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# Requirement for Matrix Metalloproteinase-9 (Gelatinase B) Expression in Metastasis by Murine Prostate Carcinoma

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**Although a number of effective therapies are available for localized prostate cancer, metastatic prostate cancer is difficult to treat and impossible to cure. Identification of the gene products that enable a prostatic carcinoma cell to metastasize should facilitate an understanding of the processes leading to metastasis. To characterize the contribution of matrix metalloproteinase-9 (MMP-9, gelatinase B or the 92-kd type IV gelatinase/collagenase) to the development of metastasis in prostate cancer, we reduced MMP-9 expression in metastatic murine prostatic carcinoma cells using a ribozyme. The ribozyme transfected cells had lower basal levels of MMP-9 as well as decreased levels after stimulation by transforming growth factor-β or phorbol 12-myristate 13-acetate when compared with the parental cells or with control transfectants. The cells with down-regulated MMP-9 were unable to form lung colonies in the experimental metastasis assay, whereas the controls and parental cells readily formed metastases. All cell types readily formed tumors after injection and down-regulation of MMP-9 did not adversely affect the rate of tumor growth. Thus, MMP-9 expression is required for hematogenous metastasis in a murine prostate model system raising the possibility that it may play an equivalent role in human prostate cancer.** ***(Am J Pathol 1998, 152:591–596)***

In individual patients it is currently not possible to predict whether metastasis has occurred from the size or other clinical parameters of the primary tumor. For patients with prostatic carcinoma, the determination of whether metastases are present has considerable clinical significance as prostate cancer can often be effectively treated if the tumor has remained confined to the gland, but for patients with metastatic disease, long term survival is rare. Thus, an understanding of the gene products that are required for metastasis in prostate cancer could be valuable both for delineation of the alterations that must occur in the cell for metastasis to occur and to lead to the identification of markers that might predict metastasis.

To determine some of the factors that are required for metastasis by prostate carcinoma cells, we turned to the mouse prostate reconstitution system developed by Thompson et al[1] in which neonatal urogenital sinus tissue, the fetal precursor of the adult prostate, was explanted, infected with a recombinant retrovirus that expresses the oncogenes *ras*$^{H}$ and *myc*, and reimplanted into the renal capsule. This procedure resulted in the formation of tumors that histologically were virtually identical to human prostatic carcinomas and that displayed characteristic murine prostate markers. These tumors were generally nonmetastatic, but when male p53 −/− or p53 +/− mice were used as the source of urogenital sinus tissue, the resultant tumors frequently gave rise to metastases in the lung, liver, bone, and mesentery.[2] Sehgal et al,[3] using cells isolated from both primary tumors and metastases generated in this way, found that the 105-kd gelatinase activity characteristic of murine matrix metalloproteinase-9 (MMP-9) or murine gelatinase B could be induced by transforming growth factor-β (TGF-β) in five of the six lines isolated from metastatic tumors but in only one of six cell cultures isolated from the primary tumors. These data correlating MMP-9 production to prostate cancer metastasis suggested that MMP-9 expression might be important for metastasis in this system and were consistent with observations linking MMP-9 to metastasis in other systems.

MMP-9, gelatinase B, or the 92-kd type IV gelatinase/collagenase is one member of a family of matrix metalloproteinases (MMPs), an enzyme family defined by a conserved catalytic domain that requires $Zn^{2+}$ for activity.[4–6] MMP-9 is capable of degrading a variety of substrates including molecules found in the extracellular matrix such as collagens IV, V, elastin, entactin, as well as casein, the precursor of tumor necrosis factor-α, and a cell surface

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molecule, galectin.[7–9] MMP-9 is secreted in an inactive form of molecular mass of 92 kd in humans and rats but of 105 kd in mice because of a 16-amino acid insert.[10,11] In addition to the widely recognized secreted form of MMP-9, Toth et al[12] have also identified a cell surface-associated form. Successive cleavages of the N-terminal region, which has inhibitory activity against the enzyme itself, results in the active 82-kd form and sometimes an active 65-kd form of the human enzyme.[13] *In vitro* treatment of pro-MMP-9 with either stromelysin, MMP-2, tissue kallikerien, matrilysin, type I collagenase,[14–19] or plasminogen activator generates the active form, but the physiological activators are unknown. The tissue inhibitors of matrix metalloproteinases, TIMPs-1, -2, -3, or -4 can inhibit MMP-9 activity, but TIMP-1 has a higher affinity for MMP-9 than does TIMP-2, and MMP-9 is frequently isolated in a complex with TIMP-1.[20–31]

