CATALYSIS OF THE 4-HYDROXYLATION OF RETINOIC ACIDS BY CYP3A7 IN HUMAN FETAL HEPATIC TISSUES

HAO CHEN,1 ALAN G. FANTEL, AND MONT R. JUCHAU

Department of Pharmacology (H.C., M.R.J.) and Department of Pediatrics (A.G.F.), School of Medicine, University of Washington, Seattle, Washington

(Received January 28, 2000; accepted May 23, 2000)

This paper is available online at http://www.dmd.org

ABSTRACT:

Cytochrome P4503A7 (CYP3A7) is the primary CYP isoform expressed in human fetal hepatic microsomes, and its potential role in human embryotoxicity has attracted considerable investigative attention. In this study, we investigated the 4-hydroxylation of highly embryotoxic and teratogenic retinoic acids (RA) as catalyzed by human fetal liver microsomes (HFLM) and demonstrated that CYP3A7 is an efficient RA hydroxylase. When all-trans-retinoic acid (tRA), 9-cis-retinoic acid (9cRA), or 13-cis-retinoic acid (13cRA) were incubated with HFLM (54–109 gestational days) plus NADPH, each of these three retinoic acids was rapidly converted to its corresponding 4-hydroxy and 4-oxo metabolites. The reactions were strongly inhibited by CO (CO.OO; 80:20) and were NADPH-dependent, indicating that the reactions were catalyzed by P450 isoenzymes. At 54 to 89 gestational days, 4-hydroxylase activities were relatively low. However, at gestational days 96 to 109, activities were much higher. Selective inhibitors were employed for elucidation of the roles of individual CYP isoenzymes in HFLM. α-Naphthoflavone, paclitaxel, and diethyldithiocarbamate showed little or no effects on HFLM-catalyzed reactions, indicating that CYP3A1, CYP1A2, CYP1B1, CYP2C8, and CYP2E1 did not play significant roles in the catalysis. By contrast, troleandomycin strongly inhibited the reaction (70–75% inhibition), suggesting that CYP3A7 was primarily responsible for the observed catalysis. It was also discovered that CYP3A7 SUPERSOMES efficiently catalyzed the 4-hydroxylations of tRA, 9cRA, and 13cRA. Because 4-hydroxylated metabolites of RA are much less potent embryotoxins and teratogens, the results indicated that the 4-hydroxylation of RA, catalyzed prenatally by CYP3A7, might play an important role in protecting the human fetus against RA-induced embryotoxicity.

All-trans-retinoic acid (tRA),2 the active metabolite of vitamin A1 (all-trans-retinol), is essential for normal human development (Means and Gudas, 1995; Soprano and Soprano, 1995). Both excesses and deficiencies of tRA, on the other hand, are now known to cause severe birth defects (Levin, 1995; Means and Gudas, 1995; Soprano and Soprano, 1995). Therefore, enzymatic regulation of levels of tRA in embryonic tissues plays an extremely important role in both normal and abnormal human embryonic development.

Levels of tRA can be regulated by oxidation of the carbon atom at the fourth position in the hydrophobic ring. Via this mechanism, tRA is converted to 4-OH-tRA, and the generated 4-OH-tRA is then further oxidized to 4-oxo-tRA. Under conditions of excessive exposure to tRA, 4-hydroxylation of tRA might be an important protective mechanism for developing embryos. First, conversion of tRA to 4-OH-tRA is irreversible; thus down-regulation of tRA becomes more efficient. Second, as compared with tRA, 4-OH-tRA and 4-oxo-tRA show far lower binding affinity to nuclear RA receptors (Repa et al., 1993). It has long been observed that the toxicity of retinoids positively correlates with their binding affinity to RA receptors (Levin, 1995). In cultured rodent embryos, it was demonstrated that the 4-oxo metabolite of tRA produced a much lesser teratogenic effect than tRA (Martini and Murray, 1999). Therefore, enzymatic regulation of levels of tRA in embryonic tissues plays an extremely important role in protecting the human fetus against RA-induced embryotoxicity.

This work was supported by National Institute of Environmental Health Sciences Grant 04041.

1 Current address: Pharmacokinetics & Drug Metabolism, PathoGenesis Corporation, 201 Elliott Ave. West, Seattle, WA 98119.

