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Estradiol down regulates the binding activity of an avian vitellogenin gene repressor (MDBP-2) and triggers a gradual demethylation of the mCpG pair of its DNA binding site

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ABSTRACT

A negative regulating protein (MDBP-2) from rooster liver nuclear extracts binds preferentially to a methylated promoter region 5'TTCACCTTmCGCTATG-AGGGGGATCATACTGG3' of the avian vitellogenin II gene (Nucleic Acids Res. 19, 1029–1034, 1991). Treatment of adult and immature roosters with estradiol results in a 90% decrease in the binding activity of MDBP-2 within three days. This corresponds to the level found in egg laying hens. The decrease in the binding activity of MDBP-2 precedes the onset of vitellogenin gene transcription. At the same time, there is a two-fold increase in the binding activity of NHP-1 (tested with the same oligonucleotide as for MDBP-2), a protein thought to be involved in the active demethylation of DNA. The methylated oligonucleotide binds either MDBP-2 or NHP-1 and there is no complex formation between the two proteins and DNA. Estradiol treatment does not change the equilibrium binding constant of MDBP-2 which is about 10^{-9}M for the methylated oligonucleotide. The early kinetics of demethylation of the mCpG pair in the binding site of MDBP-2 was studied by means of genomic sequencing. A low level of demethylation of mCpG starts gradually on both DNA strands already 4 hours after estradiol treatment during the lag phase of vitellogenin mRNA synthesis. It is concluded that the lowering of the binding activity of MDBP-2 may have a stronger effect on the derepression of the gene than the slow demethylation of MDBP-2 DNA binding site. The role of the methylated CpG is to assure a high binding affinity of the repressor to DNA.

INTRODUCTION

In the liver of immature chickens there is following a single injection of estradiol a demethylation of several mCpGs inside and outside the vitellogenin gene (1–4). At the same time there is a binding of the 5' and 3' end flanking region of the gene to the nuclear matrix (5) and the appearance of DNAse I hypersensitive sites in the promoter region (6, 7) (see Fig. 1). Among all mCpGs tested the one situated at the nucleotide position +10 from the start of the gene, becomes demethylated in a tissue and expression specific manner (4). A precise study of changes in the patterns of protein-DNA interactions in intact hepatocytes expressing and not expressing vitellogenin II gene was carried out by means of *in vivo* genomic footprinting with dimethylsulfate (4). The results indicated that in the DNAse I hypersensitive site containing the CpG there is a marked change in the pattern of protein-DNA interactions (4). The nature of the cis-acting element covering this region of the gene was studied by means of *in vitro* transcription competition experiments (8). The results strongly suggested that the sequence covering this region is a negative cis-acting element and that a protein binds preferentially to the methylated DNA (8). These results were confirmed by testing the fractionated liver nuclear extracts by gel shift assays (9). A protein, called the methylated DNA binding protein (MDBP-2) represses the *in vitro* expression of vitellogenin gene (9). The present *in vivo* studies with mature and immature roosters show that the repressor (MDBP-2) binding activity is down regulated by a single injection of estradiol. At the same time the mCpG situated in the binding site of MDBP-2 becomes gradually demethylated starting a few hours after estradiol treatment. Both the start of the demethylation and the down regulation of the MDBP-2 binding activity precedes the onset of vitellogenin II gene transcription.

MATERIALS AND METHODS

Chemicals

Heparin-Sepharose was purchased from Pharmacia. ^{32}P-alpha dATP and ^{32}P gamma ATP (3000 Ci/mmole) were obtained from Amersham. 17-β-estradiol was purchased from Serva (Heidelberg).

Hormone Treatment of Animals

Mature and immature white Leghorn roosters (300–350 g body weight) were injected for the time indicated with a single intramuscular injection of 17-β- estradiol, or other steroid hormones, (40 mg/kg) dissolved in propyleneglycol (40 mg/ml).

Preparation of Nuclear Extracts

The preparation of nuclear extracts from hen and rooster livers was carried out as described by Gorski et al. (10) and Sierra (11) with the following modifications: for hen liver and estradiol treated roosters the buffer contained 1.9 M sucrose and for the non treated animals the buffer contained 2 M sucrose. The entire preparation procedure was carried out within 9–10 hours. Aliquots of the dialysed samples were stored frozen in liquid nitrogen or at -80°C. The protein concentration was determined according to the method of Bradford (13) using bovine serum albumin as a standard.