The importance of MMP-9 in metastasis was underscored by results in a rat sarcoma model system. Bernhard et al[32] noted that rat embryo fibroblasts transformed by $ras^H$ plus *myc* were metastatic and secreted MMP-9, but that the same cells transformed by *ras* plus adenovirus E1A were nonmetastatic and did not secrete MMP-9. Both cell types were equally tumorigenic. Introduction of E1A expression vectors into metastatic transformed rat embryo fibroblasts inhibited both metastasis and MMP-9 expression. E1A will also block metastasis by murine mammary cells.[33] To establish the role of MMP-9 in this system, Bernhard et al[34] introduced an expression vector for MMP-9 into the nonmetastatic $ras^H$ plus E1A transformants. This led to metastasis in the experimental metastasis assay. In complementary work, Hua and Muschel[35] showed that inhibition of MMP-9 expression using an expression vector for a ribozyme against MMP-9 in the fibroblasts that were transformed by $ras^H$ plus *myc* blocked experimental metastasis but did not measurably alter tumorigenicity. These data established the importance of MMP-9 in metastasis in a rat sarcoma model system as well as its independence from tumorigenicity and led us to ask whether inhibition of MMP-9 expression would affect metastasis by the cell lines isolated from the metastatic tumors generated in the mouse prostate reconstitution system.

## Materials and Methods

### Plasmids

MMP-9 ribozyme and the control hammerhead expression vectors were constructed by subcloning DNA fragments coding for the ribozyme directed against MMP-9 or for a hammerhead structure into the expression vector pRC/CMV (Invitrogen, San Diego, CA) as described in Hua and Muschel.[35]

### Cell Culture

Murine prostate carcinoma cells, the metastatic (148–1,LMD) and primary cells (148–1,PA), were generated as described in Thompson et al.[2] Cell cultures were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin, and streptomycin. Metastatic (148–1,LMD) cells were transfected with 30 $\mu$g of pRC/CMV, pRC/CMV with the MMP-9 ribozyme, or the control hammerhead construct by the calcium phosphate precipitation method as described previously (Muschel et al, 1985).[36] The parental cells already contain one copy of the neomycin resistance gene because of the homologous recombination to eliminate p53. The parental cells retained neomycin resistance at 600 to 700 $\mu$g/ml. Introduction of the ribozyme-expressing plasmids was accomplished by selection after transfection for greatly enhanced resistance to geneticin. Selection was with geneticin at 1.4 mg/ml. Individual colonies were isolated using cloning cylinders. Clones AR 1.51 and AR 1.52 were isolated after transfection of 148–1,LMD with the expression vector for the MMP-9 ribozyme, and subclones AAS 1.22 and AAS 1.74 were isolated after transfection of the same cells with the expression vector for the hammerhead control ribozyme.

### Zymography

Gelatin substrate gel electrophoresis was accomplished as previously described.[32] To prepare conditioned medium, cells were washed with serum-free medium and resupplied with fresh serum-free media. They were treated with TGF-$\beta$ (80 pmol/L) or with TPA (50 ng/ml). After 24 hours, the serum-free conditioned media was harvested, adjusted for cell number, centrifuged at 1500 $\times$ *g* to remove cellular debris, and concentrated to 650 $\mu$l (Centriprep-10 concentrator, Millipore, Burlington, MA). Concentrated media was electrophoresed under nonreducing conditions and without heating through a 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel containing 0.1% porcine gelatin (Sigma, St. Louis, MO). Gels were washed in 0.05 mol/L Tris (pH 7.4), 2% Triton X-100, 0.2 m of NaCl, and 0.02% $NaN_3$. Positive controls were human recombinant MMP-9 generously provided by R. Fridman (Wayne State University, Detroit, MI) or conditioned medium from 2.10.10 cells from transformed rat embryo fibroblasts.[37] Gels were stained in 0.2% Coomassie blue for 1 hour and destained in 20% (v/v) methanol and 10% (v/v) acetic acid. The clear bands represent gelatinase activity.

