# Thyroid Hormone-, Carbohydrate, and Age-Dependent Regulation of a Methylation Site in the Hepatic S14 Gene

**N. C. W. Wong*, Harold L. Schwartz, Kevin Strait, and Jack H. Oppenheimer**

Department of Medicine
Division Of Endocrinology and Metabolism
University of Minnesota
Minneapolis, Minnesota 55455

**The rat hepatic S14 gene has served as a model of thyroid hormone regulation of gene expression. Earlier studies of the S14-containing chromatin region demonstrated that a cytosine residue at position 625 (C-625) in the 3′ untranslated exon was hypermethylated in hepatic DNA derived from hypothyroid animals. This observation was consistent with the markedly reduced level of expression of the S14 gene in these rats. The current studies have extended these observations to groups of rats in various thyroidal states. By using the restriction enzyme *Hha*I, the percent demethylation of this site was quantitated (hypothyroid, 9.3%; euthyroid, 19.2%; hyperthyroid, 66.6%). Moreover, the level of methylation was shown to be reversible as the thyroidal state was altered. Our data also indicate that these changes are probably independent of *de novo* DNA synthesis. Kinetic studies of the demethylation of this cytosine residue after $T_3$ administration showed no change for at least 1 day and maximal change after about 4 days. This contrasts with the significant rise in S14 mRNA evident within 30 min and suggests that demethylation plays no role in the acute induction of this gene by $T_3$. Carbohydrate feeding, another stimulus of S14 expression, similarly caused the demethylation of this cytosine residue. Earlier studies had demonstrated that mRNA S14 expression was not detectable in rat pups before about 20 days of age and continued to rise through the first year of life. Consistent with those findings, S-14 C-625 was fully methylated up to 15 days of age. Progressive demethylation then occurred up to 12 months of age. These results indicate that increased demethylation of a specific site in the 3′ untranslated region of the S14 gene, possibly resulting from augmented excision repair processes, is correlated with increased expression of the gene. (Molecular Endocrinology 3: 645–650, 1989)**

0888-8809/89/0645-0650$02.00/0
Molecular Endocrinology


## INTRODUCTION

The rapid response of hepatic mRNA S-14 to both the administration of thyroid hormone and dietary carbohydrate makes this gene a convenient model for the study of hepatic gene regulation by these stimuli (1). In examining the methylation status of the S14 gene in various tissues with the use of isoschizomers *Hpa*II/*Msp*I and *Hha*I and *Cfo*II, we showed that the gene was hypomethylated in those tissues in which the gene was expressed (2). However, although the methylation state of most of the cytosine residues of the S14 gene was independent of thyroidal status, a specific sequence recognized by the *Hha*I and *Cfo*I isoschizomers situated in the second untranslated exon of the S14 gene appeared to be substantially hypomethylated in the livers of euthyroid compared to hypothyroid animals. Comparison of the restriction enzyme maps of the S14 gene with the published nucleotide sequence of the S14 gene (3) indicates that the hormone-sensitive site is situated at nucleotide 625 (C-625) of the S-14 cDNA (Fig. 1).

In general, hypomethylation of genes is associated with increased expression of such genes, although specific exceptions to this rule have been reported (4–8). The role of hormones in determining the methylation

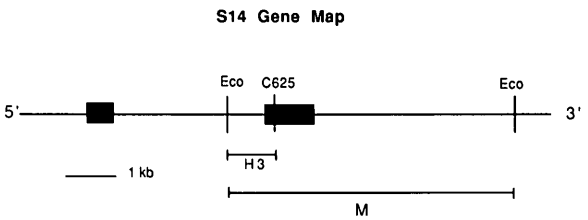


**Fig. 1.** Map of Rat Hepatic Gene S14

The gene consists of two exons (*black boxes*) and one intervening sequence (3). Cytosine 625 is located near the 5′ end of the second exon. H3, approximately 1 kb in length, is the product of restriction with *Eco*RI followed by *Hha*I. M, about 6.0 kb, is the larger *Eco*RI fragment from which H3 is cut by *Hha*I.

status of genes has also received recent attention. Thus, studies from several laboratories have suggested that estrogen administration leads to an irreversible hypomethylation of the vitellogenin gene (9–11). Although the evidence indicated that the reduction in methylation state occurred too slowly to be involved in the hormonal induction of vitellogenin (9, 10, 12), Jost and co-workers (9, 13) suggested that this process may play a role in the more rapid response of this gene to a secondary challenge with estrogen.