2 Abbreviations used are: tRA, all-trans-retinoic acid; RA, retinoic acid; CYP, cytochrome P-450 monoxygenase; HFLM, human fetal liver microsomes; HALM, human adult liver microsomes; 9cRA, 9-cis-retinoic acid; 13cRA, 13-cis-retinoic acid; 4-OH-tRA, 4-hydroxy-trans-retinoic acid; 4-oxo-tRA, 4-oxo-all-trans-retinoic acid; 4-OH-9cRA, 4-hydroxy-9-cis-retinoic acid; 4-oxo-9cRA; 4-OH-13cRA, 4-hydroxy-13-cis-retinoic acid; 4-oxo-13cRA, 4-oxo-13-cis-retinoic acid; TAO, troleandomycin; PAC, paclitaxel; ANF, α-naphthoflavone.

Send reprint requests to: Prof. M. R. Juchau, Department of Pharmacology, School of Medicine, Box 357280, University of Washington, Seattle, WA 98195. E-mail: juchau@u.washington.edu
The solvents utilized were of the highest purity commercially available. From Aldrich Chemical Co. (Milwaukee, WI) or from Sigma. Reagents and from Gentest Corp. (Woburn, MA). All other chemicals were purchased either.

4-hydroxy standards were highly consistent with those reported in the literature. 4-oxo-tRA, 4-oxo-9cRA, and 4-oxo-13cRA, were gifts from Dr. Eva-Maria Gutknecht and Mr. Co. (St. Louis, MO). Metabolite standard compounds, 4-oxo-tRA, 4-oxo-9cRA, and 4-oxo-13cRA, were detected. Duration of gestation appeared to have a major influence on the generation of 4-OH-RAs and 4-oxo-RAs were readily achieved. Duration of gestation appeared to have a major influence on the generation of 4-OH-RAs and 4-oxo-RAs were readily achieved. The reaction was initiated by the addition of NADPH (1 mM) to incubation vessels, and the incubation was continued for an additional 10 min. The velocity of the reaction was linear during the first 20 min of incubation. The total volume of the incubation mixture was 1 ml. At the end of the incubation, the reaction was terminated by addition of 0.4 ml of ice-cold n-butanol/methanol (95/5, v/v). Retinoids were extracted with n-butanol/methanol and were separated by centrifugation (16,000 g for 30 min at 4°C). The organic phase was collected and stored at −80°C for HPLC analyses. To prevent autoxidation and isomerization of retinoids, butylated hydroxytoluene (0.5 μmol) was added to the incubation vessels and the incubation and extraction of RA and its metabolites were completed in a dark room with only yellow light. For determinations of kinetic parameters, 1.2 to 35 μM concentrations of RA were used. \( K_m \) and \( V_{max} \) were determined by linear regression of the raw data obtained from Eadie-Hofstee plots.

Inhibition of 4-Hydroxylation Catalyzed by HFLM, HALM, or CYP SUPERSONES. Experiments designed to assess inhibition by carbon monoxide followed the same procedures described above except that the incubations were in an atmosphere of CO (80%) and O₂ (20%). The ratio and flow rate of gases were regulated with a gas regulator, and incubations opened to air (\( N_2 : O_2 \), 80:20) served as controls. For chemical inhibition, inhibitors were added to incubation vessels and were preincubated with substrate plus HFLM, HALM, or CYP SUPERSONES for 3 min at 37°C before the reactions were initiated by adding NADPH. For mechanism-based inhibition, inhibitors were preincubated with HFLM, HALM, or CYP SUPERSONES and NADPH for 15 min at 37°C, and the reactions were initiated by addition of substrate. Termination of the reactions and extraction of RA and its metabolites followed the same procedures as those described above. As suggested in the literature (Masimirembwa et al., 1999), a concentration of 10 μM CYP inhibitor was chosen for single concentration studies. Additional concentrations were also tested as indicated. Incubations without inhibitors served as controls. For heat inactivation experiments, suspensions of HFLM or CYP SUPERSONES were heated at 100°C for 3 min before addition to incubation vessels.

