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Radioimmunoassay of rat leptin: sexual dimorphism reversed from humans

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Adipose tissue secretes leptin, which interacts with receptors in the hypothalamus. In rodent models of obesity, leptin increases metabolism and decreases food intake, which helps to maintain normal body composition. Accurate and precise methods to quantitate circulating leptin concentrations are needed for physiological studies. We developed an RIA to measure leptin in rat plasma, serum, or adipocyte culture fluids. The working range of the assay, defined by the detection limit and the highest calibrator, was 0.5–50 μg/L. Recovery of 1.6–11.6 μg/L leptin added to serum was 92–103%. The rat leptin RIA correlated well with a previously developed mouse RIA when rat plasma was assayed with both methods ($r = 0.94$), but the mouse leptin assay underestimated rat leptin in plasma. Within- and between-run CVs were 2.4% to 5.7%. Plasma leptin concentrations correlated directly with percentage of body fat, and correlation improved when the results were separated by gender ($r = 0.796$, $P < 0.001$ for males; $r = 0.710$, $P < 0.001$ for females). Leptin concentrations were generally higher in male rats than in females; plasma leptin increased 0.60 μg/L for each percentage of increase in body fat for males but only 0.22 μg/L for females. We conclude that rat serum/plasma leptin concentrations are accurately and precisely measured with this new RIA.

In the few years since leptin was first characterized as the product of the *obese* gene deficient in the *ob/ob* mouse *[1]*, considerable knowledge of leptin physiology has been obtained. Mice given exogenous leptin have reduced food intake and increased energy expenditure *[2–4]*. A receptor, with several splice-variant forms, has been identified and found in many rodent tissues (reviewed in *[5]*).

Considerable effort has been directed at understanding the role of leptin in human obesity. Plasma concentrations of leptin are increased in obese humans and strongly correlate with the degree of adiposity as expressed by percentage of body fat or body mass index *[6–9]*. Leptin concentrations show a diurnal pattern, with nocturnal concentrations increased in comparison with daytime concentrations *[10]*. The diurnal pattern is disrupted by fasting; leptin concentrations decrease dramatically in response to fasting beyond the overnight period (>12 h) *[11–13]*. Insulin homeostasis appears to influence leptin metabolism, as evidenced by the increased leptin concentrations in insulin-resistant subjects *[14]*, but acute changes in insulin concentrations during several-hours-long hyperinsulinemic clamps have minimal effect on leptin plasma concentrations *[15, 16]*. The half-life of circulating leptin (~25 min) is constant over a range of adiposity *[17]*. Several studies have shown that plasma leptin concentrations are appropriate for the degree of subjects' adiposity in anorexia nervosa *[18]*, in the wasting syndrome of HIV infection *[19]*, and in lean long-distance runners *[20]*. Strenuous exercise of a few hours has little effect on plasma leptin concentrations, but prolonged exercise decreases plasma leptin concentrations *[20, 21]*. Circulating leptin is bound to high-molecular-mass proteins to various degrees in human and rodent plasma *[22, 23]*. Several potential physiological roles for leptin have been identified; some evidence exists that plasma leptin concentrations may influence energy expenditure in children *[24]* and predict weight gain in young women *[25]*. Plasma leptin concentrations rose dramatically in hamsters receiving exogenous endotoxin or cytokines, suggesting a role for leptin in the anorexia of infection *[26]*. Administration of exogenous leptin to mice hastened female reproductive maturity and may have triggered puberty *[27]*. Leptin also blunted the neuroendocrine response to starvation, suggesting that leptin may modulate the physiological response to starvation *[28]*.

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The rapid accumulation of knowledge concerning leptin physiology has been importantly aided by the availability of a commercial RIA for human leptin *[8]*. Rodents, particularly rats, are frequently used as models in endocrine and metabolic studies, but efforts to use these species for studies of leptin physiology have been hampered by the lack of a commercially available assay for rat leptin. Rat leptin cross-reacts <2% in the commercial assay for human leptin (Gingerich, unpublished findings) and, therefore, cannot be accurately measured with that method. The sequence of rat leptin has been predicted from the sequence of a cDNA *[29]*, and rat leptin has been successfully cloned for recombinant production *[30]*. These tools have allowed the generation of antibodies specific for rat leptin in guinea pigs, which has provided, along with recombinant rat leptin to formulate calibrators and for labeling to make tracer, the critical components of an RIA. This study examines the suitability of the newly developed RIA for rat leptin for use in studies of leptin physiology.