### Northern Blotting

Total cellular RNA (60 $\mu$g) or polyadenylated RNA (5 $\mu$g) (direct mRNA mini kit, Qiagen, Chatsworth, CA) was electrophoresed in a formaldehyde/agarose gel and transferred to a Hybond-$N^+$ membrane (Amersham, Arlington Heights, IL). RNA blot hybridization was performed with a $^{32}$P-labeled probe derived from the 1.3-kb *Eco*RI insert fragment released from cDNA clone p8P2a[37] or from the ribosomal protein rPL32 as an internal standard.[38,39] A probe that hybridizes to the ribozyme was derived from the 146-bp insert fragment released from pRC/CMV

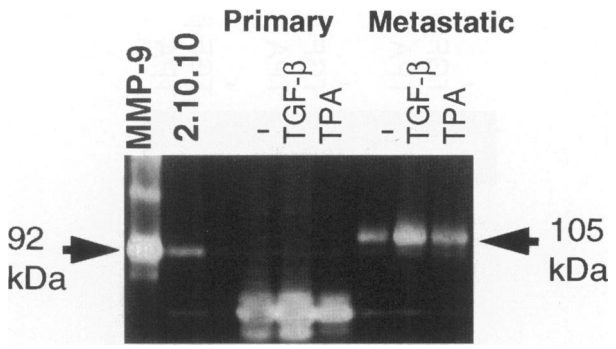


**Figure 1.** Gelatinolytic activity is present in conditioned media from metastatic murine prostate carcinoma cells. Concentrated, conditioned medium from cells derived from a primary prostate carcinoma 148–1,PA or from a metastatic tumor 148–1,LMD were subjected to gelatin substrate gel electrophoresis. The cells were treated for 24 hours with TGF-β or with TPA as indicated.

MMP-9 ribozyme construct by digestion with *Sac*I and *Apa*I.

### *Lung Colonization, Metastasis, and Tumorigenicity Assays*

Four- to six-week-old NCr-nu/nu mice were obtained from Taconic Farms (Germantown, NY) and housed aseptically. On the day of injection, cells were washed in phosphate-buffered saline, trypsinized, resuspended in serum-free Dulbecco's modified Eagle's medium, and microscopically examined to ensure single cell suspensions. For metastasis studies, mice were injected in the lateral tail vein with $5 \times 10^4$ single cells/0.1 ml. Animals were sacrificed when exhibiting labored breathing or at 4 weeks. Lungs were fixed in 10% (w/v) formalin. A dissecting microscope was used to count the lung tumors for evidence of metastasis. Tumorigenicity was assessed after injection of $5 \times 10^5$ subcutaneously in the flanks of 4- to 6-week-old male nu/nu mice. Six tumors were measured for each point. The tumor volume was determined by measurement of the tumor in three dimensions with calipers at the indicated times.

## *Results*

Cells derived either from a primary tumor or from a metastatic tumor generated in the mouse prostate reconstitution system were evaluated for expression of MMP-9.[3] Conditioned medium from the cells isolated from a primary tumor (148.1,PA) showed little basal expression of the 105-kd gelatinase activity characteristic of murine MMP-9, and there was no increase after exposure of the cells to either TGF-β or TPA (Figure 1). In contrast, the conditioned medium from the cells from the metastasis (148.1,LMD) contained detectable gelatinolytic activity at 105 kd after 24 hours, and the amount of gelatinase released increased after exposure of the cells to either TGF-β or to TPA. The conditioned medium from the primary cells had a high level of gelatinolytic activity in the range of 72 kd likely to be MMP-2, but this activity was not seen in the medium from the metastatic cells. Thus, we chose to use the 148.1,LMD prostatic carcinoma cells isolated from a metastasis as a vehicle to test whether down-regulation of MMP-9 would have an effect on metastatic potential.