HPLC Procedures. A IP column (4.6 × 250 mm; Ultrasphere, Beckman Instruments, Berkeley, CA) was used for identification and quantitation of the various RAs and their 4-hydroxy and 4-oxo metabolites. The solvent delivery system for HFLC consisted of two model 100 A dual piston Beckman pumps and was interfaced with a SPD-10A UV detector (Shimadzu Scientific Instruments Inc., Columbia, MD) (set at a wavelength of 354 nm) and a Shimadzu C-RSA Chromatopac data processor. The HPLC system was equipped with a Beckman mixing chamber and manual injector. Analytical eluents consisted of solvent A (acetonitrile: H₂O: acetic acid, 49:75:4; 95:25:0.5, v/v) and solvent B (acetonitrile: H₂O: acetic acid, 90:10:0.04, v/v), both containing 10 mM ammonium acetate. The HPLC elution conditions were as follows: 80% solvent A plus 20% solvent B with a flow rate of 0.4 ml/min for 12 min; then increased to 1.5 ml/min for another 12 min. A 90% dilution with HPLC eluent (20% A plus 80% B) of the supernatant before injection on the HPLC column was performed. A 5 μM CYP inhibitor was added to incubation vessels and the incubation and extraction of RA and its metabolites were completed in a dark room with only yellow light. For determinations of kinetic parameters, 1.2 to 35 μM concentrations of RA were used. \( K_m \) and \( V_{max} \) were determined by linear regression of the raw data obtained from Eadie-Hofstee plots.

Statistical Analyses. All experimental data were expressed as means ± S.D. for three or four experimental measurements. A Microsoft Excel statistics package (version 5.0; Microsoft Corp., Redmond, WA) was used for all statistical analyses.

Results

Figure 1 presents typical HPLC chromatograms demonstrating separations of authentic tRA and its 4-hydroxy and 4-oxo metabolites (A), organic extracts of incubation of tRA plus HFLM (B), and organic extracts of incubation of tRA without HFLM (C). The chromatograms clearly show that the generation of 4-OH-tRA and 4-oxo-tRA were HFLM-dependent. Parent substrate retinoids and retinoid metabolites were well separated from each other; thus quantitative measurements of 4-OH-RAs and 4-oxo-RAs were readily achieved.

Figure 2 exhibits generation of 4-OH- and 4-oxo-tRA catalyzed by HFLM or by HALM. Interestingly, the primary metabolite detected was 4-OH-tRA and only minimal amounts of 4-oxo-tRA were detected. Duration of gestation appeared to have a major
influence on the 4-hydroxylase activity of HFLM. For catalysis of conversion of tRA to 4-OH-tRA, specific activities of HFLM at gestational days 54, 73, 87, and 89 were approximately 150 pmol/min/mg of protein, which were comparable with that of HALM. However, the specific activities of HFLM at gestational days 96, 105, and 109 were approximately 10- to 15-fold higher than those measured in HALM.

In Table 1, specific activities of HFLM (from 109 days of gestation) and HALM in catalysis of 4-hydroxylations of tRA, 9cRA, and 13cRA are compared. For all substrates used, HFLM was much more efficient than HALM in catalysis of the 4-hydroxylation of RA. For HFLM-catalyzed reactions, the 4-hydroxylation of 9cRA was approximately 3- and 5-fold more efficient than for tRA or 13cRA. For HALM-catalyzed reactions, tRA and 9cRA appeared to be better substrates than 13cRA.

Figure 3 presents linear relationships between incubation time and generation of 4-OH-tRA catalyzed by HFLM or HALM. Velocities of the reactions also increased linearly with increasing concentrations of protein for both HFLM and HALM.

Figure 4 shows Eadie-Hofstee plots for conversions of tRA to 4-OH-tRA catalyzed by HFLM (A) and HALM (B). $K_m$ and $V_{max}$ for the HFLM-catalyzed reaction were 1.27 µM and 2400 pmol/min/mg of protein, respectively ($r^2 = 0.94$). $K_m$ and $V_{max}$ for the HALM-catalyzed reaction were 1.55 µM and 560 pmol/min/mg of protein, respectively ($r^2 = 0.83$).

