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Early reversible induction of leukotriene synthesis in chicken myelomonocytic cells transformed by a temperature-sensitive mutant of avian leukemia virus E26

(*myb* oncogene/macrophages)

ANDREAS J. R. HABENICHT*†, MATTHIAS GOERIG*, DIETRICH E. R. ROTHE*, ELISABETH SPECHT*, REINHARD ZIEGLER*, JOHN A. GLOMSET‡, AND THOMAS GRAF§

*University of Heidelberg, 69-Heidelberg, Bergheimerstrasse 58, Federal Republic of Germany; ‡Howard Hughes Medical Institute SL-15, Regional Primate Research Center SJ-50, University of Washington, Seattle, WA 98195; and §European Molecular Biology Laboratory, 69-Heidelberg, Meyerhofstrasse 1, Federal Republic of Germany

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ABSTRACT **We used chicken myelomonocytic cells transformed by a temperature-sensitive mutant of the *myb/ets* oncogene-containing avian leukemia virus E26 to study the regulation of leukotriene (LT) synthesis during macrophage differentiation. Cells exposed to arachidonic acid and the Ca^{2+} ionophore 23187 produced up to 180 times more LTs at the nonpermissive temperature (42°C) than at the permissive temperature (37°C). Induction of LT synthesis was detectable within 2 hr after temperature shift, whereas conventional macrophage markers became evident after 2–3 days. *N*-Formylmethionylleucylphenylalanine, opsonized zymosan, and complement factor C5a induced LT synthesis in temperature-sensitive mutant-transformed cells only when the cells were maintained at 42°C, and this effect was blocked by pertussis toxin. When cells were kept at 42°C for 48 hr and then shifted back to 37°C to induce retrodifferentiation, LT synthesis rates declined within 8 hr and reached near control values within 36 hr. Retrodifferentiation also led to decreased LT synthesis in response to *N*-formylmethionylleucylphenylalanine, opsonized zymosan, and C5a. These results indicate that activation of the 5-lipoxygenase pathway is a very early event in the macrophage differentiation pathway that is directly or indirectly controlled by the temperature-sensitive v-myb protein.**

Leukocytes have been shown to synthesize leukotrienes (LTs) (1) in response either to unspecific stimuli, such as Ca^{2+} ionophores, or to specific agonists, such as the chemotactic peptide *N*-formylmethionylleucylphenylalanine (fMLP), opsonized zymosan, and complement factor C5a (2–5). The current view of the regulation of LT synthesis holds that these agonists stimulate the release of arachidonic acid (Δ_4Ach) from endogenous phospholipid stores and also activate the 5-lipoxygenase that initiates the conversion of Δ_4Ach into LTs (2, 5). Whereas the mechanisms of Δ_4Ach release have received considerable attention, very little is known about the mechanisms that regulate other components of the LT response system including the 5-lipoxygenase and the other enzymes of LT synthesis. One way to identify these mechanisms is to study the up-regulation of LT synthesis during leukocyte differentiation (6–8).

A particularly promising approach for the study of otherwise scarce hematopoietic precursors is the use of avian leukemia retroviruses to produce homogeneous populations of transformed bone marrow cells (9). Thus, infection of chicken bone marrow cells with the *myb/ets* oncogene-containing E26 virus yields populations of transformed macrophage precursors (10). Moreover, mutants of E26 virus have been isolated that are temperature sensitive (ts) for transformation of myelomonocytic cells and that have a lesion in their *myb* oncogene (10, 11). When cells transformed by such mutants are shifted from the permissive temperature (37°C) to the nonpermissive temperature (42°C), they differentiate into resting cells that express functional characteristics of macrophages, including adherence to the culture dish and phagocytosis of bacteria. They undergo a change in the pattern of cell surface antigen expression consisting of disappearance of myeloblast-specific antigens and appearance of macrophage-specific antigens (10). Importantly, time-lapse photography has demonstrated that the differentiation of these cells into macrophages is a reversible process (12). Thus, ts-mutant-transformed cells provide a reversible macrophage differentiation system that circumvents many of the problems encountered with other differentiation systems.