In the following studies, we have extended our initial observations by examining the following issues. 1) Since we had previously compared only the euthyroid and hypothyroid states, we now assessed the effects of hyperthyroidism on the methylation of hepatic S-14 C-625 and compared the results to those in the euthyroid and hypothyroid animals. 2) Since previously reported instances of hormone-induced demethylation reflected an essentially irreversible process, we were interested in determining whether this was also the case for $T_3$-induced S-14 C-625 demethylation. 3) In an effort to relate demethylation to the induction of mRNA S14 we compared the kinetics of accumulation of the demethylated C-625 to the accumulation of mRNA S14 in response to thyroid hormone. 4) We examined the possibility that dietary carbohydrate could also cause increased demethylation of C-625. 5) Lastly, in view of previous studies showing both age-related changes in the expression of hepatic gene S14 (14, 15) and alterations in the methylation state of other genes during development (4, 5), we investigated the methylation state of C-625 as a function of age.

## RESULTS

Although our previous studies had indicated a marked increase in the methylation of C-625 in the livers of hypothyroid animals, the effect of excess thyroid hormone administration had not been evaluated. Accordingly, we undertook studies to compare the methylation status of C-625 in animals rendered hyperthyroid by chronic administration of $T_3$ to that in hypothyroid and unmanipulated euthyroid rats. In these studies, animals of comparable weight (150–250 g) were used, but no effort was made to age-match the groups. The results, illustrated in Fig. 2, confirmed our previous findings with respect to the greater degree of demethylation of C-625 in the euthyroid animals compared to that in the hypothyroid rats. In addition, administration of excess thyroid hormone clearly resulted in further demethylation. Quantitation by computer-assisted videodensitometry suggested that approximately 9% of S-14 C-625 was demethylated in the hypothyroid animals, 25% in euthyroid animals, and 60% in hyperthyroid animals.

The finding of a greater degree of C-625 methylation in euthyroid animals after they had been rendered hypothyroid suggested that the demethylation process was at least partially reversible. This was surprising in light of other examples of hormonally induced demethylation (3, 9, 12). However, since the hypothyroid animals were not age-matched to the euthyroid group, we could not rule out the possibility that the decreased

