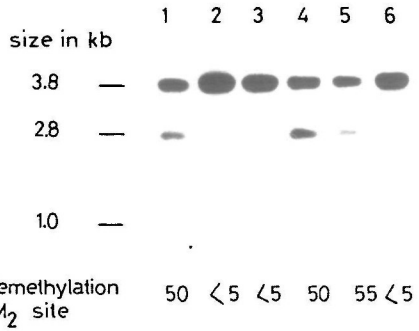

Fig. 5
Effect of inhibition of DNA synthesis on the estrogen-dependent demethylation of site M_2.
Genomic DNA (10 µg) from groups of 5 chickens treated with (lane 1) Ara C + estrogen (5 days); (lane 2) Ara C alone (5 days); (lane 3) hydroxyurea alone (5 days); (lane 4) hydroxyurea + estrogen (5 days); (lane 5) estrogen alone (5 days) and (lane 6) control, were digested sequentially with HpaII and BamHI, fragments were separated on a 1% agarose gel, and blotted to nitrocellulose paper and the filters hybridised with ^{32}P-labelled pVT981. The presence of bands at 2.8 kb and 1.0 kb indicate hypomethylation at the M_2 HpaII site. Shorter exposures of the same blot (and others) were measured on a CAMAG densitometer, and the extent of demethylation calculated, % demethylation = $\frac{\text{intensity of bands 2.8 + 1 kb}}{\text{intensity of bands 3.8 + 2.8 + 1 kb}} \times 100$

DISCUSSION

A. Methylation and Gene Expression

Experiments designed to investigate the possible role of DNA methylation in the control of gene expression fall into two broad categories, those on chromosomal cellular genes, and those on extrachromosomal elements, such as viruses and transfected plasmid DNAs. Whereas those experiments of the latter category have provided good evidence for a role for DNA methylation in extra-chromosomal and viral gene expression, the role of DNA methylation in the expression of cellular genes remains obscure.

There are now several cases of hormone-dependent demethylation events described in the literature (17,26,35,36). In these cases the time course of demethylation is inconsistent with a simple cause/effect relationship with transcription. If the results from these systems are generally applicable, then the

question of the role of DNA methylation in gene expression and the mechanism of establishment of methylation patterns are addressable. We have presented data concerning the latter question in this paper.

B. Mechanisms of Establishment of Tissue-Specific DNA Methylation Patterns

There are two possible mechanisms by which DNA can become demethylated. The first, and currently accepted mechanism involves the site-specific binding of proteins to key sites on the genome, which locally block repair methylase activity during replication. By inferring the existence of tissue-specific DNA binding proteins each with appropriate binding specificity, the tissue-specific DNA methylation patterns reported in the literature are readily explainable. This model predicts that demethylation would be dependent on DNA replication (2).

An alternative mechanism invokes an active enzymatic demethylation process, involving either removal of the methyl groups from methylated cytosine or removal of the methylated cytosine itself. This process may or may not be dependent upon replication. There is currently no evidence in the literature to exclude either possibility. "Passive" demethylation has, until recently, been held to be the likely mechanism of establishment of methy lation patterns (37), although no firm evidence in support of this conjecture has been forthcoming. However, a cytosine demethylase has been recently detected in murine erythroleukaemia cells (38) and a demethylase activity has been inferred by Colgen et al. (39) based on the observation that an MspI site 2.5 kb from the 5' end of the chicken ApoVLDLII gene becomes demethylated at a rate inconsistent with the "passive" demethylation model.

C. Role of the Estrogen-Dependent Demethylation of Site M_2 in Vitellogenin Gene Expression

We have demonstrated in this paper that neither the estrogen-dependent activation of the chicken vitellogenin gene, nor a demethylation event which occurs at a HpaII site 611 bp from the 5' end of the gene, are dependent on DNA synthesis. On the basis of these data we consider the demethylation event an active process, and therefore the product of an enzymatic activity.

The question of site specificity of such a demethylase is raised by the uniqueness of the demethylated site. We are unable to say whether the specificity lies at the sequence level, or is a product of substrate (DNA)conformation. The time course of demethylation also presents problems of interpretation. The delayed appearance of the fully demethylated site is, perhaps, surprising and may be due to the fact that the demethylase itself needs to be induced by estrogen. We have attempted to assay cytosine demethylase activity by the method of Gjerset and Martin (38) in extracts from control and estrogen-induced chicken liver nuclei, but have detected no appropriate increase in the low levels of demethylase present in nuclei (Jost, unpublished data). One possibility which may explain both the site specificity and the delayed time course involves the estrogen-receptor complex. The site-specific binding of this complex to the region of the M_2 site may induce the formation of a suitable substrate conformation for the demethylase. This newly formed substrate may be masked by the receptor complex whilst it remains bound; however, as the receptor leaves its binding site, the M_2 site may become available to the demethylase. We have recently obtained evidence for the presence of a receptor-binding site on the M_2 site (Jost, manuscript submitted for publication) which is consistent with this latter possibility. This interpretation is supported by the presence of estrogen-dependent chromatin changes in this region of the genome (35). The presence of a DNAse I-hypersensitive site very close to the M_2 site, whose appearance and disappearance correlates with the presumptive time course of the interaction of the estrogen receptor with this region *in vivo* (designated HS-C by Burch and Weintraub), also supports the notion that the estrogen receptor may play an indirect role in the demethylation of the M_2 site. The presence of the same HS-C site in oviduct chromatin may therefore be crucial in the explanation of the highly demethylated M_2 site in oviduct DNA.

Finally, what is the role, if any, of this demethylation event in the control of the chicken vitellogenin gene? From a consideration of the kinetics of demethylation it seems unlikely that it plays a role in the activation of the gene.

It seems possible that the new methylation state facilitates the secondary stimulation of the gene, and might therefore play a role in the so-called "memory effect", whereby subsequent doses of estrogen produce a more rapid and more profound response in the gene than the initial dose (14,15). Nonetheless, the role of this particular demethylation event remains obscure.

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