In the present study we used ts-mutant-transformed cells to investigate the relation between LT synthesis and macrophage differentiation. To compare the results obtained with ts-mutant-transformed cells with those obtained with differentiated macrophages, parallel experiments were performed with freshly isolated chicken bone marrow macrophages and with a macrophage cell line (HD-11) transformed by the v-*myc* oncogene-containing MC29 virus (10, 13).

MATERIALS AND METHODS

Materials. Culture media were obtained from GIBCO; antibodies against leukotrienes C_4 and B_4 (LTC_4 and LTB_4, respectively) were from NEN and Amersham; fMLP was from Calbiochem. All other reagents were from Sigma Chemie, Munich.

Cell Culture. The source of the ts21 E26 and wild-type (wt) E26 virus transformed cells and the culture conditions has been described (10). Cells were seeded at $0.5–1.0 \times 10^6$ cells per ml and maintained at densities that did not exceed 2×10^6 cells per ml. Normal chicken bone marrow macrophages and the HD-11 cell line transformed with the v-*myc* oncogene-containing MC29 virus were cultured as described (13).

Standard Incubation Conditions. Incubation conditions were precisely as described by Borgeat and Samuelsson (14). From 10^6 to 10^7 cells per ml were taken up in Ringer's solution

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Abbreviations: Δ_4Ach, arachidonic acid; fMLP, *N*-formylmethionylleucylphenylalanine; LT, leukotriene; LTB_4 and LTC_4, leukotrienes B_4 and C_4, respectively; PT, *Bordetella pertussis* toxin; ts, temperature sensitive; wt, wild type.

†To whom reprint requests should be addressed.

(14) containing 10 mM L-cysteine and incubated for 15 min with 50 μM Δ_4Ach in 5 μl of ethanol per ml (carrier) and 20 μM ionophore 23187 in 5 μl of ethanol per ml. In other experiments fMLP in 10 μl of dimethyl sulfoxide, zymosan at 2 mg/ml in 10 μl of isotonic phosphate-buffered saline (PBS), or highly purified porcine desarginine-complement factor C5a (15, 16) in 100 μl of PBS was added. The reaction was started by addition of ionophore or agonists and stopped after 10 min with ethanol.

Reversed-Phase HPLC. LTs were extracted (17) and chromatography was performed as described (18) on μ-Bondapak TM C_{18} columns (Waters; 3.9 × 300 mm; 5-μm spheres) using the solvent system methanol/H_2O/acetic acid, pH 5.7, [65/35/0.02 (vol/vol)] at a flow rate of 1 ml/min. Retention time was 23 min for LTC_4 and 35 min for LTB_4. Overall recovery for LTB_4 was 35–51% and for LTC_4 was 38–50%. Determination of the concentration of LTs was performed by radioimmunoassay as described (19). Results are presented as data from two dishes (means ± SD).

RESULTS

Ca^{2+} Ionophore and Δ_4Ach Synergistically Induce LT Synthesis in ts-Mutant-Transformed Cells Maintained at 42°C But Not at 37°C. To determine the optimal conditions of LT synthesis, ts-mutant-transformed cells were maintained for 48 hr at 42°C to induce their differentiation into macrophages (10). We then measured the synthesis rates of LTB_4 and LTC_4, with respect to the concentration of Δ_4Ach, the concentration of ionophore 23187, and the incubation time in the assay. Maximal rates of LT synthesis occurred in the presence of 20–50 μM Δ_4Ach and 10–20 μM ionophore (Fig. 1 *A* and *B* and data not shown). Elevated synthesis of LTs could be detected within 2 min, and maximal rates were observed after 10 min, which declined thereafter for LTB_4 or remained constant for up to 60 min for LTC_4 (Fig. 1 *C* and *D*). Ionophore or Δ_4Ach alone also induced synthesis of small amounts of LTs (Fig. 1). When the ionophore was added with Δ_4Ach, synergistic and maximal LT synthesis was observed (Fig. 1 *A* and *B*). These incubation conditions were identical with those used by Borgeat and Samuelsson (14) and were used in all further experiments in which receptor-independent LT synthesis was to be measured.