Materials and Methods

Animal studies. Sprague–Dawley, Long–Evans, Zucker, and Fischer 344 rats of both sexes were purchased from Harlan Sprague–Dawley. CD Sprague–Dawley rats were obtained from Charles Rivers Laboratories. Rats were housed in groups of five under 12 h of alternating light (0600–1800 h) and dark (1800–0600 h) and allowed free access to Purina rat chow and water for 2 weeks until killed. The animal facility where these studies were performed is a fully accredited, institutional member of the American Association for the Accreditation of Laboratory Animal Care and provides a committee that approved the protocol used.

Method for body fat measurement. Rats were killed by decapitation between 0700 and 0800 h, and trunk blood was collected into 5-mL tubes containing 72 USP units of sodium heparin (Vacutainer Tube; Becton Dickinson) that were placed on ice until separation of plasma by centrifugation at 4 °C. Plasma was stored at −20 °C for 30–45 days before analysis. Carcasses were placed into plastic bags and frozen at −20 °C for 1–15 days and then shipped to Covance Laboratories for measurement of total body fat by Soxhlet extraction *[31]*. Briefly, the entire rat is ground into fine particles and weighed into a cellulose thimble containing sodium sulfate. The thimble is dried to remove excess moisture and pentane is dripped through the sample to remove the fat. The extract is then evaporated, dried, and weighed.

Adipocyte isolation and culture. Epididymal fat deposits were removed under aseptic conditions from adult male Sprague–Dawley rats under halothane anesthesia. The adipocytes were isolated by collagenase digestion according to the Rodbell procedure *[32]* with minor modifications. Fat pads were minced with scissors in buffer, pH 7.4, containing 5 mmol/L D-glucose, 20 g/L bovine serum albumin, 135 mmol/L NaCl, 2.2 mmol/L $CaCl_2$, 1.25 mmol/L $MgSO_4$, 0.45 mmol/L KH_2PO_4, 2.17 mmol/L Na_2HPO_4, and 10 mmol/L HEPES and digested in the same buffer in the presence of 2.5 mg/2 mL buffer per gram of tissue type II collagenase (specific activity, 456 kU/g; Sigma Chemical Co.) at 37 °C with gentle shaking at 60 cycles/min for 45 min. Isolated adipocytes were separated from undigested tissue by filtration through a 400-μm nylon mesh and washed three times. The adipocytes were resuspended in 5 mmol/L glucose culture medium (Life Technologies, Inc.), supplemented with 50 mL/L fetal bovine serum, and then incubated for 30 min at 37 °C before being plated in Matrigel-coated (Becton Dickinson) culture plates (Fisher Scientific). One hundred fifty microliters of the adipocyte suspension (2:1 ratio of packed cells to media) was placed in each well with 2 mL of culture medium supplemented with 50 mL/L fetal bovine serum. The cells were maintained in an incubator at 37 °C in CO_2-enriched (60 mL/L) air. Samples (300 μL) for leptin assay were collected after 24–96 h in culture.

Measurement of plasma/serum leptin. A new RIA was developed at Linco Research that readily detected leptin concentrations in rat plasma or serum. The assay is based on a polyclonal antibody raised against recombinant rat leptin in guinea pigs and on calibrators and ^{125}I-labeled tracer prepared from recombinant rat leptin. Calibrators (0.5, 1, 2, 5, 10, 20, and 50 μg/L) or specimens were pipetted in duplicate into tubes at 100 μL each and mixed with anti-leptin antibody (100 μL). After incubation for 18–24 h at room temperature (disequilibrium assay format), 100 μL of ^{125}I-tracer was added to each tube, and incubation continued for another 18–24 h. Cold precipitating antibody (1.0 mL; anti-guinea pig IgG, raised in goats) was added to all tubes and incubated for 20 min at 4 °C to precipitate the antibody/leptin complex. Centrifugation for 20 min at 2500*g* at 4 °C yielded visible pellets; the supernatants were decanted, and the radioactivity in the pellets was counted. Log values of calibrators were plotted vs the calibrator-bound counts/zero calibrator-bound counts (B/Bo) to generate a curve for calculation of unknowns. Leptin was measured in plasma and culture fluids with an assay specific for mouse leptin (Linco Research), which has been evaluated previously *[33]*.