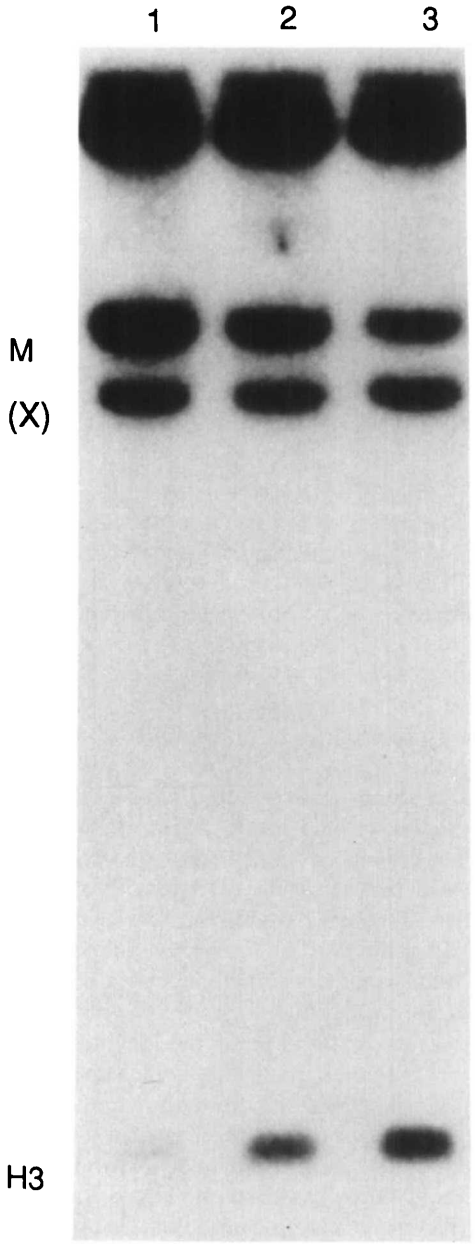


**Fig. 2.** Effect of Thyroidal State on the Methylation of C625

Euthyroid animals were rendered hypothyroid by treatment with 0.025% methimazole or hyperthyroid by daily injection of 15 $\mu$g $T_3$/100 g BW for 7 days. Isolated nuclei were treated with *Eco*RI followed by *Hha*I as described in *Materials and Methods*. Twenty micrograms of DNA were loaded into each lane. The intensity of H3 is inversely proportional to the degree of demethylation of cytosine at nucleotide position 625 of the S-14 cDNA (3). M represents the larger *Eco*I fragment from which H3 is produced by the action of *Hha*I. Lane 1, Hypothyroid; lane 2, euthyroid; lane 3, hyperthyroid. The *third band from the top* [(X)] appears in only a minority of samples and probably represents microheterogeneity of the DNA.

methylation in the euthyroid rats was a function of the maturation of the animal and not a direct action of the hormone. Thus, one could postulate that C-625 is most highly methylated in younger rats and that the degree of methylation lessens with age. Methimazole-induced hypothyroidism may simply arrest this developmental process.

To evaluate this possibility, the following experiment was performed. All animals studied were obtained from one shipment of euthyroid animals, weighing 150–175 g. Four euthyroid rats were assigned to each of four treatment groups. One group was immediately killed (group A). Two groups (groups B and C) began 3 weeks of treatment with methimazole. Group C received seven daily injections of 15 μg $T_3$/100 g BW during the third week of methimazole treatment. The fourth group (group D) remained as age-matched euthyroid unmanipulated controls. As is evident from the data listed in Table 1, little change in the degree of methylation occurs over the course of the 3 weeks of treatment in euthyroid rats. Initially, about 19% of the cytosine sites were unmethylated (group A), and this only increased to 26% in the euthyroid group maintained for an additional 3 weeks (group D). This change was not statistically significant. However, treatment with methimazole and the resulting hypothyroidism led to a significant reduction in the number of cytosine residues free of methylation to 9% of the total. On the other hand, hyperthyroidism induced by daily injections of $T_3$ for 7 days led to a marked increase in demethylation, with almost 70% of these sites having been cleaved (group C). These findings thus indicate that the thyroid hormone-induced changes in methylation status are reversible and, therefore, at least in part independent of the effects of the maturational process.

Of considerable interest was the question of whether the demethylation of C-625 is causally related to the induction of mRNA S14 by $T_3$. If the demethylation process were causally related to the initial appearance of mRNA S14, one might anticipate that the demethylation of C-625 would precede the appearance of mRNA S14 after the injection of $T_3$. The time course of demethylation was, therefore, determined (Fig. 3). This clearly showed that the appearance of mRNA S14 preceded the demethylation of S14 C-625. The initial changes in the methylation state were not consistently observed until 24 h after $T_3$ treatment, and maximal demethylation was not achieved until about 4 days after

**Table 1.** Effect of Thyroid Hormone on Methylation State of C-625

| Group | S14 C-625 (% Demethylation) | Liver DNA (μg/Liver) |
|---|---|---|
| A. Euthyroid, Killed Immediately | 19.2 ± 1.4 | 19.4 ± 1.4 |
| B. PTU, 3 weeks | 9.0 ± 0.8[a] | 20.1 ± 2.0 |
| C. PTU + $T_3$ (15 μg/100 g BW) | 66.6 ± 1.7[a] | 25.0 ± 1.3 |
| D. Euthyroid, 3 weeks | 25.8 ± 4.4 | 25.9 ± 1.0 |

Values are the mean ± SE (n = 4/group). PTU, Propylthiouracil.
[a] Significantly different from group A ($P < 0.05$).

