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The Localization of Vasoactive Intestinal Peptide (VIP)-Like Immunoreactivity in Gonadotropes of the Rat Anterior Pituitary

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ABSTRACT—The anterior pituitary gland has recently been shown to contain a number of bioactive peptides other than inherent pituitary hormones. One of these peptides is vasoactive intestinal peptide (VIP). The localization of VIP has not known and so the present study was undertaken to determine which type of pituitary cell contains this peptide. The adult rat anterior pituitary was immunohistochemically examined with two VIP antisera. One of these antisera successfully stained some pituitary cells. Both the double immunostaining technique and the flip-flop section method revealed VIP-like immunoreactivity, mainly in gonadotropes. This immunostaining was lost when the anti-VIP was preabsorbed with synthetic VIP or with pituitary adenylyl cyclase activating peptide (PACAP), 68% of the 1-28 portion of which is homologous to VIP. When tested with anti-PACAP, however, no immunoreactivity was observed in anterior pituitary tissue. These results indicate that VIP is localized in gonadotropes in the anterior pituitary. The physiological significance of such localization of VIP is discussed in relation to the regulation of PRL secretion.

INTRODUCTION

Vasoactive intestinal peptide (VIP) was first isolated from the intestine (Said and Mutt, 1970). Subsequently this peptide was found in other tissues such as the nervous system (Emson *et al.*, 1979; Said and Rosenberg, 1976). In addition to the role of this peptide as a neurotransmitter in the brain, evidence indicates that VIP is involved also in regulation of hormone release from the anterior pituitary (Mezey and Kiss, 1985). This possibility is substantiated by the fact that hypophysial portal blood of the rat contains a high concentration of VIP (Said and Porter, 1979; Shimatsu *et al.*, 1981). VIP has been shown to stimulate prolactin (PRL) release from the anterior pituitary both *in vivo* (Kato *et al.*, 1978) and *in vitro* (Ruberg *et al.*, 1978; Samson *et al.*, 1980; Shaar *et al.*, 1979). The presence of immunoreactive VIP (Shimatsu *et al.*, 1981) and its mRNA (Houben and Deneff, 1994) within the anterior pituitary itself raised the possibility that this peptide regulates pituitary cells through a paracrine or autocrine mechanism. Morel *et al.* (1982) reported the presence of VIP immunoreactivity in PRL cells of the rat pituitary. However, not all investigators have been successful in finding VIP immunoreactivity in the anterior pituitary of the rat. For example, Lam *et al.* (1989) could not detect any immunoreactive VIP cells in the anterior pituitary of rats. But, according to their report, VIP cells were immunostained only when rats were made

hypothyroidal. In contrast, more recently, Carrillo and Phelps (1992) succeeded in demonstrating the presence of many VIP-containing cells in intact rat pituitaries. They observed, however, that there was little topological relationship between these immunoreactive VIP cells and PRL cells. In view of these diverse observations, the present immunohistochemical study was undertaken to localize VIP immunoreactivity in the anterior pituitary of the rat. Because several peptides have amino acid sequences similar to that of VIP, special attention was paid to the specificity of the immunohistochemical staining.

MATERIALS AND METHODS

Animals

One month old Sprague-Dawley rats were used. The rats were housed in a light (lights on from 06:00 to 18:00) and temperature controlled room and were given standard laboratory chow and water *ad libitum*.