Statistics. All quantitative results are reported as means ± SD. Statistical significance was assessed in paired and unpaired data by Student's *t*-test; $P < 0.05$ was considered significant. ANCOVA analysis was performed with STATA software (Stata Corp.). Relationships between continuous variables were evaluated by Spearman correlation.

Results

Limit of detection. The lowest measurable concentration of leptin was determined by repeated within-run analysis of

the 0.5 μg/L calibrator and of a buffer solution containing no leptin. The ranges of the concentrations, expressed as means ± 2 SD, determined for the two solutions were 0.37 to 0.77 μg/L and −0.12 to 0.32 μg/L, respectively; because these ranges do not overlap, the assay will differentiate a specimen with a leptin concentration of 0.5 μg/L from 0 in 95% of single measurements.

Analytical recovery. Various amounts of rat leptin were added to a rat serum pool (endogenous leptin, 1.6 μg/L), and the leptin concentration was determined in replicate (n = 4) assays. Recovery ranged from 92% to 103% at added concentrations of 1.6 to 11.6 μg/L (Fig. 1).

Comparison with mouse-specific leptin assay. Leptin concentrations were determined in plasma from 12 adult male Sprague–Dawley rats (weight range, 391–571 g) for comparison with results determined with a homologous assay designed for measurement of mouse leptin (calibrators and tracer made from recombinant mouse leptin, antibody made to mouse leptin; Fig. 2); this assay has been evaluated previously *[33]*. On average, mouse assay results (4.2 ± 2.0 μg/L) were 67% of the rat assay values (6.3 ± 3.4 μg/L), but linear regression analysis of the results (mouse leptin assay = 0.55 rat leptin assay + 0.7 μg/L) showed that the correlation of the two assays was highly significant ($r = 0.94$, $P < 0.001$). Leptin concentrations were also determined in 39 samples of media collected from primary cultures of rat adipocytes, by both assays (Fig. 3). Mouse assay results (1.9 ± 1.2 g/L) were 86% of rat assay values (2.1 ± 1.2 μg/L), and this difference was due entirely to a set bias between the assays (mouse leptin assay = 1.028 rat leptin assay − 0.3 μg/L). As with plasma, the correlation of the two methods was highly significant ($r = 0.97$, $P < 0.0001$).

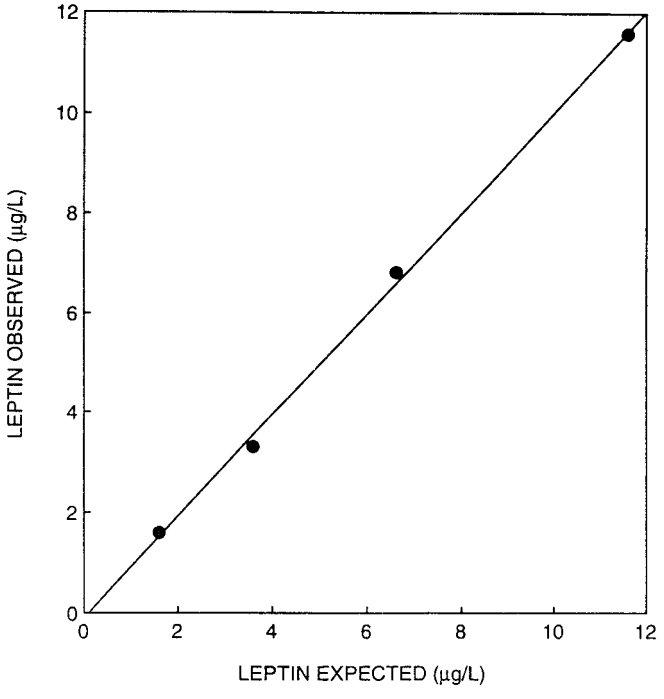

Fig. 1. Recovery of leptin added to a rat serum pool with an endogenous leptin concentration of 1.6 μg/L.