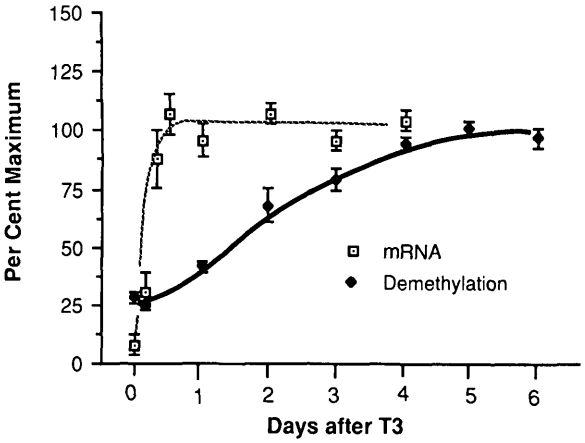


**Fig. 3.** Time Course of Demethylation of Cytosine-625 after $T_3$ Administration to Hypothyroid Animals

$T_3$ (200 μg/100 g BW) was injected ip daily, and groups of four rats were killed at the indicated intervals. Data represent the mean and SE for each group.

$T_3$ injection. These findings contrast with the rapid induction of mRNA S14, commencing approximately 20 min after $T_3$ administration (16), and therefore negate the possibility that the demethylation process plays a significant role in the initial induction of the S14 gene.

Since carbohydrate administration also results in the induction of mRNA S14 (1) we tested the effect of this stimulus on the demethylation of C-625 in euthyroid animals maintained on a high carbohydrate fat-free (lipogenic) diet for 4 days. This treatment resulted in an increase in C-625 demethylation from 20.8 ± 1.6% (mean ± SE) in chow-fed rats to 47.0 ± 4.6% in carbohydrate-fed animals (n = 7/group).

Earlier studies from our laboratory demonstrated that S14 mRNA levels were almost undetected from birth to 15 days of age, rose sharply as the rat pup was weaned to normal chow between 17 and 25 days (14), and continued to rise throughout the first year of life (15). We have now examined the methylation status of S14 C-625 as a function of age. Consistent with the virtual absence of S14 gene expression during the first 2 weeks of life, this site was totally methylated at birth and at 15 days of age (Table 2). However, during the next 2 weeks, during which a sharp rise in mRNA S14 levels was observed (15), approximately 20% of the cytosine residues at C-625 were demethylated. Progressive demethylation of C-625 continued during the year, so that by 12 months about half of the cytosine residues at this site were susceptible to the action of *Hha*I. The results of studies with $T_3$ and carbohydrate together with the age-related effects indicate a strong association between the methylation status of C-625 and expression of the S14 gene. Since the alterations in gene expression precede changes in methylation, our results imply that gene expression determines the methylation status of this cytosine residue.

## DISCUSSION

The results of the current series of experiments have confirmed and extended our previous findings that the

**Table 2.** Effect of Age on C-625 Methylation in Euthyroid Rats

| Age | % Demethylation | mRNA S14[a] |
|---|---|---|
| 0 Day | ND | |
| 15 Days | ND | 0.005 ± 0.001 (6) |
| 1 Month | 19.8 ± 0.2 (3) | 0.93 ± 0.33 (13) |
| 2 Months | 34.4 ± 3.8 (3) | 1.67 ± 0.26 (9) |
| 4 Months | 39.6 ± 2.7 (3) | 1.73 ± 0.34 (3) |
| 12 Months | 47.7 ± 3.9 (3) | 4.48 ± 1.13 (4) |