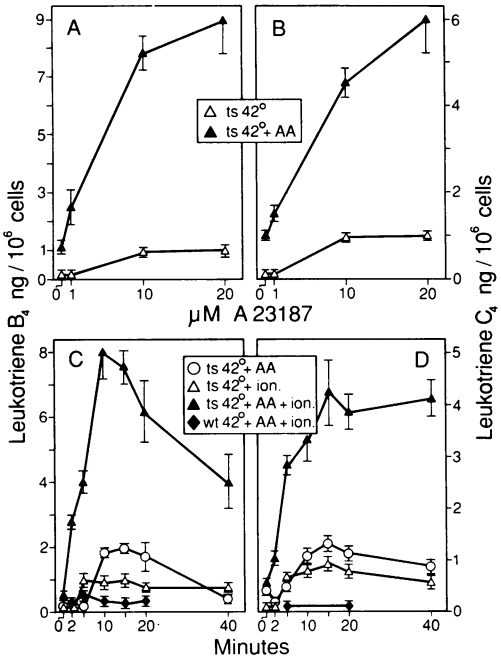

FIG. 1. LT synthesis in ts-mutant-transformed cells in response to Ca^{2+} ionophore and Δ_4Ach (AA). Ts-mutant- or wt-virus-transformed cells were maintained at 42°C for 48 hr. (*A* and *B*) Parallel cultures were incubated with or without 50 μM Δ_4Ach and Ca^{2+} ionophore. (*A*) LTB_4. (*B*) LTC_4. (*C* and *D*) Other cultures were incubated under standard conditions as indicated. (*C*) LTB_4. (*D*) LTC_4.

Table 1. Ca^{2+} ionophore, fMLP, and opsonized zymosan induce LTB_4 synthesis in chicken bone marrow macrophages and HD-11 macrophages

Additions	LTB_4, ng per 10^6 cells	
	Bone marrow macrophages	HD-11 macrophages
Ionophore 23187 + Δ_4Ach	19.3 ± 2.1	26.9 ± 2.2
fMLP + Δ_4Ach	18.1 ± 1.5	13.2 ± 1.0
Zymosan + Δ_4Ach	15.7 ± 1.8	9.8 ± 0.1

Parallel cultures were incubated under standard conditions or with 10 nM fMLP or opsonized zymosan at 2 mg/ml for 10 min in the presence of 50 μM Δ_4Ach.

As shown in Table 1 normal chicken bone marrow macrophages and HD-11 macrophages produced amounts of LTs that were similar to those produced by differentiating ts-mutant-transformed cells. In contrast, ts-mutant-transformed cells kept at 37°C or cells that had been transformed with wt E26 virus and maintained at 37°C or 42°C did not synthesize significant amounts of LTs (Fig. 1 *C* and *D*, Fig. 2, and Table 2). The production of LTs required the continuous synthesis of RNA and protein since both actinomycin D and cycloheximide inhibited the appearance of the 5-lipoxygenase pathway (Table 2).

Up-Regulation of the 5-Lipoxygenase Pathway Is a Very Early Event During Macrophage Differentiation. The time course of appearance of the 5-lipoxygenase pathway during macrophage differentiation in ts-mutant-transformed cells is shown in Fig. 2. Maintenance of the cells at 42°C for as short a time as 2 hr led to a significant increase in the rates of synthesis of both LTs, and synthesis rates increased sharply thereafter. In contrast, when ts-mutant-transformed cells were maintained at 37°C or wt-virus transformed cells were maintained at 42°C for up to 72 hr, no significant LT synthesis was observed (Fig. 2 and Table 2). Up-regulation of LT synthesis was the earliest event so far observed in their macrophage differentiation pathway because adherence of the cells to the culture dish did not occur before 48 hr (10). Moreover, appearance of macrophage functions, such as phagocytosis, and macrophage-specific surface antigens did not occur before 72 hr after temperature upshift (10).

fMLP, Opsonized Zymosan, and C5a Stimulate LT Synthesis in ts-Mutant-Transformed Cells at 42°C. To test the ability of several agonists to induce LT synthesis, we induced

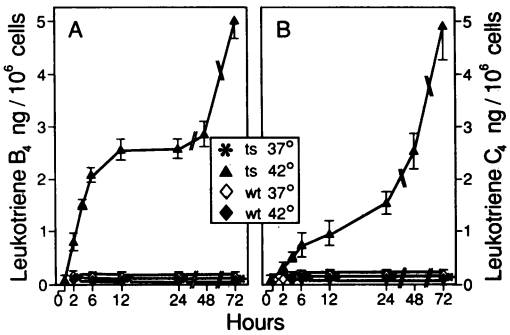

FIG. 2. Kinetics of induction of 5-lipoxygenase in ts-mutant- or wt-virus-transformed cells. Ts-mutant- or wt-virus-transformed cells were maintained at 37°C or shifted to 42°C. At various times thereafter cultures were incubated under standard conditions. (*A*) LTB_4. (*B*) LTC_4.

