Transcriptional Suppression of the Transferrin Gene by Hypolipidemic Peroxisome Proliferators

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Activation of gene expression by hypolipidemic peroxisome proliferators (e.g. native and substituted long chain fatty acids, aryloxyalkanoic fibrate drugs) is accompanied by transcriptional suppression of liver transferrin gene in treated animals or human hepatoma cell line. Transcriptional suppression of liver transferrin by hypolipidemic peroxisome proliferators results from (a) displacement of hepatic nuclear factor (HNF)-4 from the transferrin promoter by nonproductive binding of the peroxisome proliferator-activated receptor-retinoic acid X receptor heterodimer to the (−76/−52) PRI promoter element of the human transferrin gene and (b) suppression of liver HNF-4 gene expression by hypolipidemic peroxisome proliferators with a concomitant decrease in its availability for binding to the transferrin PRI promoter element. HNF-4 gene suppression and its displacement from the transferrin promoter results in eliciting HNF-4-enhanced transcription of transferrin. Liver transferrin suppression by hypolipidemic peroxisome proliferators may result in reduced iron availability as well as modulation of transferrin-induced differentiation processes. Transcriptional suppression of HNF-4-enhanced liver genes (e.g. apolipoprotein C-III, transferrin) may complement the pleiotropic biological effect exerted by hypolipidemic peroxisome proliferators.

Transferrin (Tf)1 is highly expressed in the adult mammalian liver and is secreted by hepatocytes into the serum where it functions as an iron transport protein and growth factor for a variety of cells (reviewed in Refs. 1 and 2). Liver Tf expression was reported to be activated by steroid hormones (3) and iron deficiency (3, 4). Tf is synthesized to a lower extent by Sertoli cells in the adult testis as well as by adult brain oligodendrocytes, astrocytes, and epithelial cells of the choroid plexus, where it is involved in the maturation of germinal cells and in central nervous system proliferation and differentiation processes.

In experiments to be reported elsewhere,2 serum iron, iron binding capacity and plasma Tf were found to be 50% reduced in rats treated by xenobiopathic amphiphatic carboxylic acids (e.g. aryloxyalkanoic acids (bezafibrate), substituted long chain di-carboxylic acids (Medica 16)) known collectively as hypolipidemic drugs/peroxisome proliferators (HD/PPs) (reviewed in Ref. 15). HD/PPs have previously been reported to activate the expression of a variety of discrete genes (e.g. peroxisomal β-oxidation genes (6), ω-oxidation P450IV genes (7), liver genes coding for thyroid hormone-dependent activities (8), and others) as a result of transcriptional activation mediated by binding of peroxisome proliferators-activated receptors (PPARs) to sequence-specific PPAR-activated response elements (PPREs) in the respective promotors (9–13).3 PPAR binding to PPREs requires the retinoic acid X receptor (RXR) for forming the high affinity PPAR-RXR heterodimer (14). The putative binding of HD/PPs to PPREs and the role of HD/PPs in initiating the binding of PPAR/RXR to PPREs still remains to be investigated (9).

Since some HD/PPs are extensively used in humans as hypolipidemic drugs (15) and since transcriptional suppression, rather than activation, mediated by the HD/PP/PPAR-RXR-PPRE transduction pathway may complement the pleiotropic biological effect exerted by HD/PPs, we became interested in elucidating the mode of action of HD/PPs as putative suppressors of liver Tf. Liver Tf gene suppression by HD/PPs will be shown here to be mediated by PPAR/RXR and to involve the HNF-4 enhancer element of the Tf gene promoter.

EXPERIMENTAL PROCEDURES

Animals and Cultures—Male albino rats weighing 150–200 g were fed with laboratory chow diet. 0.25% (w/w) Medica 16 was added to their diet where indicated. Hep G2, CV-1, and COS-7 cells were cultured in Dulbecco’s modified Eagle’s media supplemented with 10% fetal calf serum with either Me2SO as vehicle or 120 μM of Medica 16 added to the culture medium where indicated.

Run-on Transcription—Hep G2 nuclei were prepared according to Ref. 16. Run-on transcription assays were carried out as described previously (17).