Tissues

Six rats were sacrificed by decapitation. The pituitary was fixed in Bouin's solution. A portion of the small intestine was also fixed to confirm the immunohistochemical characteristics of the anti-VIP employed in this study. After overnight fixation the pituitaries were dehydrated in ethanol and embedded in Paraplast. Serial sections of the pituitary were cut transversely at a thickness of 2 μ m. In order to compare the localization of VIP-producing cells and other types of hormone-producing cells, we employed two methods: one was the flip-flop (mirror) section technique (Dada *et al.*, 1983), the other was double immunostaining of a single section. In this study, a pair of flip-flop sections were first mounted on slides and the next section was taken for double immunostaining. Sectioning in this manner was

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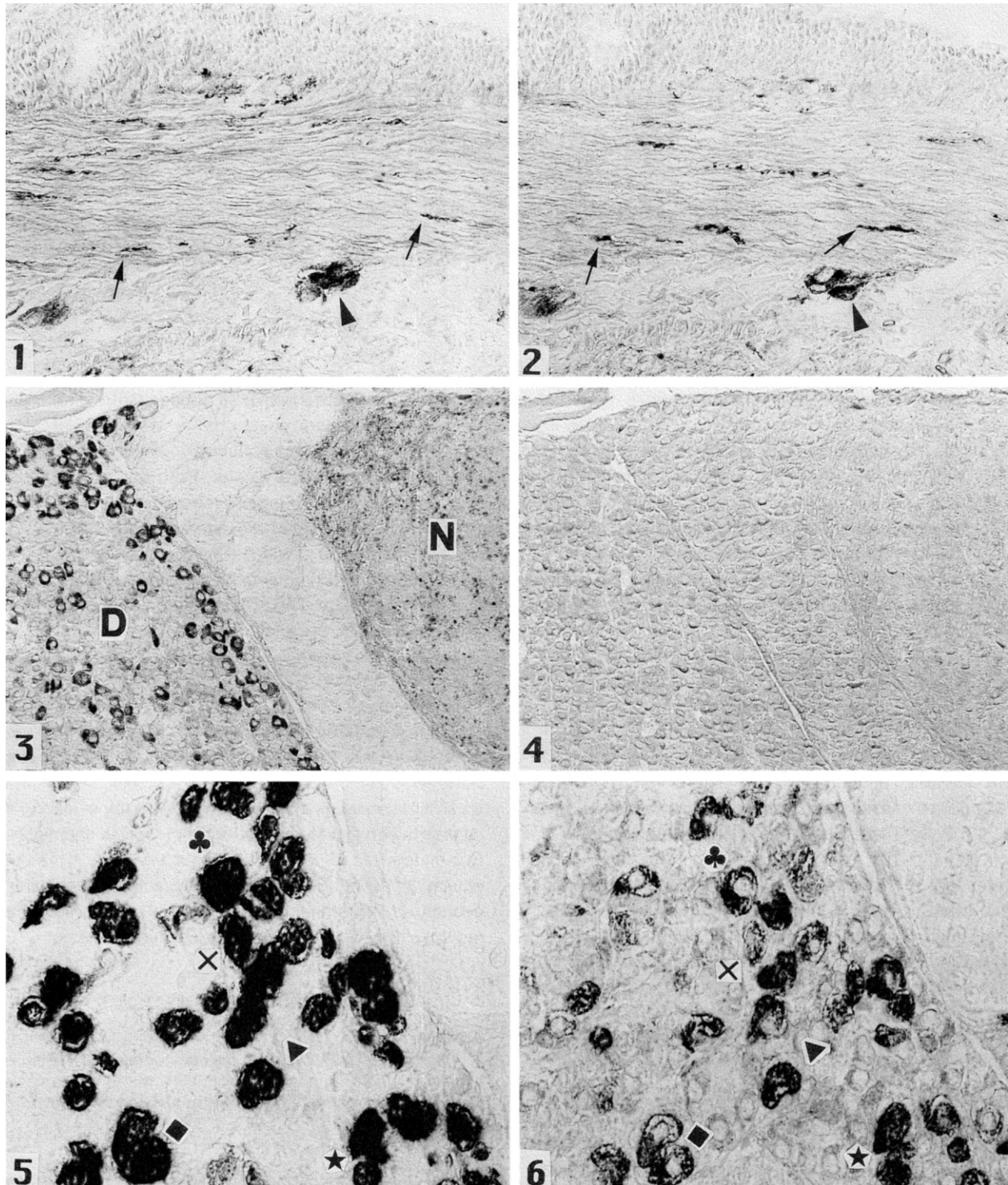


Fig. 1. Transverse section of rat small intestine immunohistochemically stained with anti-VIP(Ito). Arrows indicate the VIP positive nerve terminals in the inner circular muscle layer. The submucous plexus is also stained (arrowhead). $\times 400$.