Table 2. Actinomycin D and cycloheximide disrupt up-regulation of the 5-lipoxygenase in ts-mutant-transformed cells

Condition(s) and addition(s)	LTB_4, ng per 10^6 cells	LTC_4, ng per 10^6 cells
ts E26 at 37°C	0.6 ± 0.4	0.5 ± 0.3
ts E26 at 42°C	10.3 ± 1.8	6.3 ± 0.8
ts E26 at 42°C + actin.	0.5 ± 0.2	0.8 ± 0.2
ts E26 at 42°C + cyclo.	0.7 ± 0.2	1.0 ± 0.2

Ts-mutant-transformed cells were maintained at 37°C or 42°C for 40 hr. Parallel cultures were incubated with actinomycin D at 5 μg/ml or cycloheximide at 10 μg/ml. Then 8 hr later, cells were incubated under standard conditions. actin., Actinomycin D; cyclo., cycloheximide.

differentiation of ts E26 cells by shifting them to 42°C for 48 hr and then measured the rates of synthesis of LT in response to agonists. In the absence of exogenous Δ_4Ach, fMLP and C5a stimulated LT synthesis in a concentration-dependent manner in response to fMLP and C5a (Fig. 3). Opsonized zymosan at 2 mg/ml also stimulated LT synthesis (see below). The effects of all three agonists on LT synthesis resembled that of the Ca^{2+} ionophore: each agonist acted synergistically with Δ_4Ach to induce synthesis of both LTs (Fig. 3, see below, and data not shown). The kinetics of fMLP-induced LT synthesis after shift of ts cells at 42°C closely resembled the kinetics of receptor-independent LT synthesis (Fig. 4). As shown in Table 3 pertussis toxin (PT) blocked the effects of fMLP and opsonized zymosan on LT synthesis but not that of the Ca^{2+} ionophore (this experiment was not performed with C5a).

Induction of the 5-Lipoxygenase Pathway and Receptor-Dependent LT Synthesis Are Reversed in ts-Mutant-Transformed Cells After the Shift from 42°C to 37°C. Studies performed in the laboratory of one of us (T.G.) have shown that differentiation of ts-mutant-transformed cells into macrophages can be reversed by shifting the temperature back to 37°C (12). Cells that have differentiated for several days follow a retrodifferentiation pathway that leads to a decrease of macrophage-specific cell surface antigens and phagocytic activity, cell rounding, and induction of cell proliferation (12). In view of these results, we sought to determine whether up-regulation of the 5-lipoxygenase pathway could be reversed by shifting the temperature from 42°C to 37°C. For this purpose, we first shifted the cells for 48 hr to 42°C, then shifted them back to 37°C for various periods of time and determined the rate of receptor-independent LT synthesis. As shown in Fig. 5, backshift of the temperature resulted in a significant decrease of the synthesis rates of LTs within 4–8 hr and to near control levels within 16–48 hr.

To determine whether receptor-dependent LT synthesis was also down-regulated during retrodifferentiation, we allowed the cells to differentiate for 48 hr, then either maintained them for another 48 hr at 42°C or shifted them back to 37°C. At various times during this temperature program, we tested the ability of the cells to produce LTs in response to fMLP, opsonized zymosan, and C5a. As is evident from Fig. 6, retrodifferentiation of mutant-transformed cells resulted in reduced LT synthesis rates in response to these agonists.