Transfection Assays—Transcriptional activity was measured in cells cultured in Dulbecco’s modified Eagle’s media supplemented with 10% fetal calf serum. Cells were transfected for 6 h with the respective CsCl-purified plasmid DNA added by calcium phosphate precipitation, washed, and further cultured for 42 h in the absence or presence of 120 μM of Medica 16 added as 1000× stock in dimethyl sulfoxide. The β-galactosidase expression vector pRSGAL (2 μg) added to each precipitate served as an internal control for transfection. When transfected with variable amounts of expression vectors, total amount of DNA was kept constant for each expression vector by supplementing with the parent pSG5 vector (18). Cells extracts were prepared by freeze-thawing and assayed for β-galactosidase and CAT activities. Results are ex-

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§ The abbreviations used are: Tf, transferrin; CAT, chloramphenicol acetyltransferase; HD/PP, hypolipidemic drug(s)/peroxisome proliferator; HNF-4, hepatic nuclear factor-4; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR-activated response element; RXR, retinoic acid X receptor; TK, thymidine kinase.

2 G. Link, manuscript in preparation.

3 R. Hertz, V. Nikodem, A. Ben-Isha, I. Berman, and J. Bar-Tana, manuscript in preparation.
pressed as fold induction relative to CAT expression observed in cells transfected with the parent pSG5 vector. Each point represents the mean of duplicate cultures differing by no more than 10%.

Gel Mobility Shift Assays—Gel mobility shift assays were carried out using rat nuclear extracts (19), in vitro synthesized transcription factors, or whole COS extracts overexpressing the respective transfected expression vectors. hRXR and hHNF-4 cDNAs cloned in pSG5 were linearized by XbaI and transcribed (Stratagene) and translated in rabbit reticulocyte lysates (Promega). COS extracts enriched with PPAR, RXR, or HNF-4 were prepared from COS-7 cells transfected for 5 h by calcium phosphate precipitation with 10 μg of pSG5, pSG5-PPAR, pSG5-RXR, pSG5-HNF-4, or selected combinations of the above plasmids. Following transfection, cells were incubated for 48 h, harvested, lysed by three cycles of freezing-thawing in 100 μl of lysis buffer (600 mM KCl, 10 mM Tris-HCl, pH 7.5, 1 mM diithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, and 1 μM leupeptin), and centrifuged at 20,000 g for 15 min; the supernatants were then aliquoted and stored at −70 °C. For gel shift assays, programmed or unprogrammed reticulocyte lysates (2 μl) or whole COS extracts (4 μg) as indicated were incubated for 20 min on ice in 11 μl Hepes (pH 7.9) containing 50 mM KCl, 1 mM diithiothreitol, 2.5 mM MgCl2, 10% glycerol, 1 μg of poly(dI-dC) in a final volume of 20 μl. 0.1 ng of the respective 32P-labeled oligonucleotide was then added, and incubation was continued for an additional 20 min at room temperature. Protein-DNA complexes were resolved on a 5% non-denaturing polyacrylamide gel in 0.5 × TBE.

Molecular Probes and Plasmid Constructs—Rat Tf cDNA (20) was provided by A. Kahn (Paris). Human Tf cDNA was as described previously (21). hTf-PPR-TK-CAT was prepared by inserting the synthetic oligonucleotide encompassing the (−73/−52)hTf-PRI promoter sequence (−5′-ACGGGAGGTCAAAGATTGCGCC-3′) flanked on either side by HindIII restriction sites into pBLCAT2 upstream of the −105-base pair TK promoter. Clones containing one copy of the PRI element were isolated, and their 5′-3′ orientation was confirmed by sequencing. (−620/−39)hTf-CAT, 5′-deleted constructs of (−620/−39)hTf-CAT, (−620/−39)hTf[(PRI) mut]-CAT, and (−620/−39)hTf[(PRI) mut]-CAT were as described previously (22). Poly(A) binding protein cDNA (24) cloned into the EcoRI site of PGEM1 (Promega) was from T. Grange (Paris). HNF-4 recombinant DNA pLEN4S (23) was from F. M. Sladek (Riverside, CA). pSG5-HNF-4expression plasmid was constructed by inserting the BamH1 fragment of pLEN4S encoding the entire 3-kilobase HNF-4 cDNA into the BamH1 site of pSG5. pSG5-mPPAR expression plasmid (9) and anti-mPPAR antiserum were from S. Green (Zeneca, Cheshire, United Kingdom). PPAR-G (12) cDNA was from E. Johnson (La Jolla, CA) and cloned into the XbaI-Smal site of pCMV4 (25). RS-hRXRα (26) was from R. Evans (La Jolla, CA). pSG5-RXR expression plasmid was constructed by inserting the EcoRI fragment of RS-hRXRα into the EcoRI site of pSG5.