Fig. 2. The consecutive section to that shown in Fig. 1, stained with anti-VIP (Biomeda). Immunoreactivity is also observed in the nerve terminals (arrows) and the submucous plexus (arrowhead). $\times 400$.

Fig. 3. Portion of the rat pituitary gland immunohistochemically stained with anti-VIP (Ito). Note many rounded immunoreactive cells in the pars distalis (D), and profiles of nerve endings in the pars nervosa (N). $\times 200$.

Fig. 4. Adjacent section to that shown in Fig. 3, stained after adsorption of anti-VIP(Ito) with 10^{-6} M of pituitary adenylate cyclase activating peptide (PACAP). No immunoreactive material is seen. $\times 200$.

Figs. 5, 6. Paired flip-flop sections of the rat pituitary, one immunostained for LH (Fig. 5) and the other stained for VIP (Fig. 6). The corresponding parts are shown by the same symbols. $\times 600$.

performed at intervals of 120 μm . In each rat 20 pairs of flip-flop sections were prepared.

Immunohistochemical procedures

The primary antisera against VIP used in this study were obtained from two different sources. One antiserum was a gift from Dr. H. Ito (Hokkaido University) and the other was commercially obtained from Biomedica (CA, USA). In both cases, synthetic porcine VIP (1-28) conjugated with BSA was used as an antigen. Rabbit anti-PACAP (1-38) was purchased from the Peptide Institute (Minoh, Japan). For the demonstration of VIP, we first compared the peroxidase-anti-peroxidase complex (PAP) method with the streptavidin-biotin-peroxidase (SAB) complex method. The SAB method was found to be better since it gave far stronger immunostaining and weaker background staining. Deparaffinized sections were sequentially treated with the following solutions for the times indicated: 3% H_2O_2 for 10 min, 10% normal goat serum for 10 min, anti-VIP for 1 hr, biotin-labeled anti-rabbit IgG for 5 min and peroxidase-labeled streptavidin for 10 min. All of these staining steps were carried out at room temperature, with each step followed by rinses with 0.02 M phosphate-buffered saline. The final reaction product was visualized with the use of 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution containing 0.001% H_2O_2 . To compare the localization of VIP and other pituitary hormones, one of the flip-flop sections was stained with anti-VIP and the other with one of the following sera by the use of the PAP technique: anti-rat PRL, anti-rat GH (gifts from Dr. K. Wakabayashi, Gunma University), anti-ovine LH β (this laboratory), anti-human ACTH, anti-human TSH β (these two antisera were supplied from NIAMDK), or anti-bovine S100 protein (Dakopatts, Netherlands) (dilution 1:1000).

For the double-immunostaining method, VIP was first visualized using DAB. After treating the sections with 0.1 M glycine buffer (pH 2.2) for 2 hr, adenyphophysial hormones were next localized with 4-chloro-1-naphthol as chromogen. The specificity of the primary antisera, except for anti-VIP, has been previously determined in this laboratory (Carbajo-Pérez *et al.*, 1989; Watanabe and Haraguchi, 1994). The immunochemical characteristics of the anti-VIP(Ito) were previously reported by Ito *et al.* (1986). We further confirmed the specificity of the two antisera to VIP using the pre-adsorption test adding synthetic porcine VIP and human PACAP were added to these antisera (1000 \times) at a concentration of 10^{-6} M.