Values are the mean ± SE. The numbers in parentheses indicate the number of animals examined. ND, Not detectable.
[a] The data for mRNA S14 are taken from the report of Jump and Oppenheimer (14).

demethylation of S-14 cytosine-625 is regulated either directly or indirectly by thyroid hormone. This residue is situated in the sequence GCGC contained within the untranslated second exon of the S14 gene. Of particular interest is the finding that the degree of demethylation progressively increases from the hypothyroid through the hyperthyroid state. Since remethylation could be demonstrated in the transition from the euthyroid to the hypothyroid state, the phenomenon was clearly reversible. The other major example of hormonally regulated DNA demethylation is that described for the vitellogenin gene under the influence of estrogen, and that effect is reported to be essentially irreversible (9, 12).

Of further interest is the progressive demethylation of C-625 in parallel with the previously demonstrated rise in levels of mRNA S14 throughout the first year of life in the rat. This developmental pattern is unusual, given that most of the gene models studied, such as the adult chicken $\beta$-globin gene, show relatively rapid onset of expression and demethylation of critical sites (4, 5). Our observation further strengthens the presumed association of demethylation of C625 and S14 gene expression.

The molecular mechanism responsible for the $T_3$ regulation of C-625 methylation remains obscure. It is widely believed that demethylation of cytosines requires new DNA synthesis (4). Wilks *et al.* (17), however, have reported that the estrogen-induced demethylation of vitellogenin in chicks is not blocked by cytosine arabinoside or hydroxyurea. Although thyroid hormone has been reported to stimulate the growth of liver and the attendant DNA synthesis (18, 19), we believe that it is unlikely that hypomethylation of C-625 can be attributed to $T_3$ stimulation of *de novo* synthesis of unmethylated DNA. Our data indicate that during the transition from hypo- to hyperthyroidism more than half of the C-625 residues in the S14 gene become demethylated. Since this is accomplished within 7 days after initiation of daily $T_3$ treatment, one would have to postulate at least a doubling of the hepatic DNA pool within this period. Measurement of total hepatic DNA in these rats demonstrated only a 20% increase over baseline hypothyroid values (Table 1). This is consistent with the rather low mitotic index reported for rat liver in both euthyroid and hyperthyroid animals (20). Further, it is also generally agreed that the rate of turnover of bulk DNA is exceedingly small. This leaves two potential alternative explanations: 1) thyroidal stimulation of a demethylase or 2) $T_3$-augmented demethylation by excision repair. Although a demethylase has been reported in murine erythroleukemic cells (21), we are unaware of communications supporting the existence of such demethylase activity in rat liver. Moreover, enzymatic removal of the methyl group is mechanistically unlikely (22). The second and in our opinion more likely possibility involves excision repair of DNA. Saluz *et al.* (13) have reported preliminary evidence indicating the probable involvement of excision repair in the estrogen-induced demethylation of specific cytosine residues in the vitellogenin gene. Further, Razin *et al.* (23) have demonstrated that 5-methylcytosine can be replaced by cytosine in differentiating Friend erythroleukemia cells, presumably by an enzymatic mechanism. It is entirely possible that $T_3$-induced augmentation of excision repair could similarly lead to demethylation of C-625. With regard to remethylation, it appears possible that the hypothyroid state facilitates the action of DNA methylase on previously unmethylated DNA.

Our finding that the rise in mRNA S14 clearly precedes demethylation of C-625 precludes a role of demethylation in the initial induction of this mRNA by $T_3$. Jost and colleagues (9, 13) have proposed that the demethylation of the vitellogenin gene by estradiol is responsible for a more rapid induction by second exposure to this hormone, the so-called memory effect. However, more recent studies have reported a dissociation between the demethylation and the conditioned response to estradiol (12). Whether the demethylation has any role in an anamnestic response to $T_3$ is currently under study in our laboratory. However, as suggested above, it is entirely possible that the demethylation is simply a reflection of increased turnover of specific portions of a gene under active transcription. As pointed out in our previous report the comparable nucleotide in the S14 gene in the spleen, a tissue in which the S14 gene is not expressed, is fully methylated (2). Our current findings that the feeding of a high carbohydrate fat-free diet, which is known to stimulate mRNA-S14, results in increased demethylation of C-625 is also consonant with the view that demethylation may reflect accelerated excision repair in actively transcribed genes. Similarly, the age-related increase in demethylation may reflect the progressive age-dependent increase in gene expression that we have previously noted (14, 15).