RESULTS

Suppression of Tf Transcription by HD/PP—The 50% reduction in plasma Tf in rats treated with Medica 16 was accompanied by a respective decrease in liver rTf mRNA (Fig. 1A), thus indicating that the HD/PP effect could be accounted for by pretranslational inhibition of Tf expression. A decrease in Tf mRNA was similarly observed in human transformed Hep G2 liver cells incubated in the presence of Medica 16 (Fig. 1B), thus pointing to the direct effect exerted by HD/PPs on cells expressing Tf.

Suppression of liver Tf mRNA by Medica 16 was accompanied by inhibition of Tf transcription rate as verified by run-on transcription assays in Hep G2 cell nuclei (Fig. 2). Incubation in the presence of Medica 16 for 72 h resulted in a pronounced inhibition of Tf transcription rate, indicating that reduction in Tf mRNA exerted by Medica 16 treatment may be ascribed to suppression of transcription of liver Tf gene by Medica 16.

Essentially similar decreases in Tf mRNA and Tf transcription rates were observed using bezafibrate (not shown), thus generalizing the observed effect to other members of the HD/PP class of compounds.

Tf Transcriptional Suppression Is Mediated by PPAR/RXR Binding to the Tf Proximal Promoter—Transfection experiments in hepatoma cells using 5′- and 3′-deleted mutants of the human Tf gene promoter have previously implicated the first 620 nucleotides of the Tf promoter in regulating the expression of the Tf gene in liver cells (22, 27, 28). These studies, complemented by expression from the mouse Tf gene promoter in transgenic mice (29, 30), by in vitro transcription assays using the G-free cassette system (31), as well as by in vitro binding assays using liver nuclear extracts (22, 32), have indicated that three distinct functional regions were involved in regulating liver Tf transcription by the (−620/−1)hTf promoter, namely, the (−76/−51)hTf-PRI element, the (−103/−83)hTf-PRII element, and an upstream promoter region within the (−620/−125)hTf promoter sequence, which modulates the activity of

![Fig. 1. The effect of Medica 16 on Tf mRNA levels in rat liver and Hep G2 cells. A, rats were treated for 5 days with Medica 16 as described under “Experimental Procedures.” Total RNA was prepared according to Ref. 43, and 25 μg were subjected to Northern blot analysis. Rat Tf mRNA was determined using the 1.4-kilobase Psrl restriction fragment of rTf cDNA (20) 32P-labeled by the random priming method (42). Representative experiment is shown out of three independent experiments. B, Hep G2 cells were cultured for 72 h as described under “Experimental Procedures” in the presence or absence of Medica 16. Total RNA was prepared according to Ref. 43, and 25 μg were subjected to Northern blot analysis. Hep G2 Tf mRNA was probed by the 1.9-kilobase BamH1-ClaI restriction fragment of hTf cDNA (21). Representative experiment is shown out of four independent experiments.](http://www.jbc.org/)