RESULTS

We first determined whether the antisera against VIP that we had stain this peptide in the small intestine. Both sera (Ito and Biomedica) were found to stain the fine nerve network in the submucous plexus and the inner circular smooth muscle coat (Figs. 1 and 2). But, as will be described below, when these antisera were applied to pituitary sections, different immunohistochemical results were obtained.

Immunostaining with anti-VIP (Ito)

Anti-VIP (Ito) was found to stain many immunoreactive cells in the normal rat anterior pituitary (Fig. 3). These immunostained VIP cells were oval to round in shape and they were larger in size than the neighboring immunonegative cells. Although these VIP-containing cells were distributed throughout the pars distalis, they were concentrated near the surface of the gland. The VIP cells situated in the dorsal region surrounding the residual lumen (called the sex zone) were more intensely stained.

Strong immunoreactivity was also observed in the pars

nervosa. Immunostained material appeared to be within the processes that resembled neurosecretory axons (Fig. 3). When anti-VIP (Ito) was preadsorbed with synthetic VIP, no immunoreactive cells were observed. A similar loss of immunostaining was also seen after adding PACAP (1-38, human) as shown in Fig. 4. When sections of the pituitary were stained with anti-PACAP (1-38, human), however, no immunoreactive cells were observed. This anti-PACAP has been shown to react intensely with the rat hypothalamic nuclei (Kimura *et al.*, 1994).

Immunostaining with anti-VIP (Biomedica)

When pituitary sections were stained with the anti-VIP (Biomedica), a few immunoreactive cells were occasionally seen in the anterior pituitary (data not shown). The number of these VIP positive cells were so few that it was very difficult to identify the cell type. No immunoreactive cells were observable in the posterior and intermediate lobes.

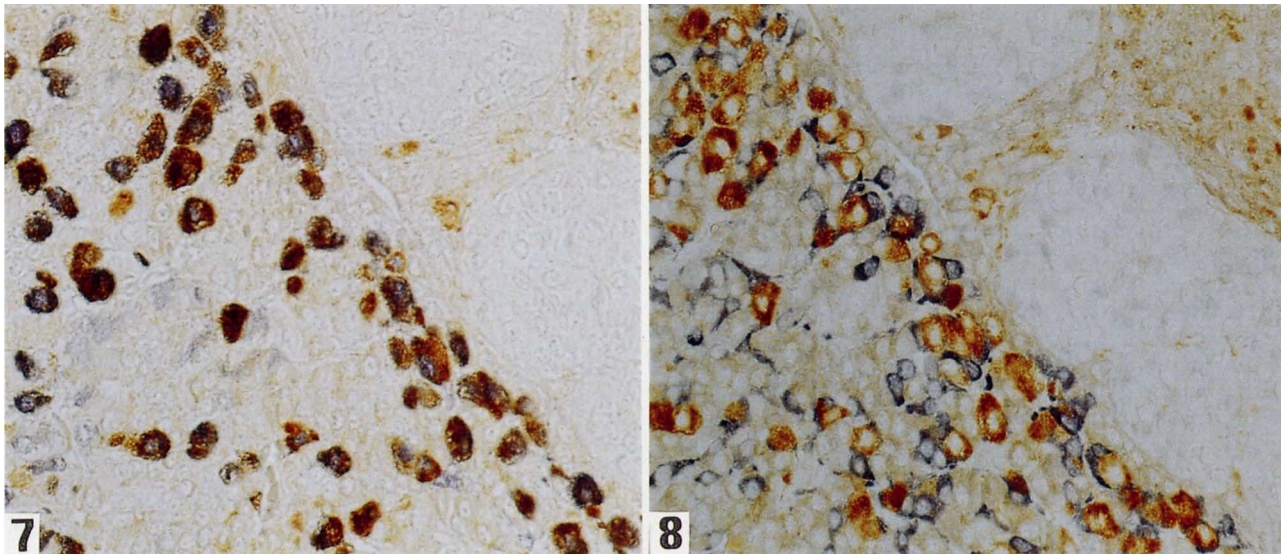
Results of flip-flop sections and double immunostaining