Augmented hypomethylation associated with increased expression of genes can be found in almost any portion of a gene (3, 4). Nevertheless, hormone-induced demethylation generally occurs in the 5′ upstream region, often close to points of regulation, as indicated by the presence of DNase 1-hypersensitive sites (10). In our model, the site of $T_3$-regulated methylation/demethylation was situated in the second exon of the S14 gene, an exon that is not translated. This appears to be a distinctly unusual site for such activity.

However, it is entirely possible that the gene contains other thyroid hormone-dependent cytosine methylation sites. The detection of C-625 depends on the fact that methylation of the internal C in the sequence GCGC blocks cleavage by *Hha*I at that site. The application of techniques for direct genomic sequencing, as described by Saluz and Jost (24), should facilitate a more comprehensive examination of the methylation sites of the S14 gene in relation to hormonal control of the expression of this gene.

## MATERIALS AND METHODS

Euthyroid male Sprague-Dawley rats, weighing 150–175 g, were purchased from BioLabs, Inc. (St Paul, MN). Rats were made hypothyroid by treatment with 0.025% methimazole in the drinking water for 3 weeks. Animals were judged to the hypothyroid when weight gain ceased. Hyperthyroidism was induced by seven daily injections of $T_3$ (15 $\mu$g/100 g BW). For the study of acute responses of the S14 gene to $T_3$, rats were injected with a receptor-saturating dose of 200 $\mu$g/100 g BW. The effect of dietary carbohydrate was studied in euthyroid rats fed a lipogenic Fat Free Test Diet (ICN Pharmaceuticals, Irvine, CA) for 4 days. This diet is free of fat and contains 60% carbohydrate by weight, all in the form of sucrose. Studies of age-related demethylation were carried out in rat pups on the day of birth, at 15 days, and at 1, 2, 4, and 12 months of age. Animals were killed by exsanguination under light ether anesthesia. The tissues were quickly excised and frozen in liquid nitrogen for storage at −80 C until needed for analysis. Total hepatic RNA was extracted as described by Jump *et al.* (16). Nuclei and DNA were isolated by procedures previously reported (2).

DNA was prepared as described by Jump *et al.* (2) with the following modification to decrease the amount of residual undigested DNA remaining at the origin of the electrophoretic gel. The isolated DNA was first digested with *Eco*RI to generate much smaller fragments before digestion with *Hha*I. This enzyme cuts the DNA at the tetrameric sequence GCGC, but only if the internal cytosine is unmethylated. The DNA fragments were separated by polyacrylamide gel electrophoresis and transferred to Zetabind (AMF), as described previously (2). Zetabind blots were prehybridized, hybridized, and washed according to the procedures outlined by the manufacturer. The blots were hybridized with the $^{32}$P-labeled S14-C2 probe as described by Jump *et al.* (2) and exposed to XAR-5 x-ray film. The analysis of the resulting autoradiographic pattern is facilitated by the relatively simple nature of the S14 gene, which consists of two exons separated by a single intron (Fig. 1). The proportion of methylated cytosine is inversely related to the intensity of the fragment labeled H3, which was determined by computer-assisted videodensitometry (25). The percent demethylation was calculated from the intensity of band H3 as a fraction of the sum of the intensities of H3 and the mother fragment, M.

### Acknowledgments

We would like to express our appreciation to Ms. Alex Iden for excellent technical assistance.

Received November 14, 1988. Revision received December 23, 1988. Accepted January 3, 1989.

Address requests for reprints to: Dr. Jack H. Oppenheimer, Box 91, University of Minnesota Health Sciences, Minneapolis, Minnesota 55455.