![Fig. 2. The effect of Medica 16 on the rate of transcription of the Tf gene in Hep G2 cells. Hep G2 cells were cultured for 72 h in the presence or absence of Medica 16, and run-on transcription assays were determined on isolated nuclei as described previously (17). Newly synthesized 32P-RNA was hybridized with the hTf cDNA plasmid linearized with BamHI restriction enzyme. The extent of hybridization was normalized to the signal obtained with poly(A) binding protein cDNA. Representative experiment is shown out of three independent experiments.](http://www.jbc.org/)
the two proximal promoter elements. Tf gene suppression by HD/PP was therefore further analyzed in Hep G2 cells transfected with the \((−620/+39)\) hTf promoter linked to a CAT reporter gene and incubated in the presence and absence of added Medica 16. The putative involvement of PPAR and RXR in the Medica 16 effect was verified by cotransfecting the cells with expression vectors for PPAR and/or RXR, respectively. As shown in Fig. 3, A and B, CAT expression from the \((−620/+39)\) hTf-CAT construct was 25–50% inhibited by incubating the cells in the presence of added Medica 16. CAT expression in the presence of Medica 16 was further inhibited by cotransfecting the cells with expression vectors for both PPAR and RXR, indicating that the PPAR-RXR heterodimer was involved in Tf gene suppression by Medica 16. Inhibition of CAT expression by Medica 16 in the absence of cotransfected PPAR and RXR may therefore be accounted for by endogenous PPAR and RXR in Hep G2 cells. It is noteworthy that similarly to other genes affected by the PPAR/RXR transduction pathway, cotransferring the cells with expression vectors for PPAR and RXR resulted in inhibition of CAT expression from the \((−620/+39)\) hTf-CAT construct even in the absence of added Medica 16. Ligand-independent transcriptional modulation of Tf (Fig. 3A) and other genes (11–13) by PPAR/RXR might reflect the presence of an endogenous PPAR activator or the constitutive capacity of the PPAR-RXR heterodimer to modulate transcription of concerned genes. The role played by Medica 16 in Tf gene suppression by PPAR/RXR was better exemplified using PPAR-G (12) (Fig. 3B), having a lower constitutive (ligand-independent) activity as compared with PPAR due, perhaps, to its lower affinity for the endogenous HD/PP ligand. These results may therefore indicate that transcriptional suppression of Tf by Medica 16 is mediated by PPAR/RXR affecting an element within the \((−620/+39)\) Tf promoter sequence.

![Fig. 3. The effect of Medica 16 and PPAR/RXR on the transcriptional activity of the human Tf promoter.](image-url)
expression of the Tf gene by PPAR/RXR and Medica 16 was further characterized by transfecting Hep G2 cells with 5'-deleted constructs of the TF promoter linked to CAT and analyzing the effect exerted by cotransfected PPAR/RXR and added Medica 16 on expression of the concerned constructs. As shown in Fig. 3C, CAT expression promoted either by the (−620/+39)hTf or (−125/+39)hTf promoter sequences, which consist of the TF-PRI as well as the TF-PRII elements, was suppressed by cotransfected PPAR/RXR in the absence or presence of Medica 16, thus pointing to a PPAR/RXR responsive element within the (−125/+39)hTf promoter sequence. Deleting the TF-PRII element resulted in a 7-fold decrease in CAT expression, reflecting the synergistic role played by TF-PRI and -II in TF transcription. However, transcriptional suppression by PPAR/RXR was still maintained in the (−82/+39)hTf-CAT construct. On the other hand, deleting both the TF and PRII elements resulted in loss of suppression by PPAR/RXR, thus indicating that transcriptional suppression of the TF gene by PPAR/RXR involves the TF-PRII element.

The role played by the TF-PRII element in transcriptional suppression of the TF gene by PPAR/RXR was further verified in Hep G2 cells transfected with (−620/+39)hTf-CAT constructs mutated in either the PRI (−620/+39)hTf(PRII mut)-CAT) or PRII (−620/+39)hTf(PRII mut)-CAT) elements (22) and cotransfected with expression vectors for PPAR and RXR. As shown in Fig. 3D, expression from the TF-PRII mutated construct was 50% inhibited as compared with the respective wild type construct. However, the PRII mutation did not interfere with transcriptional suppression by PPAR/RXR. Expression from the TF-PRII mutated construct was 90% inhibited as compared with the wild type construct and could not be further suppressed by PPAR/RXR. Hence, suppression of the liver TF gene by PPAR/RXR appears to be specifically mediated by the TF-PRII element.

Transfection studies were complemented by studying binding of PPAR and/or RXR to the hTF-PRI sequence using mobility shift analysis. As shown in Fig. 4A, the PPAR-RXR heterodimer transcribed and translated in vitro in rabbit reticulocytes, but not the respective individual receptors, indeed binds to the hTF-PRI element. Similarly, PPAR derived from transfected COS cells (Fig. 4B) and complemented by endogenous or transfected RXR specifically binds to the hTF-PRI element and may be supershifted by anti-mPPAR antibody.