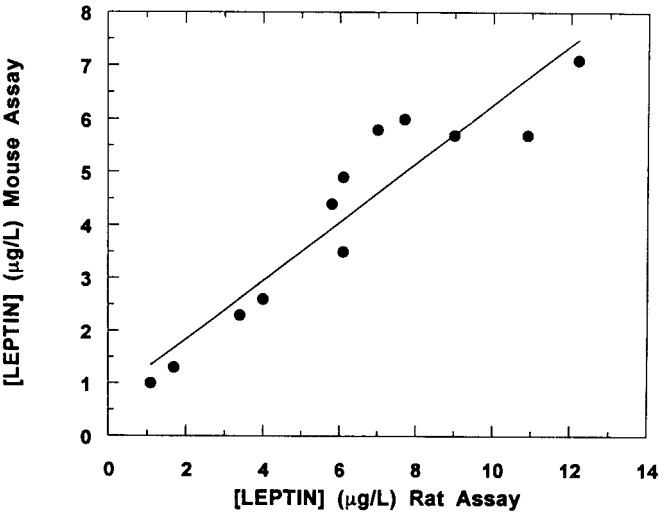

Fig. 2. Leptin concentrations in plasma of 12 male Sprague–Dawley rats measured with the rat leptin RIA, compared with results from a mouse leptin RIA.

Linear dilution. Recovery of rat leptin on dilution was assessed with four pools of Zucker rat plasma with initial concentrations of 15.8, 16.0, 15.1, and 14.6 μg/L. Dilution of these pools with buffer (1.33-, 2-, and 4-fold) resulted in near 100% recovery of leptin concentrations at all dilutions (Table 1).

Precision. Within- and between-assay precision was assessed by repeated analysis of four rat serum pools

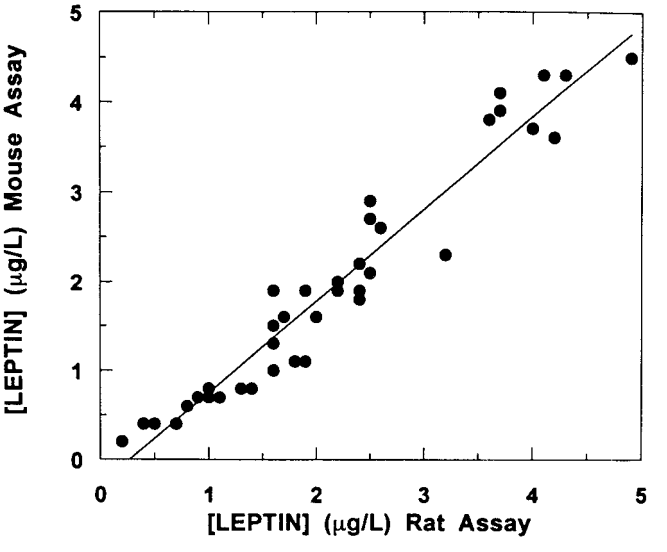

Fig. 3. Leptin concentrations in media from primary cultures of rat adipocytes measured with the rat leptin RIA, compared with results from a mouse leptin RIA.

Table 1. Plasma dilution linearity of rat leptin RIA.

Sample volume[a], μL	Measured/expected, %[b]
100	100[c]
75	103 ± 5
50	101 ± 5
25	96 ± 7

[a] Sample volume made up to 100 μL by the addition of buffer.
[b] Mean of results for four plasma specimens, after conversion to a percentage of starting concentration. All dilutions were prepared in duplicate.
[c] Starting concentrations were defined as 100%.

containing 1.6–11.6 μg/L leptin. CVs ranged from 2.4% to 4.6% within runs and from 4.8% to 5.7% between runs (Table 2).

Stability. Stability of leptin in plasma was assessed in two pools, one of which had been supplemented with recombinant rat leptin to raise the leptin concentration to ~6 μg/L. Leptin in both plasma pools was very stable for up to 7 days at 4 °C or at room temperature, but the pool that had been supplemented with recombinant leptin lost marked amounts of leptin after 3 days at 37 °C (Table 3). The other pool, with a lower concentration of entirely endogenous leptin, was stable at 37 °C for 3 days. Five freeze–thaw cycles had little effect on plasma leptin concentrations (starting concentrations, 2.4 and 5.8 μg/L; ending concentrations, 2.3 and 5.7 μg/L, respectively). Two months of storage of six specimens, at concentrations from 1.9 to 58.1 μg/L, at −20 °C was without effect on measured leptin concentrations; the stored specimens had 105% ± 9% of the initial leptin concentrations.