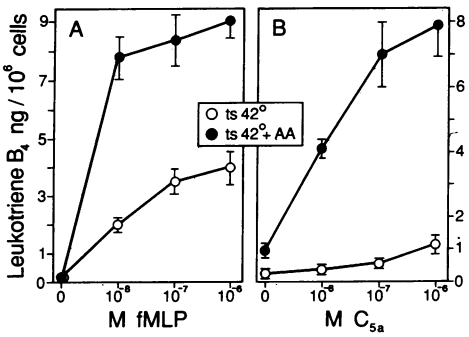

FIG. 3. fMLP and C5a-dependent LTB_4 synthesis in ts-mutant-transformed cells in the presence and absence of Δ_4Ach (AA). Ts-mutant-transformed cells were shifted to 42°C for 48 hr. Cultures were incubated with various concentrations of fMLP (*A*) or C5a (*B*) with or without 50 μM Δ_4Ach for 10 min, and synthesis rates of LTB_4 were determined.

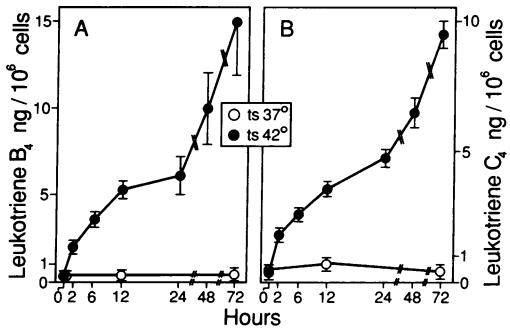

FIG. 4. Kinetics of fMLP-dependent LT synthesis in ts-mutant-transformed cells. Ts-mutant-transformed cells were shifted to 42°C and LT synthesis rates were determined at various times thereafter in response to 50 μM Δ_4Ach and 10 nM fMLP. (*A*) LTB_4. (*B*) LTC_4.

DISCUSSION

The results of the present investigation demonstrate that macrophage differentiation of ts-mutant-transformed chicken myelomonocytic cells is associated with early up-regulation of the 5-lipoxygenase pathway, that this up-regulation is associated with increased receptor-dependent LT production, and that retrodifferentiation is associated with early down-regulation of the 5-lipoxygenase pathway and also with down-regulation of receptor-dependent LT synthesis.

Several lines of evidence suggest that the results obtained in ts-mutant-transformed myeloid cells can be used as a model for leukocyte development. Undifferentiated normal myeloid precursor cells express high levels of the cellular homologue of the viral *myb* gene (c-*myb*) whereas differentiated macrophages do not (20, 21). Normal bone marrow macrophages and HD-11 macrophages produce amounts of LTs that are similar to those produced by differentiating ts-mutant-transformed cells (2, 4). Dixon *et al.* (22) reported that HL-60 cells that were induced to differentiate into neutrophils contained significant amounts of 5-lipoxygenase mRNA whereas undifferentiated control cells did not. Furthermore, we found that HL-60 cells induced to differentiate into macrophages by phorbol esters also up-regulate the 5-lipoxygenase pathway (23).

The observations that the Ca^{2+} ionophore, fMLP, opsonized zymosan, and C5a act synergistically with Δ_4Ach to induce LT production in ts-mutant-transformed cells and that

Table 3. PT prevents fMLP and zymosan-dependent but not Ca^{2+} ionophore-dependent LTB_4 synthesis

Addition(s)	LTB_4, ng per 10^6 cells
Δ_4Ach	1.3 ± 0.1
Ionophore + Δ_4Ach	12.9 ± 0.9
Ionophore + Δ_4Ach + PT	12.2 ± 0.7
fMLP + Δ_4Ach	11.1 ± 0.1
fMLP + Δ_4Ach + PT	4.1 ± 0.3
Zymosan + Δ_4Ach	9.8 ± 0.1
Zymosan + Δ_4Ach + PT	3.5 ± 0.3

HD-11 macrophages were incubated with PT at 500 ng/ml for 60 min, then incubated under standard conditions or with opsonized zymosan at 2 mg/ml or 10 nM fMLP in the presence of 50 μM Δ_4Ach.