Fractionation of Nuclear Extracts

Nuclear extracts were fractionated by FPLC on Heparin-Sepharose columns by step elution gradients (0.1 to 1 M KCl). The buffer contained 20 mM Hepes pH 7.9, 5 mM $MgCl_2$, 0.2 mM EDTA, 10% (v/v) glycerol, 2 mM beta mercapto-ethanol and 2 mM benzamidine. Individual fractions were concentrated by means of Centriprep 30 concentrators from Amicon.

NHP-1 and MDBP-2 were eluted with 0.3 M and 0.5 M KCl respectively.

Gel Mobility Shift Assays

These were carried out as described by Pawlak et al. (9). Upon autoradiography, the protein-DNA complexes were cut out of the gel and counted for radioactivity in a scintillation counter.

Genomic Sequencing

Total DNA from purified liver nuclei was prepared according to Saluz and Jost (12) and genomic sequencing of total unfractionated nuclear DNA was carried out by linear amplification with Taq polymerase (12, 13).

RNA Extraction and Hybridisation

Total RNA was prepared from pieces of frozen tissues according to Chomczynski and Sacchi (14). The amount of vitellogenin mRNA in the total RNA preparation was determined by dot blot hybridisation on nitrocellulose filters as described by Thomas (15). Nick translated plasmids pVT22 and pVT344 bearing 5 Kb of avian vitellogenin cDNA were used as a probe (16). For the controls the same amounts of total RNA from the liver of adult roosters (where the vitellogenin gene is not expressed) was hybridised under the same conditions as for the RNA prepared from the immature roosters treated with estradiol. The values of the controls were subtracted from the test values obtained for each time point. Upon autoradiography, the filters were cut and counted for radioactivity in a scintillation counter.

RESULTS

Estradiol down regulates the binding activity of MDBP-2 in adult rooster

We have compared the level of MDBP-2 binding activity in adult roosters and egg laying hens. Fig. 2 shows that in fractionated liver nuclear extracts there is a much higher binding activity of MDBP-2 in roosters than in egg laying hens. In moulting hens (low level of estradiol in the blood and low level of vitellogenin gene expression) there is about the same level of binding activity of repressor as in the roosters, however the difference in their pattern of proteolytic clipping has not yet been explained, (see Figs. 1 and 8 of reference 9, where the nuclear extracts were prepared from adult moulting hens and roosters). These observations suggest that estradiol may control the level of the repressor MDBP-2 binding activity in the liver. The results shown in Fig. 2 confirm this hypothesis: a single injection of estradiol to adult roosters resulted in a dramatic decrease in the binding activity of MDBP-2. The differences observed above are not due to an artefact of the extraction of nuclei with 0.4 M $(NH_4)_2SO_4$. The same results were obtained by using higher concentrations of KCl (1 M) or even 4 M of guanidium hydrochloride. The possible translocation of MDBP-2 from the nuclei to the cytoplasm has been investigated in adult roosters treated with estradiol. In fractionated cytoplasmic extracts of adult rooster the MDBP-2 binding activity could barely be detected and estradiol had no effect on the level of MDBP-2 (datas not shown).

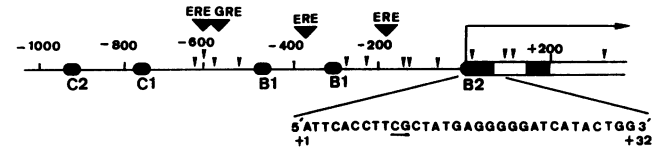

Fig. 1. Map of the promoter region of avian vitellogenin II gene. The full ellipses are the DNAse I hypersensitive regions B_1, B_2, C_1, and C_2 which appear upon estradiol treatment (6, 7). The small arrow heads represent the CpGs. ERE is the estrogen response element and GRE is the glucorticoid response element. The closed and open boxes represent the exon and introns respectively.