Figure 5 shows effects of CO and various other CYP-selective
inhibitors (10 μM) on the HFLM-catalyzed conversion of tRA to 4-OH-tRA. Incubation with CO (CO:O₂, 80:20) resulted in approximately a 90% reduction in the rate of the reaction. α-Naphthoflavone (ANF; selective for CYP family 1 isoenzymes) and paclitaxel (selective for CYP2C8) exhibited moderate inhibitory effects (approximately 25–30% and 10 to 15% inhibition, respectively) on the reaction, whereas diethyldithiocarbamate (selective for CYP2E1) showed no effect. By contrast, troleandomycin (TAO; highly selective for CYP3A isoenzymes) showed an impressive inhibitory effect (70–75% inhibition).

### Table 1

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Substrate</th>
<th>4-OH-RA pmol/min/mg protein</th>
<th>4-oxo-RA pmol/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFLM</td>
<td>tRA</td>
<td>587 ± 23</td>
<td>106 ± 68</td>
</tr>
<tr>
<td>HALM</td>
<td>tRA</td>
<td>105 ± 24</td>
<td>38 ± 7</td>
</tr>
<tr>
<td>HFLM</td>
<td>9cRA</td>
<td>1596 ± 172</td>
<td>343 ± 12</td>
</tr>
<tr>
<td>HALM</td>
<td>9cRA</td>
<td>143 ± 75</td>
<td>35 ± 5</td>
</tr>
<tr>
<td>HFLM</td>
<td>13cRA</td>
<td>291 ± 74</td>
<td>131 ± 43</td>
</tr>
<tr>
<td>HALM</td>
<td>13cRA</td>
<td>38 ± 19</td>
<td>55 ± 18</td>
</tr>
</tbody>
</table>

RA substrates (7 μM) were preincubated with microsomes at 37°C for 5 min. Gestational age of HFLM was 109 days. Reactions were initiated by additions of NADPH (1 mM) and were continued for 10 min. Experiments were conducted in a darkened room with only yellow light to prevent both photoisomerization and autoxidation. For details, see Materials and Methods.
To investigate its inhibition specificity, ANF was added to incubation vessels containing CYP3A7 SUPERSOMES plus tRA, and the results are shown in Fig. 6A. At a lower concentration (1 μM), ANF did not inhibit the reaction. However, at higher concentrations (10–100 μM), ANF inhibited CYP3A7-catalyzed reaction by approximately 20 to 25%. Interestingly, inhibition by 100 μM ANF was not significantly greater than inhibition by 10 μM ANF. Because inhibition by TAO does not conclusively indicate catalysis by CYP3A isoforms, we tested TAO inhibition of RA 4-hydroxylation by heterologously expressed CYP3A7, CYP3A4, and CYP3A5, and the results are presented in Fig. 6B. Extensive inhibition (60–70% at 10 μM) by TAO was exhibited for each isoform. Inhibition specificity of PAC also was tested by adding PAC (10 μM) to the CYP3A7-catalyzed reaction but no statistically significant inhibition was observed.

To investigate preliminarily the potential importance of CYP26 for the reaction, human prenatal brain microsomes [CYP26 was reportedly expressed extensively in human prenatal brain microsomes (Trofimova-Griffin and Juchau, 1998)] were incubated with tRA. Only minimal amounts of 4-OH-tRA were detected (results not shown) from incubations with brain microsomes when compared with the HFLM-catalyzed reaction.

**Fig. 6.** Effects of varying concentrations of ANF on CYP3A7 SUPERSOMES-catalyzed conversion of tRA (35 μM) to 4-OH-tRA (A), and effects of varying concentrations of TAO on CYP3A7 (○), CYP3A5 (△), or CYP3A4 (□) SUPERSOMES-catalyzed conversion of tRA (35 μM) to 4-OH-tRA (B).

Values of controls in B were 525 ± 37 pmol/min/nmol of CYP (CYP3A7), 273 ± 17 pmol/min/nmol of CYP (CYP3A5), and 236 ± 24 pmol/min/nmol of CYP (CYP3A4). Results are means ± S.D. of three to four measurements. For details, see Materials and Methods.
Table 2 presents kinetic constants for HFLM- (pooled from 96–109 days of gestation) and CYP3A7 SUPERSOMES-catalyzed 4-hydroxylation of tRA, 9cRA, and 13cRA. For both HFLM- and CYP3A7 SUPERSOMES-catalyzed reactions, conversion of tRA to 4-OH-tRA exhibited the lowest $K_m$ and conversion of 13cRA to 4-OH-13cRA exhibited the highest $K_m$. In both cases, conversion of 9cRA to 4-OH-9cRA exhibited the greatest $V_{max}$ and conversion of 13cRA to 4-OH-13cRA exhibited the lowest $V_{max}$.