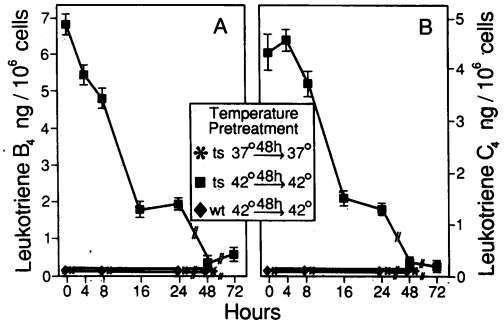

FIG. 5. Kinetics of inactivation of the 5-lipoxygenase pathway in ts-mutant-transformed cells. Ts-mutant-transformed cells were maintained at 37°C or 42°C and control wt-transformed cells were shifted to 42°C for 48 hr (temperature pretreatment program). At 48 hr all cultures were shifted to 37°C (time 0). At various times thereafter the cultures were incubated under standard conditions. (*A*) LTB_4. (*B*) LTC_4.

the effects of the agonists are inhibited by PT whereas the effect of Ca^{2+} ionophore is not indicate that these agonists activate the 5-lipoxygenase through receptors that are coupled to guanine nucleotide-binding proteins. These results are consistent with and extend previous findings in blood neutrophils by Borgeat and Samuelsson (14) and in peritoneal mouse macrophages by Tripp *et al.* (24) who showed that the Ca^{2+} ionophore acted synergistically with Δ_4Ach to produce LTs. Moreover, Clancy *et al.* (16) reported that C5a and fMLP stimulated formation of LTB_4 from exogenous Δ_4Ach in blood neutrophils. In contrast to other lipoxygenases, 5-lipoxygenase requires Ca^{2+} for maximal activity (2, 5). Our observation that LT synthesis is greatly stimulated by the Ca^{2+} ionophore is consistent with the interpretation that the synergistic activity with Δ_4Ach of fMLP, zymosan, and C5a depends on a Ca^{2+}-dependent activation of the 5-lipoxygenase.

The present study raises an important question: What are the primary events that lead to up-regulation and down-regulation of the 5-lipoxygenase pathway in differentiating and dedifferentiating macrophages? Macrophage differentiation in ts-mutant-transformed cells is triggered by the inactivation of the v-*myb* oncogene product, a nuclear protein with DNA binding activity (11). Sequence analysis of ts21, the E26 mutant clone used in this study, revealed that it has a lesion in the *myb* gene (L. Frykberg, B. Vennström, H. Beug, and T.G., unpublished results). The capacity of the myb protein to block macrophage differentiation and even to reverse the differentiated macrophage phenotype of both v-*myc*-transformed and

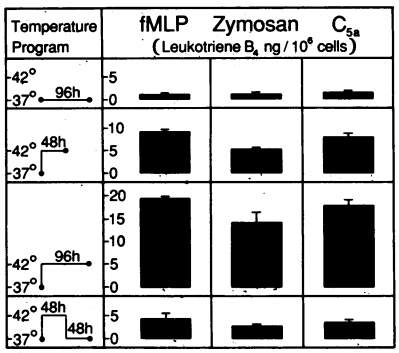

FIG. 6. Down-regulation of receptor-dependent LTB_4 synthesis in ts-mutant-transformed cells. Ts-mutant-transformed cells were shifted to 42°C for 48 hr. Then cultures were either maintained at 42°C for another 48 hr or shifted back to 37°C. At various times during this temperature program LTB_4 synthesis was determined in response to 10 nM fMLP, or opsonized zymosan at 2 mg/ml, or 0.1 μM C5a in the presence of 50 μM AA.

normal bone marrow macrophages was demonstrated by using ts mutants of E26 virus in doubly infected cells (12, 25). To determine which are the primary events that lead to up-regulation of the 5-lipoxygenase pathway it will be necessary to identify the gene(s) that are regulated by the v-myb protein in ts-mutant-transformed cells and, by analogy, is regulated by the c-myb protein in normal blood cell development. The finding that both the induction and the inhibition of the 5-lipoxygenase pathway are the earliest events so far described in the temperature-induced differentiation of mutant-transformed cells raises the possibility that genes that encode enzymes involved in LT synthesis are among the targets of the *myb* gene product. Alternatively, inactivation of the v-myb protein affects genes that induce the differentiation of the cells into macrophages and indirectly up-regulate the activity of 5-lipoxygenase. That both transcriptional and translational activities are required for up-regulation of the 5-lipoxygenase pathway is indicated by the ability of both actinomycin D and cycloheximide to disrupt this up-regulation. Further studies on the molecular biology of ts-mutant-transformed cells and the availability of cDNA probes for the 5-lipoxygenase gene (22, 26) should allow us to answer these questions.

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