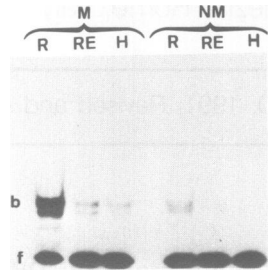

Fig. 2. Gel mobility shift assay of the Heparin-Sepharose fraction eluted with 0.5 M KCl (containing the MDBP-2) with endlabelled oligonucleotide 5' TTCA-CCTTCGCTATGAGGGGGATCATACTGGCA 3' (in duplex form) covering the nucleotide positions +2 to +34 of the avian vitellogenin promoter region. **Lanes R, RE** and **H** represent adult roosters, estradiol treated adult roosters and egg laying hens respectively. **M** stands for the methylated oligonucleotide and **NM** for the non methylated one. The protein-DNA complexes are labelled **b** and **f** is the free DNA.

Kinetics of the down regulation of the MDBP-2 binding activity in immature roosters

Immature roosters (300–350 g) were given a single injection of estradiol (40 mg/kg body weight). For each time point indicated in Fig. 3, nuclei of 4–5 rooster livers were prepared, extracted and fractionated by FPLC on Heparin-Sepharose columns (see Methods). The fraction eluted with 0.5 M KCl containing all repressor activity was tested by gel mobility shift assay using the double stranded radioactively labelled methylated oligonucleotide 5'TTCACCTTmCGCTATGAGGGGGAT-CATACTGG 3' (only upper strand is shown) as a substrate. Fig. 3 shows that already four hours post estradiol treatment there is a 50% decrease in the binding activity of MDBP-2 and 3 days later there is only about 10% of the initial binding activity. The

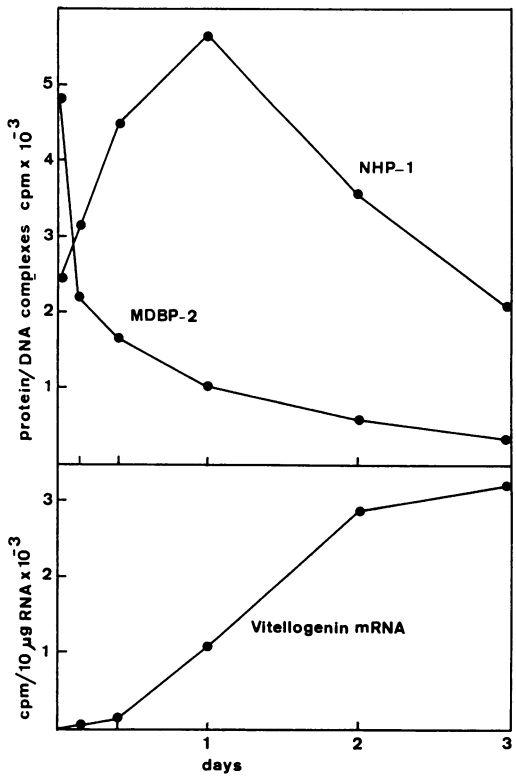

Fig. 3. *In vivo* kinetics of the down regulation of MDBP-2 binding activity by estradiol. At the time indicated nuclei were isolated, fractionated by FPLC on Heparin-Sepharose and tested by gel mobility shift assays as described in Methods. The fraction eluted from Heparin-Sepharose with 0.5 M KCl contained the MDBP-2 binding activity whereas NHP-1 was present in the 0.3 M KCl fraction. Total RNA was isolated from the livers and tested by dot blot hybridisation for vitellogenin mRNA sequence.

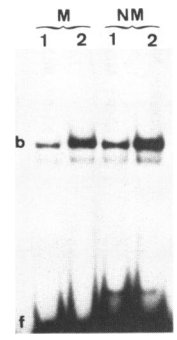

Fig. 4. Gel mobility shift assay of the Heparin-Sepharose fraction 0.3 M (containing NHP-1) with the same oligonucleotide as in Fig. 3. Lanes 1 are the NHP-1 present in non treated immature roosters and **lanes 2** the NHP-1 in immature roosters 24 hours post estradiol treatment. **M** stands for the methylated oligonucleotide and **NM** is the same non methylated oligonucleotide.