**Discussion**

HFLM exhibited excellent catalytic activities for conversion of three RAs (tRA, 9cRA, and 13cRA) to their corresponding 4-hydroxy and 4-oxo metabolites. The results suggest that such 4-hydroxylase activity might have important physiological and toxicological significance. As mentioned before, excessive amounts of RAs are highly embryotoxic and teratogenic, but their 4-hydroxy metabolites are less toxic. Consequently, levels of 4-hydroxylase activity could be an important determinant of the susceptibility of embryos to RA toxicity. Prenatal hepatic tissues from 54 to 89 gestational days showed much lower 4-hydroxylase activities than those obtained at later gestational stages, suggesting that early gestation embryos might be much more sensitive than later gestation fetuses to the toxic effects of RAs. This appears to be in harmony with the current concept that prenatal humans appear to be more sensitive to RA-induced toxicity during the embryonic period (before 70 gestational days) (Lammer et al., 1985). Therefore, the level of 4-hydroxylase activity in prenatal tissues would appear to be a logical index of susceptibility to RA-induced embryotoxicity and teratogenicity.

RA 4-hydroxylase activities measured at gestational days 54 to 89 were lower than expected based on results from our previous studies with warfarin as a substrate (Yang et al., 1994). This may be substrate related (e.g., endogenous competitive inhibitors for hydroxylation of RA during earlier gestation) or due to known interindividual variations in fetal CYP3A7 levels. Estimates of levels of CYP3A4 isoforms in livers of both adult and fetal human tissues (Hakkola et al., 1998; Juchau et al., 1998; de Wildt et al., 1999) have indicated considerable interindividual variations. In general terms, CYP3A7 is estimated to account for approximately 50% (30–85%) of total P450 in human fetal hepatic microsomes with only traces of CYP3A4 and CYP3A5. In human adult hepatic microsomes, CYP3A4 is estimated to account for approximately 30% of total P450 with only traces of CYP3A7 and CYP3A5. Total P450 in adult preparations, however, is approximately 1.5-fold higher than in fetal preparations. Thus, total levels of all CYP3A isoforms in fetal and adult preparations would be expected to within the same range but with the aforementioned high interindividual variability.

CYP3A7 appeared to be primarily responsible for the highly efficient catalysis of 4-hydroxylation of RA in HFLM. First, CYP3A7 is by far the most dominant CYP isoform expressed in human prenatal hepatic microsomes (Kitada et al., 1991; Kitada and Kamataki, 1994; Schuetz et al., 1994). Second, TAO, the highly selective inhibitor for members of CYP3A subfamily, very effectively and similarly inhibited the reactions catalyzed by both HFLM and heterologously expressed CYP3A7, indicating that the majority of the observed 4-hydroxylation activity was due to catalysis by CYP3A7. Third, cDNA-expressed human CYP3A7 exhibited impressive catalytic activities for the same reactions. In addition, cDNA-expressed CYP3A7 exhibited very low $K_m$ and high $V_{max}$ values with each of the three retinoid substrates studied, tRA, 9cRA, and 13cRA, and values obtained with cDNA-expressed CYP3A7 exhibited a good correlation and were consistent with those obtained with HFLM.