In order to determine which type of cell contains VIP in the pituitary, we performed the flip-flop section and double immunostaining methods using the anti-VIP (Ito). Figures 5 and 6 show a pair of flip-flop sections of the pituitary stained with the two different antisera. A comparison of these two sections made it clear that most, but not, all immunoreactive VIP cells were found to contain LH. The colocalization of VIP and LH was also demonstrated by the double immunostaining technique. As shown in Fig. 7, the different colors of the reaction products for VIP and LH were simultaneously found in oval-shaped gonadotropes. Such colocalization, on the other hand, was not seen with VIP and PRL (Fig. 8).

DISCUSSION

Since VIP was found in the nerve endings of the hypothalamus (Besson *et al.*, 1978; Emson *et al.*, 1978; Giachetti *et al.*, 1977) and in hypophysial portal blood (Said and Porter, 1979), many investigators have drawn attention to the possible synthesis of this peptide within the pituitary. Early study by Van Noorden *et al.* (1979) failed to reveal immunoreactive VIP in the anterior lobe of the rat pituitary though much was observed in the posterior lobe. Arnaout *et al.* (1986) provided the first biochemical evidence for VIP synthesis in the anterior pituitary.

The actual presence of VIP mRNA was demonstrated within the pituitary by Segerson *et al.* (1989). There is, however, a marked variation in the immunocytochemical results on the site of VIP production in the anterior pituitary. The ultrastructural observation that VIP was localized in anterior pituitary cells (Morel *et al.*, 1982) was not confirmed by subsequent immunohistochemical studies at the light microscopic level. Some investigators observed no VIP cells (Lam *et al.*, 1989) or only a few VIP cells (Köves *et al.*, 1990; Steel *et al.*, 1989) in the normal rat anterior pituitary. An increase in the number of VIP cells could be found only after



Figs. 7, 8. Two consecutive sections of the pituitary doubly immunostained with anti-VIP (Ito) and anti-LH (Fig. 7), or anti-PRL (Fig. 8). Note that the majority of VIP labeled cells (brown) are also labeled with LH (purple) in Fig. 7. The distribution of VIP cells (brown) and PRL cells (purple) is independent as shown in Fig. 8. $\times 400$.

rats were treated with estrogens (Lam *et al.*, 1989; Steel *et al.*, 1989) or substances that cause the hypothyroidal state (Köves *et al.*, 1990). Other investigators (Carrillo and Phelps, 1992), on the other hand, have observed many VIP cells without any endocrinological manipulation as shown in the present study. What might be the reasons for these marked discrepancies? We here consider differences in the immunological characteristics of the primary antisera to VIP and differences in the subsequent histochemical procedures. VIP consists of 28 amino acids residues. An antiserum to such a small peptide is not easily generated by injection of simply emulsified antigen though Steel *et al.* (1989) succeeded in preparing antibody in this way. Yanaihara *et al.* (1977) prepared antiserum after adsorption of the antigen with PVP. Other investigators conjugated VIP with bovine thyroglobulin (Carrillo and Phelps, 1992; Lam *et al.*, 1989) or serum albumin by the use of carbodiimide (Ito *et al.*, 1986). The discrepancies of immunohistochemical results presently reported may be explained by different ways of antibody production.