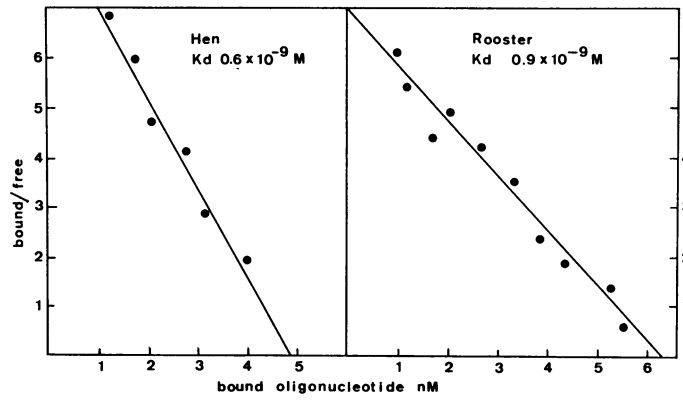

Fig. 5. Equilibrium binding constant of MDBP-2 with the methylated oligonucleotide 5′ TTCACCTTmCGCTATGAGGGGGATCATACTGG 3′ (in duplex form). MDBP-2 eluted from Heparin-Sepharose (2–6 µg) was titrated with increasing concentrations of ^{32}P labelled oligonucleotide in the presence of 1 µg of *E. coli* DNA in a total volume of 10 µl. After 20 min incubation at room temperature, the complex was separated from the free oligonucleotide on a 6% acrylamide native gel. After autoradiography the free and bound oligonucleotides were cut out of the gel and counted for radioactivity. The Kd was determined from a Scatchard plot.

decrease in the binding activity precedes the onset of vitellogenin mRNA synthesis. For comparison we tested another protein, NHP-1, a ubiquitous nuclear protein that binds with high affinity (Kd, 10^{-11}M) to different DNA sequences containing the motif RRYR, including the oligonucleotide binding MDBP-2, (17). NHP-1 was eluted from Heparin-Sepharose column with 0.3 M KCl and was tested by gel mobility shift assay with the same double stranded labelled methylated oligonucleotide. In striking contrast to MDBP-2 the NHP-1 binding activity increased over a 2-fold within 24 hours and then returns to the initial level 3 days later.

As shown in Fig. 4 the NHP-1 binds the non methylated oligonucleotide slightly better than the methylated oligonucleotide, whereas MDBP-2 preferentially binds to the methylated oligonucleotide (Fig. 2).

The equilibrium binding constant of MDBP-2 in rooster and egg laying hens

The differences in binding activity of MDBP-2 in hen and rooster could reflect either a drop in the concentration of the repressor protein resulting from degradation, covalent modifications or sequestration or could be a consequence of a change in the binding affinity of the protein for its methylated DNA substrate. The latter hypothesis was tested by measuring the equilibrium binding constant. The determination of the Kd was carried out as previously described for NHP-1 (17). Under the same experimental conditions, preparations from hens and adult roosters gave apparent Kd of about 0.6×10^{-9}M and 0.9×10^{-9}M respectively for the methylated oligonucleotide (Fig. 5). Estradiol treatment of mature roosters did not change the Kd of MDBP-2. Because of the low affinity of MDBP-2 for the non methylated oligonucleotide and the detection limits of the gel shift assays it was difficult to determine accurately the Kd for the non methylated DNA which was estimated to be around 5×10^{-7}M (results not shown).

MDBP-2 and NHP-1 do not bind simultaneously the same oligonucleotide

When incubated separately MDBP-2 and NHP-1 bind to the same methylated oligonucleotide (Fig. 2 and 4). However, MDBP-2 and NHP-1 are not capable to bind to the same oligonucleotide at the same time (Fig. 6) and there is no larger complex formed between MDBP-2 and NHP-1. A preincubation of the oligonucleotide with either MDBP-2 or NHP-1 results in some

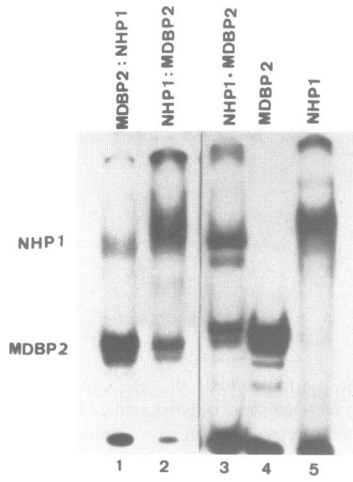

Fig. 6. Gel mobility shift assay of the endlabeled 5'TTCACCTTmCGCTATG-AGGGGGATCATACTGG3' with MDBP-2 (lane 4), NHP-1 (lane 5) or a combination of both (lanes 1−3). For **lane 1**, the oligonucleotide was preincubated for 15 min with MDBP-2 (4 μg) followed by the addition of NHP-1 (4 μg), the incubation was continued for another 15 min. For **lane 2** the reverse experiment was done, NHP-1 was added first to the reaction mixture followed by MDBP-2. For **lane 3**, NHP-1 and MDBP-2 were incubated at the same time for 30 min at 25°C. The reaction mixture was analysed on a 5% polyacrylamide gel.