HNF-4 Displacement from the hTF-PRII Element by PPAR/RXR.—The hTF gene in the liver system has recently been reported to be transactivated by HNF-4 and to bind HNF-4 and PPAR/RXR to the hTF-PRII element (Fig. 3A). Transcriptional suppression of the TF gene by PPAR/RXR mediated by the TF-PRII element (Fig. 3) could therefore be ascribed to PPAR/RXR interference with HNF-4 transcriptional activation of the liver TF gene.

PPAR/RXR interference with HNF-4 binding to the TF-PRII element was studied by comparing the TF-PRII binding affinities of HNF-4 and PPAR/RXR using gel shift assays. As shown in Fig. 5, both HNF-4 and PPAR/RXR could bind to the hTF-PRII element sequence with apparent binding affinities of 2.7 ± 0.3 and 3.6 ± 0.6 nM hTF-PRII (mean ± S.D. for three independent experiments), respectively, thus indicating that the two receptors could compete for binding to the concerned element. Their functional interaction with the hTF-PRII element in the context of the heterologous thymidine kinase promoter was analyzed in CV-1 cells transfected with a hTF-PRII-TK-CAT construct consisting of the hTF-PRII element in front of the thymidine kinase promoter (Fig. 6). Cotransfecting these cells with either HNF-4 or PPAR/RXR resulted in 10- and ×4-fold activation of CAT expression, indicating that PPAR/RXR binding to the hTF-PRII element in the context of the heterologous TK promoter may result in its transactivation.
The decrease in plasma Tf observed in rats treated with hypolipidemic peroxisome proliferators was shown here to be accompanied by a decrease in liver Tf mRNA and to result from transcriptional suppression of the liver Tf gene as verified by run-on transcription assays in liver nuclei derived from Hep G2 cells. Since the level of plasma Tf is dominated by liver Tf expression and secretion, transcriptional suppression of liver Tf by HD/PP may account for the reduced plasma Tf levels in treated animals. Furthermore, since Tf transcriptional suppression was similarly observed in human Hep G2 cells incubated in the presence of added HD/PP, Tf suppression could be relevant to dyslipoproteinemic patients treated with HD/PP.

Transcriptional suppression of liver Tf gene by Medica 16 was found here to be related to HNF-4-enhanced transcription of the Tf gene and ascribed to displacement of HNF-4 from the Tf promoter by nonproductive binding of PPAR/RXR to the Tf-PRI (HNF-4 enhancer) element. The PPAR-RXR heterodimer behaves in this respect similarly to other previously reported transcription factors, e.g. ARP-1, which may compete with HNF-4 for binding to HNF-4 enhancer elements (33, 34). The extent of inhibition of Tf transcription by PPAR/RXR in a specific cell type may therefore be expected to reflect the pre-
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TABLE I

| Gene                  | Promoter position | Direct repeat | Reference |
|-----------------------|-------------------|---------------|-----------|
| rAOX                  | (-570/-558)       | TGACCT T TGTCCT | 11        |
| rBifunctional enzyme  | (-2399/-2927)     | TGGACT A TTACCT | 36, 37    |
| rFABP                 | (-69/-56)         | TGGCTT TGGCCCT | 13        |
| Rabbit CYP4A6         | (-650/-662)       | TGGCC T TGGCCCT | 12        |
| rHMG-CoA synthase     | (-104/-92)        | AGACCT T TGGCCCT | 38        |
| rMalic enzyme         | (-462/-450)       | GGACCT G TGGCCCT | Footnote 3|
| rApo C-III            | (-85/-73)         | TGACCT T TGACCA | 17        |
| hTransferrin          | (-82/-70)         | TGGCC T TGGCCA | 17        |
| Consensus             | (-56/-68)         | CAATCT T TGACCT |           |
|                       |                   | ↓[A]CT ↓ T[CC] |           |