Correlation of plasma leptin with percentage of body fat. Leptin concentrations were measured in the plasma of 44 male and 35 female Sprague–Dawley rats, ages 24 days to 3.5 months; after death, the body fat content of the rats was determined by whole-body chemical analysis. There was a significant correlation of plasma leptin values with percentage of body fat for the combined population ($r = 0.636$, $P < 0.001$), and correlation was improved by separating the results according to gender ($r = 0.796$, $P < 0.001$, and $r = 0.710$, $P < 0.001$, for males and females, respectively; Fig. 4). Interestingly, males generally had higher leptin concentrations in relation to body fat content than females, with the distinction much more apparent at a percentage of body fats >7% (Fig. 4). Leptin plasma concentrations increased 0.60 μg/L for each percentage of increase in body fat in males but only 0.22 μg/L for each percentage of increase in body fat in females. By ANCOVA the relationships of leptin concentration to percentage of body fat were significantly different in males vs females ($P < 0.0001$).

Table 2. Variation within- and between-assays.

		CV, %	
Sample	Mean, μg/L[a]	Within-Run	Between-Run
1	1.6	2.4	4.8
2	3.3	4.1	3.0
3	6.8	2.0	5.7
4	11.6	4.6	5.7

[a] From 10 replicates of each serum sample in four separate assays.

Table 3. Effect of storage at various temperatures on rat plasma leptin.

	Leptin concentration (μg/L)[a]					
Temp	Day 0	Day 1	Day 2	Day 3	Day 5	Day 7
4 °C	2.4	2.3	2.4	2.0	2.3	2.1
	5.8	5.9	6.1	6.3	6.2	6.2
Ambient	2.4	2.1	2.3	2.3	2.5	2.2
	5.8	6.1	6.2	6.3	5.7	5.4
37 °C	1.0	0.9	1.0	1.1		
	5.8	5.7	4.5	2.7		

[a] Each result is the mean of leptin determinations in duplicate aliquots.

Plasma leptin and strain of rats. Typical plasma leptin concentrations varied widely in different strains of rats at age 2 months (Fig. 5). Obese Zucker rats had the highest plasma leptin concentrations, and Sprague–Dawley rats had the lowest. Where comparative data were available, female rats tended to have lower leptin concentrations than males, despite the fact that the females in this comparison were older (males, 2 months; females, 3 months).

Discussion

This new RIA provides accurate and precise analysis of rat serum/plasma leptin. The working range of the assay, defined by the detection limit of 0.5 μg/L and the concentration of the highest calibrator (50 μg/L), effectively covers the range of plasma leptin concentrations seen in both lean, young animals and those in adult rats of strains studied as models of genetic obesity. Leptin con-

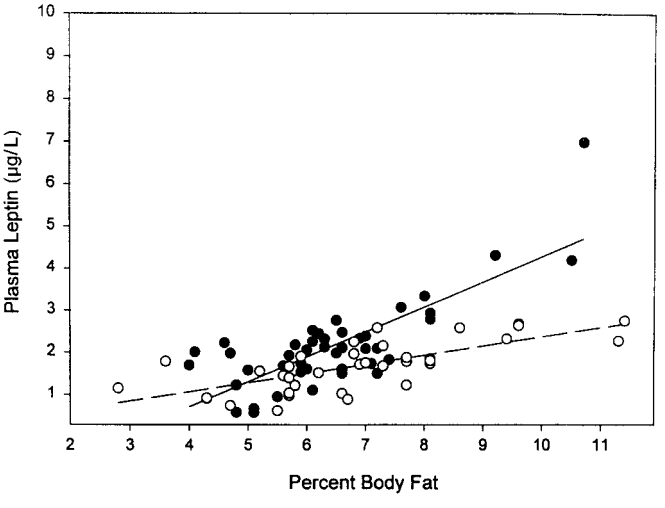

Fig. 4. Correlation of plasma leptin concentrations with percentage of body fat in 44 male (●) and 35 female (○) Sprague–Dawley rats.