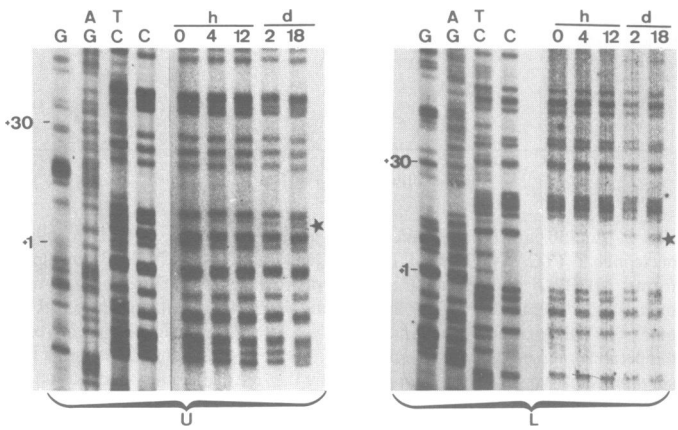

Fig. 7. *In vivo* early kinetics of hypomethylation of the mCpG in the binding site of MDBP-2 following estradiol injection to immature roosters. The experimental conditions were the same as for the one in **Fig. 3**. The state of methylation of the CpG was determined by genomic sequencing (12, 13). **U** and **L** represent the genomic sequence of the upper and lower strand of DNA. All lanes of the genomic DNA represent the cytosine specific reaction. The control **lanes G, A, T,** and **C** are shown for orientation. The CpG at nucleotide position +10 is marked by the star.

inhibition of the binding of the second protein (Fig. 6, lanes 1 & 2). These results were confirmed by the more sensitive technique of surface plasmon resonance (18) (data not shown). Taken together the results indicated that the binding of MDBP-2 and NHP-1 to the same oligonucleotide is mutually exclusive.

Early kinetics of demethylation of the mCpG in the binding site of MDBP-2

Previous genomic sequencing experiments showed that the demethylation of mCpGs situated in the estrogen and glucocorticoid response elements of vitellogenin gene occured with kinetics similar to the rate of vitellogenin mRNA synthesis (3). Demethylation started initially on the lower DNA strand and the demethylation of the complementary DNA strand lagged 24 hrs behind (3). Using the same technique we studied the early kinetics of demethylation of the mCpG situated in the MDBP-2 binding site. Fig. 7 shows that the first traces of demethylation occur on both DNA strands already 4 hrs post estradiol treatment. This site becomes fully demethylated about 10− 18 days post estradiol treatment and an injection of estradiol at that time results in an immediate transcription of vitellogenin gene without any lag period (memory effect, 19, 20, 21).

DISCUSSION

The interplay of several parameters is necessary for the hormonal control of vitellogenin II gene expression. We have studied *in vivo* the dynamic changes of a repressor binding activity and the state of methylation of its binding site. Temporal changes of these two parameters could possibly explain the transition from the silent to the active state of the gene and its modulation during the ovulation cycle of the hen (Table 1). In immature chicks or adult rooster the liver vitellogenin gene is silent. The concentration of estradiol in the blood is low (22), most of the CpGs in the promoter region are methylated (2−4) and the level of the repressor MDBP-2 is very high ensuring this way the silencing of the gene. In maturing hens, 2 weeks before the onset of egg laying there is a very large and transient increase in the concentration of estradiol in the blood (from 50 to 350 pg/ml) (23). Similarly in immature roosters a single injection of estradiol results also in a transient very high level of estradiol in the blood (24). This pulse of estradiol seems to be necessary for the hypomethylation of DNA (3, 4) as well as the down regulation of the binding activity of the repressor MDBP-2. These two parameters combined with the low affinity of MDBP-2 for the non methylated DNA could contribute to the maximum stimulation of vitellogenin gene transcription by the estradiol receptor. During the moulting period or the egg laying pause

Table 1. Possible role of MDBP-2 and DNA methylation on the regulation of avian vitellogenin II gene.