Hua and Muschel[35] had previously shown that the introduction of an expression vector for a ribozyme directed against the rat MMP-9 could down-regulate MMP-9 expression in rat cells. This ribozyme consists of a hammerhead-type catalytic site flanked by 15 bases (8 on the 5′ and 7 on the 3′ side) complementary to the rat MMP-9 mRNA. Thirteen of these 15 bases are identical between rats and mice suggesting that this ribozyme might also be effective against murine MMP-9. After transfection of this vector into parental 148.1,LMD cells, 11 transfected clones were isolated; six showed decreased levels of released MMP-9 gelatinase activity. Transfection with an expression vector that codes for a ribozyme hammerhead structure flanked by the sense MMP-9 sequence, which would not target MMP-9, was used as a control. Of the six clones isolated after transfection with the control ribozyme expression vector, none showed decreased levels of 105-kd-released gelatinase activity compared with the parental cells. Two clones transfected with the MMP-9 ribozyme and two with the control ribozyme, were selected for additional analysis. These clones were shown to contain the plasmid DNA using Southern blotting (data not shown). Figure 2A shows that mRNA corresponding to the ribozyme could be detected in the transfectants but not in the parental cells. MMP-9 mRNA could not be detected in the cells transfected with the MMP-9 ribozyme under the same conditions in which MMP-9 mRNA could be found in the clones transfected with the control ribozyme (Figure 2B). Increased levels of MMP-9 mRNA were seen in the control cells after treatment with either TGF-β or TPA, but MMP-9 mRNA was still not detected in either of the two clones transfected with the expression vector for the MMP-9 ribozyme. Loading in each lane was shown to be equivalent by reprobing the blot for rPL32 mRNA, a gene coding for a ribosomal protein.[38,39] Thus, introduction of the ribozyme directed against rat MMP-9 resulted in deceased levels of MMP-9 mRNA.

The decreased levels of MMP-9 mRNA in cells transfected with the ribozyme against MMP-9 were reflected in decreased levels of MMP-9 gelatinase released into the medium. (Figure 3). Gelatinase activity at 105 kd as detected by substrate gel electrophoresis indicated that the parental 148.1,LMD cells and the control transfectants secreted equivalent amounts of 105-kd gelatinase activity, but conditioned medium from the two ribozyme transfected clones had 5- to 10-fold less. After stimulation with either TGF-β or TPA, the parental and control cells released greater amounts of MMP-9 activity into the medium. The ribozyme transfected cells also released greater amounts of gelatinase activity after stimulation, but the absolute level was still considerably reduced in comparison with the controls. No lower molecular mass bands indicative of activation were seen in any case.

To determine the effect of lowered MMP-9 expression on the behavior of these prostatic carcinoma cells, we