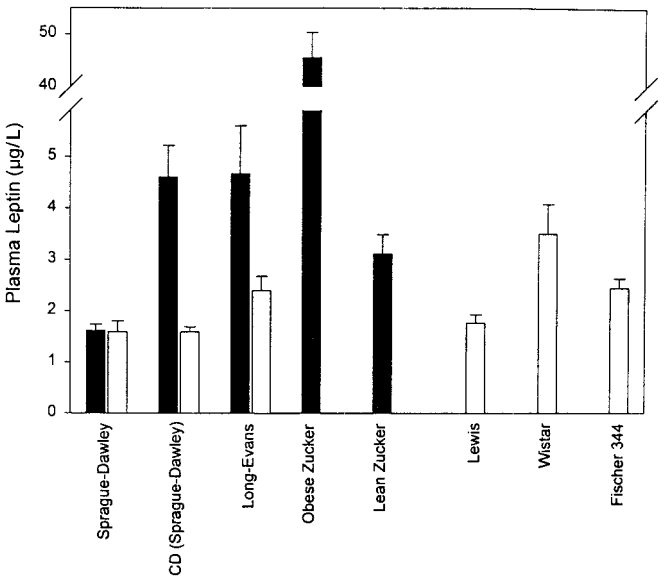

Fig. 5. Plasma leptin concentrations in various strains of rats.

centrations measured with the new procedure increased systematically and directly with percent body fat; because leptin production and plasma concentrations are known to increase with increases in adiposity in humans and mice *[6–9, 33]*, it is important that the assay accurately reflects the same relationship in rats.

Plasma leptin concentrations had been measured previously in only humans and mice, and measurements in mice sometimes used methods that generated semiquantitative results, such as immunostaining of Western blots *[6–8, 33–36]*. Nevertheless, the available data suggest that plasma leptin concentrations are very similar in humans and rats after the degree of adiposity of the subjects is taken into account. Reported plasma concentrations in lean humans (<15% body fat in men and <25% in women) *[37]* were 1–16 μg/L *[6–9]*. Plasma concentrations in the rats in this study were lower (0.5–6 μg/L) but appropriate for their low percentage of body fat compared with humans and were similar to concentrations in humans with a low percentage of body fat due to anorexia nervosa *[18]* or the wasting syndrome of HIV infection *[19]*. Plasma leptin concentrations in mice are 2–11 μg/L, as determined by RIA *[33, 34]*.

As in humans, male and female rats have different relationships of plasma leptin to percentage of body fat; but in contrast to humans, plasma leptin concentrations in females increase at a lower rate with increasing adiposity than in males. Several studies have shown that leptin concentrations rise 0.4–3 times as fast in women as in men, as the percentage of body fat increases *[38–41]*. The basis of the higher plasma leptin concentrations in women is not known, but evidence suggests that the dimorphism may be associated with puberty, because leptin concentrations in girls but not boys rise during puberty *[42]*. However, leptin concentrations do not decrease with menopause, which suggests that reproductive hormone status is not responsible for the sexual dimorphism *[38]*. Why rats and humans differ with respect to the effect of sexual dimorphism is not known, but the two species differ dramatically with respect to the sexual dimorphism of body fat content. Whereas human females have higher body fat content than males, the opposite is true for rats *[43]*. The dichotomy in the sexual dimorphism of leptin in rats compared with humans may be related to differences in the distribution and extent of fat stores.

Leptin circulates in both rodents and humans in free and protein-bound forms *[22, 23]*. The binding proteins have not been fully characterized, but they are heterogeneous, and a portion of the binding may involve disulfide linkages *[22]*. The presence of multiple forms of leptin in plasma raises the possibility that an RIA may variably measure the forms present. The human leptin RIA appears to effectively measure all forms present in human plasma, based on comparison of RIA results with quantitation on Western blots, which likely detects all forms because of denaturation of all noncovalent binding and reduction of disulfide bonds *[8]*. Comparable experiments to examine the efficacy of the rat leptin RIA to measure all circulating forms are not possible, because the very low concentrations of leptin in rat plasma defeat the sensitivity of the Western blot method. Characterization of the ability of the rat RIA to quantitate the various forms will depend on the development of methods for the physical separation of rat plasma leptin forms.

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