	Immature chicks or adult roosters	Egg laying hens	Hens in egg laying pause or moulting
Estradiol level in the blood	very low (1−5 pg/ml)	high (54 pg/ml)	low (18 pg/ml)
Vitellogenin gene expression	silent	very high	low
CpG, nucleotide position +10	methylated	hypomethylated	hypomethylated
Kd of MDBP-2	10^{-9}M on m. DNA	10^{-8}M on nm DNA	10^{-8}M on nm DNA
Concentration of nuclear MDBP-2	high	10×lower	high

The values of estradiol/ml blood are taken from reference 22, m DNA is methylated DNA and nm DNA the non methylated DNA.

there is a decrease in the vitellogenin gene expression (Jost, unpublished observation) and a lower concentration of estradiol is observed in the blood (22). An increase in the MDBP-2 binding activity will result (about a 10-fold) while the state of methylation of DNA will remain unchanged. This way the low affinity of MDBP-2 for the non methylated DNA will be compensated by a higher concentration of the repressor and will result in the down regulation of vitellogenin gene without turning it off completely. It is noteworthy that in organs that do not express vitellogenin gene there is always a high concentration of MDBP-2 (9). Another interesting alternative interpretation is suggested by the elegant work of Boyes and Bird (26). They showed that DNA methylation inhibits transcription indirectly via a methyl CpG binding protein (MeCP-1). In this case it only suffices to lower the concentration of MeCP-1 to achieve *in vivo* and *in vitro* gene activation in the absence of DNA demethylation. Similarly, from recent *in vivo* competition studies Levine et al. (27) concluded that a co-transfection of a methylated gene with non transcribed methylated DNA could overcome the inhibition of transcription. The ability of methylated DNA to overcome the inhibition of the methylated gene seems to reflect a competition for a repressing factor. In our case the lowering of the concentration of the repressor could have the same effect and the slow demethylation of the MDBP-2 binding site may be a side effect of the gene activation which may play a minor role during the primary stimulation of vitellogenin gene by estradiol. The full demethylation of this and other sites could facilitate the secondary activation of the gene (memory effect). In conclusion, the down regulation of MDBP-2 may have a stronger effect on the relieve of the repression than the gradual demethylation that occurs during the primary stimulation of vitellogenin gene. The presence of the methylated CpGs is important to maintain a high affinity binding of the repressor MDBP-2 to DNA.

The mechanism of the down regulation of the binding activity of MDBP-2 is at present not known. It could reflect either an inhibition of the transcription of MDBP-2 gene or a change in the turnover of MDBP-2 mRNA or protein. The possibility of a change in the state of glycosylation of MDBP-2 following estradiol treatment has been investigated. Estradiol does not change the state of glycosylation of MDBP-2 (Jost, unpublished results). MDBP-2 is most probably not related to other down regulating factors previously described for amphibian and avian vitellogenin genes (28, 29) since there is no sequence homology between their binding sites. Furthermore, no specific MDBP-2 binding activity could be detected in fractionated Xenopus laevis liver nuclear extracts (Jost, unpublished results).

The rapid and transient increase in the binding activity of NHP-1 is occuring during the lag phase of vitellogenin gene activation when the mCpGs become hypomethylated. A similar higher binding activity of NHP-1 was also recently observed for the estrogen response element situated 600 bp upstream of the transcription start site of vitellogenin gene (25). It was previously shown that NHP-1 is ubiquitous and sequence non specific except for the CpG dinucleotide. *In vitro* studies indicate that affinity purified NHP-1 may induce nicks around the mCpG of a synthetic methylated oligonucleotide (17, 30). Therefore, NHP-1 could be part of an enzyme complex responsible for the active removal of methyl cytosine at specific sites on the DNA. Such active mechanism has already been suggested for other systems (31, 32). It is therefore conceivable that the lowering of the MDBP-2 binding activity is instrumental to the binding of NHP-1 to the MDBP-2 binding site and the start of the active demethylation of the mCpG.

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