Gene Promoter position Direct repeat Reference

vailing content of concerned transcription factors and their respective binding affinities for the Tf-PRI element. Since the liver system is highly enriched in HNF-4 as compared with chicken ovalbumin upstream promoter transcription factor or ARP-1 (34), transfection with PPAR/RXR, or activating the endogenous PPAR/RXR by HD/PP or using both intervention modes results in Tf suppression. However, in cell types where the Tf-PRI function is dominated by suppressive transcription factors, HD/PP may exert an apparent transactivation of transcription mediated by displacing the concerned suppressive transcription factors by nonproductive binding of PPAR/RXR. The resultant effect in a specific cell type may be further confounded by competition for RXR between some of the concerned transcription factors in addition to competing for the same promoter element. It should be pointed out, however, that generalizing the mode of action of HD/PP as verified here in cells transiently transfected with Tf-promoted CAT constructs to the endogenous Tf promoter still remains to be complemented by studying the chromatin context of the endogenous gene as well as the role played by additional regulatory sequences of the Tf promoter not present in the transiently transfected promoter constructs used here.

In addition to suppressing Tf transcription by PPAR/RXR binding to the Tf-PRI element, HD/PP may suppress Tf transcription by suppressing HNF-4 expression. Suppression of HNF-4 transcription by HD/PP has previously been verified by showing that HNF-4 transcription rates and transcript and protein levels were significantly reduced in livers of treated animals (17). These previous results have been confirmed here in the context of the Tf gene by showing that the availability of HNF-4 for binding to the hTf-PRI element was significantly reduced in liver nuclear extracts derived from treated animals. Since HNF-4 is positively modulated by HNF-4 itself (35), suppression of HNF-4 gene expression by HD/PP may perhaps result as well from PPAR/RXR binding and displacement of HNF-4 from its putative enhancer in the HNF-4 gene promoter.

The increasing list of promoter elements reported to bind PPAR/RXR and be involved in transcriptional modulation of various genes by PPAR/RXR may call for updating the PPRE consensus sequence. As shown in Table I, PPRE sequences consist of a direct repeat separated by one-nucleotide spacer (DR-1). However, only half of the nucleotides within each repeat sequence are strictly conserved, while others vary considerably. Hence, additional cis and trans parameters other than those dictated by the direct repeat and/or the PPAR-RXR heterodimer, respectively, are presumably involved in binding and transactivation mediated by the PPAR/RXR-PPRE transduction pathway. Indeed, in the enoyl-CoA hydratase (37) and the malic enzyme genes, half repeats adjacent to the PPRE direct repeat were shown to modulate PPAR/RXR binding to PPRE or transactivation of PPRE-promoted transcription. In two other cases (e.g. P450IV (12) and malic enzyme), additional distant upstream promoter sequences were shown to be involved in modulating the PPAR effect. Furthermore, putative PPAR/RXR interacting proteins similar to those recently reported for the thyroid hormone nuclear receptor (39) may modulate transcriptional activity driven by the PPAR-PPRE basal unit.

Transcriptional suppression of Tf by HD/PP is essentially similar to that recently reported for the liver apolipoprotein C-III gene (17). In both, the suppressive effect appears to be related to HNF-4-enhanced transcription of the concerned gene and to result from displacement of HNF-4 by PPAR/RXR from the HNF-4 element of the concerned gene together with HNF-4 suppression by HD/PP, resulting in its reduced liver availability. Transcriptional suppression by HD/PP therefore complements transcriptional transactivation induced by HD/PP, thus extending the scope of effects of HD/PP as pleiotropic gene modulators. Other HNF-4-activated genes should similarly be considered as candidates for transcriptional suppression by HD/PP.

The functional significance of Tf suppression by xenobiotic HD/PP still remains to be investigated. Tf suppression could result in a decrease in iron availability and, if not compensated by increase in iron saturation or in Tf receptors, could lead to anemia. Slight anemia has indeed been observed in rats under conditions of subchronic treatment with HD/PP. Since HNF-4 has recently been shown to mediate transcriptional activation of the erythropoietin gene by hypoxia (40), Tf suppression by HD/PP could be complemented by erythropoetin suppression similarly mediated by the PPAR/RXR transduction pathway. Moreover, Tf ferric reduction catalyzed by the plasma membrane NADH reductase has recently been reported to initiate intracellular alkalinization and mitogenic growth (reviewed in Ref. 1). Tf suppression by endogenous activators of the PPAR-PPRE transduction pathway could therefore be biologically significant in differentiation processes modulated by Tf availability.

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