In most immunohistochemical studies hitherto performed on the anterior pituitary, only a single source of anti-VIP was employed. Steel *et al.* (1989), on the other hand, compared several different sources of antisera to VIP. According to these investigators there was a uniform immunostaining result irrespective of the source of the antiserum. In the present study, on the other hand, there was a striking difference in the staining characteristics between the two antisera. As far as the intestine is concerned, the two antisera used in this study gave a similar pattern of immunostaining. With both antisera, VIP immunoreactive nerve fibers and cell bodies were demonstrated in the myenteric and submucous plexuses, as described by Fehér and Léránth (1983). When applied to pituitary tissue, however, only anti-VIP (Ito) reacted with some

group of cells. The anti-VIP (Ito) used in our study is reported to bind strongly with 7-28 VIP but react poorly with the 18-28 fragment (Ito *et al.*, 1986). In this regard, this antiserum has an immunochemical nature similar to that of the one (#581) produced by Yanaihara (Yanaihara *et al.*, 1977). Although anti-VIP (Ito) stained many immunoreactive VIP cells as shown in this study, anti-VIP #581 was found to stain only a few cells per section (Steel *et al.*, 1989). At present we have no explanation for this difference between their and our immunohistochemical results.

In addition to the primary antiserum, discrepancies of immunohistochemical staining may also be explained in terms of the differences in staining techniques employed. Shi *et al.* (1988) compared the staining performance of the following three methods; avidin-biotin complex (ABC), peroxidase-anti-peroxidase (PAP) and streptavidin-peroxidase (SP) methods. According to them, the SP technique gave the most intense immunoreaction and least background staining. Although they reported less intense immunoreaction if streptavidin-biotin (SAB) complex was employed, our study showed that VIP cells were demonstrated only when the SAB method was used. Thus the type of peroxidase complex may have much to do with the final immunohistochemical results.

In the present study, VIP-like immunoreactivity in the anterior pituitary was lost after adsorption with PACAP(1-38). This is not surprising because of considering that residues 14-23 in PACAP are identical to those in VIP. The result raises the question as to which of these two peptides is actually immunostained by the anti-VIP (Ito). Available data indicate that PACAP is not contained in anterior pituitary cells (Kimura *et al.*, 1994) though it was occasionally seen within some neuronal processes (Mikkelsen *et al.*, 1995). In the present study an antiserum against PACAP failed to stain any anterior

pituitary cells. Accordingly, we can draw the conclusion that anti-VIP (Ito) actually immunostains VIP.

A large body of evidence indicates that VIP plays a regulatory role in the regulation of PRL secretion (Denef *et al.*, 1984; Enjalbert *et al.*, 1980; Frawley and Neill, 1981; Hagen *et al.*, 1986; Kaji *et al.*, 1985; Kato *et al.*, 1978; Lam and Reichlin, 1989; Nagy *et al.*, 1988; Ruberg *et al.*, 1978; Shaar *et al.*, 1979). The question as to what type of anterior pituitary cell contains VIP is important in terms of the manner of this PRL regulation. Some investigators observed that VIP is contained in PRL cells in normal rats (Morel *et al.*, 1982) or estrogen-treated rats (Köves *et al.*, 1990; Steel *et al.*, 1989). Lam *et al.* (1989) and Carrillo and Phelps (1992), on the other hand, claimed that VIP is localized in cells other than PRL cells. These authors did not give any definite conclusion as to the cell type that possessed immunoreactive VIP. According to Lam *et al.* (1989) immunoreactive VIP cells are stellate. They further stated that VIP cells are rather rare in the normal anterior pituitary. In contrast, the VIP cells observed by Carrillo and Phelps (1992) were abundant in number and polygonal to oval in shape. Their immunohistological results, therefore, compare well with those of the present study. The flip-flop section technique and double immunostaining employed in our study showed that most, but not all, VIP cells contained LH.

The storage of VIP in gonadotropes and its release from them may be advantageous for a rapid control of PRL secretion since round LH cells are closely surrounded by cup-shaped PRL cells (Nakane, 1970). In fact, Denef and Andries (1983) have provided *in vitro* experimental data showing a possible control of PRL release by gonadotropes. They demonstrated that LHRH-stimulated PRL release was observed only when a purified population of gonadotropes was allowed to form cell aggregates with PRL cells. Further cytological studies are needed to analyze how PRL secretion is regulated by VIP which is released from the closely associated LH/FSH cells.

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