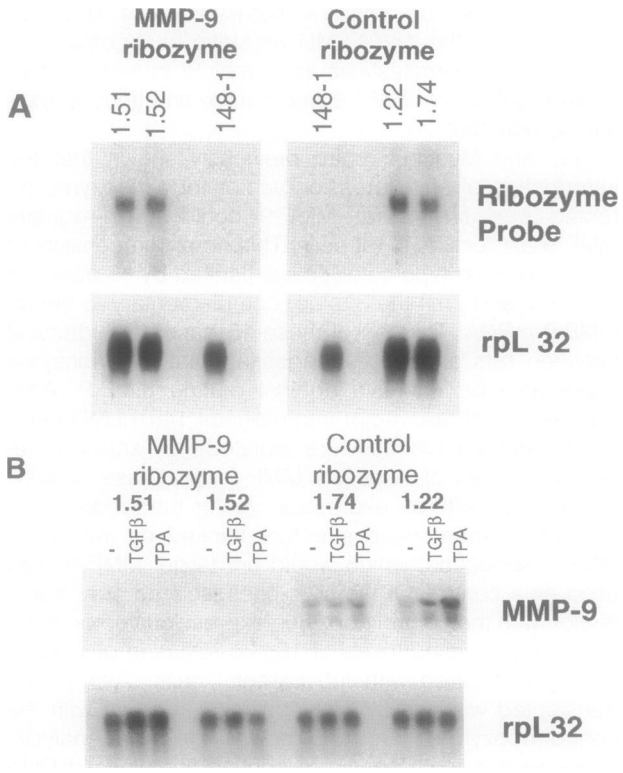


**Figure 2.** The MMP-9 ribozyme results in decreased MMP-9 mRNA expression. **A:** Expression of the ribozymes in transfected cells. Poly(A) plus RNA was isolated from the parental cells 148–1, from cells transfected with the control ribozyme 1.22 and 1.74, and from the MMP-9 ribozyme 1.51 and 1.52. The **upper panel** shows the autoradiograph after hybridization with a probe derived from the ribozyme expression vector, whereas the **lower panel** shows a loading control with the same blot reprobed using rPL32. **B:** Expression of MMP-9 mRNA in the ribozyme-transfected cells. Total RNA (10 μg) from each of the indicated cells treated with TGF-β or with TPA were subjected to Northern blotting with an MMP-9 probe. The **upper panel** shows this autoradiograph, and the **lower panel** shows the autoradiograph after reprobing with a loading control rPL32.

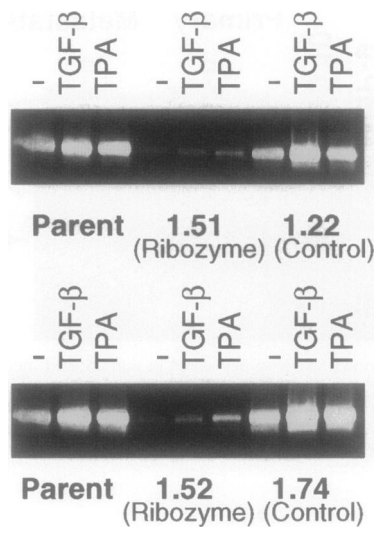


**Figure 3.** Ninety-two-kd gelatinase activity is reduced in conditioned medium from ribozyme-transfected cells. Conditioned medium was collected from cells treated with the indicated agent and subjected to gelatin substrate gel electrophoresis.

tested their metastatic potential using the lung colonization assay (Table 1). After injection of $5 \times 10^5$ cells into the tail veins of female nude mice, both the parental cells and the controls gave rise to lung colonies (22 to 31 per mouse), but injection of the same number of MMP-9 ribozyme transfected cells gave rise to at most 1 to 3 per mouse. Although the *ras*$^H$ plus *myc*-transformed prostate cells derived from the murine prostate reconstitution system have been reported to be androgen independent, we also wished to determine whether the cells would respond differently after injection in a male mouse.[40] The experimental metastasis assay was performed using male nude mice. In this case the controls and parental cells again readily gave rise to lung colonization, from 28 to 35 nodules/mouse, whereas only 3 to 4 nodules/mouse were seen after injection of the clones with down-regulated MMP-9. Thus, metastasis was inhibited by down-regulation of MMP-9.

Tumor growth rate was evaluated after subcutaneous injection in male nude mice with no significant differences seen regardless of their MMP-9 expression (Figure 4).

## *Discussion*

The metastatic potential of the prostate cells with reduced MMP-9 was drastically reduced compared with either control transfected clones or the parental, metastatic cells. In contrast, tumor growth was unaffected. The finding that MMP-9 contributes to metastasis in a murine prostate model system leads to the question of whether MMP-9 might be involved in tumor progression in human

**Table 1.** Effect of MMP-9 Ribozyme on Experimental Metastasis

| Cell type | Cell lines | Male (lung nodules/ mouse ± SD) | Female (lung nodules/ mouse ± SD) |
|---|---|---|---|
| Parental | 148-1,LMD | 29 ± 8.5 | 26.2 ± 5.3 |
| Control ribozyme | AAS 1.22 | 37 ± 11 | 22.2 ± 6.7 |
| | AAS 1.74 | 38 ± 8 | 31.8 ± 9.9 |
| MMP-9 Ribozyme | AR 1.51 | 3.25 ± 2.5 | 0.63 ± 0.3 |
| | AR 1.52 | 3.0 ± 2.2 | 1.5 ± 0.5 |

Cells ($5 \times 10^4$) from the parental population, cells from two MMP-9 ribozyme transfected clones AR1.51 and AR1.52, and from two control transfectants AAS 1.22 and AAS 1.74 were each injected intravenously into male or female nude mice. After 4 weeks, the animals were sacrificed and the number of lung nodules scored. The results shown are from one experiment with five mice in each group. Similar results were obtained in two repeat experiments. The range of nodule number in all experiments for the cells in the parental group was from 12 to 55, for the control ribozyme cells from 22 to 82, and for the MMP-9 ribozyme cells from 0 to 3. The difference between the results from the control ribozyme and the MMP-9 ribozyme groups is significant to $P < 0.001$ by the Mann-Whitney test.