This work was supported by NIH Grant AM-19812 (to J.H.O.).

* Recipient of a Medical Research Council of Canada Fellowship and an Alberta Heritage Fund Grant. Present address: Department of Medicine and Medical Biochemistry, University of Calgary, Calgary, Alberta, Canada.

## REFERENCES

1. Oppenheimer JH, Schwartz HL, Mariash CN, Kinlaw WB, Wong NCW, Freake HC 1987 Advance in our understanding of thyroid hormone action at the cellular level. Endocr Rev 8:288–308
2. Jump DB, Wong NCW, Oppenheimer JH 1987 Chromatin structure and methylation state of a thyroid hormone-responsive gene in rat liver. J Biol Chem 262:778–784
3. Liaw CW, Towle HC 1984 Characterization of a thyroid hormone-responsive gene from rat. J Biol Chem 259:7253–7260
4. Doerfler W 1983 DNA methylation and gene activity. Annu Rev Biochem 52:93–124
5. Razin A, Cedar H, Riggs AD (eds) 1984 DNA Methylation. Springer-Verlag, New York
6. Gerber-Huber S, May FEB, Westley BR, Felber BK, Hosbach HA, Andres AC, Ryfell GU 1983 In contrast to other *Xenopus* genes the estrogen-inducible vitellogenin genes are expressed when fully methylated. Cell 33:43–51
7. Macleod D, Bird A 1983 Transcription in oocytes of highly methylated rDNA from *Xenopus laevis* sperm. Nature 306:200–203
8. Fradin A, Manley JL, Prives CL 1982 Methylation of simian virus 40 Hpa II sites affects late, but not early, viral gene expression. Proc Natl Acad Sci USA 79:5142–5146
9. Wilks AF, Cozens PJ, Mattaj IW, Jost JP 1982 Estrogen induces a demethylation of other 5′ end region of the chicken vitellogenin gene. Proc Natl Acad Sci USA 79:4252–4255
10. Burch JBE, Weintraub H 1983 Temporal order of chromatin structural changes associated with activation of the major chicken vitellogenin gene. Cell 33:65–76
11. Meijlink FC, Philipson JN, Gruber M, Ab G 1983 Methylation of the chicken vitellogenin gene: influence of estradiol administration. Nucleic Acids Res 11:1361–1373
12. Burch JBE, Evans MI 1986 Chromatin structural transitions and the phenomenon of vitellogenin gene memory in chickens. Mol Cell Biol 6:1886–1893
13. Saluz HP, Jiricny J, Jost JP 1986 Genomic sequencing reveals a positive correlation between the kinetics of strand specific DNA demethylation of the overlapping estradiol/glucocorticoid-receptor binding sites and the rate of avian vitellogenin mRNA synthesis. Proc Natl Acad Sci USA 83:7167–7171
14. Perez-Castillo A, Schwartz HL, Oppenheimer JH 1987 Rat hepatic mRNA-S14 and lipogenic enzymes during weaning: role of S14 in lipogenesis. Am J Physiol 253:E536–E542
15. Jump DB, Oppenheimer JH 1985 High basal expression and 3,5,3′-triiodothyronine regulation of messenger ribonucleic acid S14 in lipogenic tissues. Endocrinology 117:2259–2266
16. Jump DB, Narayan P, Towle HC, Oppenheimer JH 1984 Rapid effects of triiogothyronine on hepatic gene expression. J Biol Chem 259:2789–2797
17. Wilks A, Seldran M, Jost JP 1984 An estrogen-dependent demethylation at the 5′ end of the chicken vitellogenin gene is independent of DNA synthesis. Nucleic Acids Res 12:1163–1177
18. Scow RO 1959 Effect of growth hormone and thyroxine on growth and chemical composition of muscle, bone and other tissues in thyroidectomized-hypophysectomized rats. Am J Physiol 196:859–865