CYP2E1, CYP2C8, CYP1B1, and CYP1A1 are reportedly also expressed in rodent and human prenatal tissues (Omiecinski et al., 1990; Chapman et al., 1994; Carpenter et al., 1996; Carpenter et al., 1997; reviewed by Hakkola et al., 1998, by Juchau et al., 1998 and by de Wildt et al., 1999). Thus, chemical inhibition was employed to investigate the possible participation of these CYP isoforms in the 4-hydroxylation of tRA. Diethyldithiocarbamate (selective inhibitor for CYP2E1) did not produce inhibitory effects on HFLM-catalyzed 4-hydroxylation of tRA at a concentration of 10 $\mu$M, thus strongly suggesting that CYP2E1 did not contribute significantly to the reactions. In addition, when CYP2E1 SUPERSOMES were incubated with tRA, 4-hydroxylase activity was negligible. When ANF (selective inhibitor for members of CYP family 1) was tested as an inhibitor of the HFLM-catalyzed 4-hydroxylation of tRA, generation of 4-OH-tRA was reduced by approximately 25 to 30%. This seemed to suggest that CYP1A1 or CYP1B1 (CYP1A2 is absent in HFLM) or both could contribute to the catalysis of the reaction. When CYP1A1 or CYP1B1 SUPERSOMES were incubated with tRA, however, neither CYP1A1 nor CYP1B1 SUPERSOMES catalyzed the reaction at a measurable rate, which is consistent with other studies (Nadin and Murray, 1999). An explanation for this apparent discrepancy is that the inhibitory effect of ANF on HFLM-catalyzed reaction was due to its nonspecific inhibition of CYP3A7. To test this idea, ANF was tested as an inhibitor of CYP3A7-catalyzed 4-hydroxylation of tRA with CYP3A7 SUPERSOMES as enzyme source. At 10 and 100 $\mu$M concentrations, ANF produced approximately 20 to 25% inhibition of the CYP3A7-catalyzed reaction. This observation clearly supported our hypothesis that the effect of ANF on HFLM-catalyzed reaction was due to its nonspecific inhibition. Therefore, CYP1A1 and CYP1B1 appeared not to play a significant role in catalysis of 4-hydroxylation of tRA in HFLM. PAC, a selective inhibitor for CYP2C8, produced approximately 10 to 15% inhibition of the HFLM-catalyzed 4-hydroxylation of tRA, and yet PAC did not inhibit the same reaction catalyzed by CYP3A7. This suggested that CYP2C8 was present in human fetal liver but did not play a major role for catalysis of 4-hydroxylation of tRA, probably due to its low concentration.

Recent studies have reported that CYP26 can catalyze 4-hydroxylation of tRA (White et al., 1996). A recent study showed that the mRNA of CYP26 was detected at relatively high levels in human prenatal tissues (Trofimova-Griffin and Juchau, 1998). A detailed investigation of the role of CYP26 in HFLM-catalyzed 4-hydroxylation of tRA was not feasible in this study because of the unavailability of selective-specific inhibitors and antibody for CYP26. Also, CYP26 SUPERSOMES are not yet available. However, the available evidence obtained in the present study suggested that CYP26 did not play a significant role in the HFLM-catalyzed 4-hydroxylation of tRA in comparison to CYP3A7. It was reported that CYP26 mRNA was expressed more extensively in human prenatal brain tissues than in the
human prenatal liver (Trofimova-Griffin and Juchau, 1998). Thus one might expect that RAs should be readily converted to their corresponding 4-hydroxy metabolites in human prenatal brain microsomes. However, when tRA was incubated with human prenatal brain microsomes plus NADPH, only extremely low levels of 4-hydroxylase activity were detected (preliminary data, not shown) when compared with the HFLM-catalyzed reaction. This observation indicated that CYP26 would not be a highly efficient catalyst for the 4-hydroxylation reaction. Second, CYP26 is reportedly active only in catalysis of 4-hydroxylation of tRA but not of 9cRA or 13cRA. As shown in this study, HFLM also efficiently catalyzed 4-hydroxylation of 9cRA and 13cRA. Therefore, CYP26 is not likely to contribute to these reactions in HFLM. Further studies of CYP26 catalysis, however, are indicated.

In summary, we have demonstrated that tRA and its steric isomers 9cRA and 13cRA can be efficiently converted to their corresponding 4-hydroxy metabolites in human fetal hepatic microsomes. CYP3A7 appeared to be the primary P450 isoform responsible for the catalysis of the reactions in human fetal hepatic microsomes, although the possibility remains for some participation of other CYP isoforms, including as yet unidentified isoforms. Taken together, the results presented here suggest quite strongly that CYP3A7 was the isoform primarily responsible for catalysis of the RA 4-hydroxylation reactions in human hepatic microsomes. Therefore, CYP3A7 may play a particularly important pharmacological/toxicological role in metabolism of RAs in human prenatal hepatic tissues in vivo.

Acknowledgments. The authors express their great appreciation to Dr. Eva-Maria Gutknecht and Pierre Weber (F. Hoffmann-La Roche LTD, Basel, Switzerland) for the standard retinoids used in this study.

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