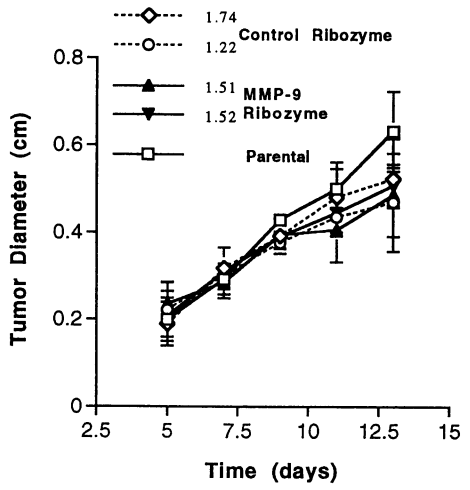


**Figure 4.** Tumor growth rate is not affected by MMP-9 expression. The diameter of the tumors formed after injection of the indicated cells was measured. The standard deviation is shown with error bars. When the error bars cannot be seen, they were smaller than the diameter of the data point.

prostate cancer. Wood et al[41] found low levels of MMP-9 mRNA in normal prostate tissue, tissues from patients with benign prostatic hypertrophy, or carcinoma with a low Gleason score (5 or less). In contrast, MMP-9 mRNA levels were highly expressed in specimens from carcinomas with high Gleason scores,[8–10] and there was a significant correlation with high MMP-9 expression and local invasion as well as survival. Elevated MMP-9 levels were found in cell cultures from prostate carcinomas with high Gleason scores,[42] and gelatinase profiles from tissue samples revealed a correlation with MMP-9 gelatinase activity and metastasis.[43] These data are consistent with MMP-9 filling a role in human prostate cancer similar to its role in the mouse reconstitution model of prostate cancer.

Other MMPs have also been implicated as playing a role in the behavior of prostate carcinoma. MMP-7 (matrilysin or PUMP-1) has been found to be overexpressed in the epithelial cells of a high frequency of human prostate carcinoma specimens and prostatic carcinoma cell lines.[5,44] Transfection of an expression vector for matrilysin into the prostatic carcinoma line DU 145 resulted in increased invasion into the diaphragm by these cells, and this correlated with invasion in the prostate itself. MMP-2 (gelatinase A or 72-kd type IV collagenase) is also overexpressed in prostatic carcinoma cells indicating that it too may play an important role in the biological behavior of this tumor.

Whether TGF-$\beta$ acts *in vivo* to stimulate MMP-9 expression and thus lead to metastatic behavior is an unresolved issue at this point. TGF-$\beta$ is expressed at higher levels in prostatic carcinoma specimens from patients with more aggressive disease.[45–48] It has also been reported that latent TGF-$\beta$ binding protein is absent from prostatic carcinoma specimens but present in normal tissues and those from patients with benign diseases.[49] It has also been suggested that response of TGF-$\beta$ receptor 1 may be altered in some prostatic carcinomas potentially leading to differential responses to TGF-$\beta$; although this does not appear to be the case in the mouse prostate reconstitution system as both receptors were present in cells derived from these tumors, and TGF-$\beta$ led to equivalent plasminogen activator inhibitor 1 stimulation in metastatic and nonmetastatic cells.[3,50] The effects of TGF-$\beta$, however, must be pleiotrophic as its effects on cell growth are likely to be independent of its effects on MMP-9 production.

These results complement our previous results in a murine sarcoma model system in which down-regulation of MMP-9 also led to a decrease in metastatic potential without a decrease in tumorigenicity or tumor growth rate. Whereas it has often been assumed that MMPs were involved in metastasis, these studies now begin to provide some evidence that MMP-9 in fact does play a role in metastasis. As MMP-9 is overexpressed in many human tumor types, these results raise the possibility that MMP-9 expression may be linked to tumor progression and to the development of metastatic potential in a variety of human cancers, including prostate cancer, and that MMP-9 expression could be a potential target for the modulation of metastatic potential.

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