19. Lee K-L, Sun S-C, Miller ON 1968 Stimulation of incorporation by triiodothyronine of thymidine-methyl-3H into hepatic DNA of the rat. Arch Biochem Biophys 125:751–757
20. Gjerset RA, Martin DW 1982 Presence of a DNA demethylating activity in the nucleus of murine erythroleukemic cells. J Biol Chem 257:8581–8583
21. Carriere R 1969 The growth of liver parenchymal nuclei and its endocrine regulation. Int Rev Cytol 25:201–277
22. Fieser LF, Fieser M 1985 Reagents for Organic Synthesis, vol 1. Wiley & Sons, New York
23. Razin A, Szyf M, Kafri T, Roll M, Giloh H, Scarpa S, Carotti D, Cantoni GL 1986 Replacement of 5-methylcytosine by cytosine: a potential mechanism for transient DNA demethylation during differentiation. Proc Natl Acad Sci USA 83:2827–2831
24. Saluz H, Jost JP 1986 Optimized genomic sequencing as a tool for the study of cytosine methylation in the regulatory region of the chicken vitellogenin II gene. Gene 42:151–157
25. Mariash CN, Seelig SA, Oppenheimer JH, Towle HC 1982 A rapid, inexpensive, quantitative technique for the analysis of two-dimensional electrophoretograms Anal Biochem 121:388–394

## Erratum

In the article "Regulation of pS2 Gene Expression by Affinity Labeling and Reversibly Binding Estrogens and Antiestrogens: Comparison of Effects on the Native Gene and on pS2–Chloroamphenicol Acetyltransferase Fusion Genes Transfected into MCF-7 Human Breast Cancer Cells," by C. A. Weaver, P. A. Springer, and B. S. Katzenellenbogen (**Molecular Endocrinology 2: 936–945, 1988**) Figure legends 1 and 5 were printed incorrectly and are reprinted below. The printer regrets the error.

**Fig. 1.** Dot Blot Hybridization Analysis of the Effects of Estrogens and Antiestrogens on pS2 RNA in MCF-7 Cells Grown in the Absence or Presence of Phenol Red

MCF-7 cells grown in CD-stripped CS in the absence (lane 1) or presence (lane 2) of phenol red for 5 days were treated with $E_2$ ($3 \times 10^{-9}$ M), KNA ($1 \times 10^{-7}$ M), OHT ($3 \times 10^{-7}$ M) or TAZ ($3 \times 10^{-7}$ M) or control vehicle (C, 0.1% ethanol) for 48 h. RNA was then isolated from MCF-7 cells and 5 $\mu$g RNA were spotted onto Gene Screen and hybridized with $^{32}$P-labeled pS2 cDNA as described in *Materials and Methods*. Blots were exposed to Dupont Cronex film with an intensifying screen overnight. Autoradiograms were developed and scanned with a densitometer.

**Fig. 5.** Assay of CAT Activity in Cell Extracts from Transfected MCF-7 Cells Grown in the Presence of Phenol Red

MCF-7 cells grown in CD-stripped serum in the presence of phenol red were transfected with Rous sarcoma virus (RSV)-CAT (lane 1); with pS2 1000-tk-CAT with the pS2 fragment in the reverse orientation relative to the tk promoter and treated with $3 \times 10^{-9}$ M $E_2$ (lane 2) or $3 \times 10^{-7}$ M OHT (lane 3); or transfected with pS2 1000-tk-CAT (correced orientation) and treated with control 0.1% ethanol (lane 4), $3 \times 10^{-9}$ M $E_2$ (lane 5), $3 \times 10^{-7}$ M OHT (lane 6), or $1 \times 10^{-7}$ M KNA (lane 7); or were transfected with the tk-CAT vector alone and treated with $3 \times 10^{-9}$ M $E_2$ (lane 8) or control ethanol vehicle (lane 9). Cell extracts were prepared and assayed for CAT activity as monitored by $^{14}$C-chloramphenicol conversion to acetylated forms. The autoradiogram was developed for 24 h.