Specific transcriptional activation \textit{in vitro} by the herpes simplex virus protein VP16

David N. Arnosti*, Chris M. Preston¹, Michael Hagmann, Walter Schaffner, R. Graham Hope¹, Gerard Laughlan¹ and Ben F. Luisi¹.*

Institut für Molekularbiologie II, Universität Zürich, Zürich, Switzerland and ¹MRC Virology Unit, Church Street, Glasgow G11 5JR, UK

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ABSTRACT

The herpes simplex virus protein VP16 interacts with cellular factors, including the protein Oct-1, to activate viral immediate early (IE) gene transcription. We have reproduced this effect by addition of purified, full-length VP16 and the DNA-binding ‘POU’ domain of Oct-1 (Oct-1/POU) to a HeLa cell \textit{in vitro} transcription system. Stimulation of transcription was dependent on the IE-specific element, TAATGARAT. In agreement with earlier observations from electrophoretic mobility shift assays, activation was not observed when Oct-2/POU, the DNA-binding domain from the Oct-2 protein, was substituted for Oct-1/POU. Single round transcription assays revealed that, together, VP16 and Oct-1/POU facilitate the assembly of pre-initiation complexes at target gene promoters.

INTRODUCTION

Upon entry into a host cell, herpes simplex virus type-1 (HSV-1) releases the tegument protein VP16 (also known as Vmw65, α-TIF or ICP25) which migrates to the nucleus, where it plays a critical role in triggering viral immediate early (IE) gene expression (1–3). VP16 recruits the endogenous DNA-binding protein, Oct-1, along with a recently characterized protein referred to as C1 (4) [thought to be analogous to CFF (5) and HCF (6,7)], and possibly other proteins, into an activating complex that assembles on specific DNA sequences (8–10). The DNA targets of this complex occur upstream of the IE viral genes and harbor the consensus sequence ATGCTAATGARAT, where R is a purine (Figure 1a, b). The target encompasses a sequence closely related to the octamer motif (ATGCAAAT) through which Oct-1 and/or Oct-2 regulate the expression of genes for histone H2B, immunoglobulins and certain small nuclear RNAs (reviewed by Kemler and Schaffner, 11). Oct-1 is a member of a family of proteins which share a highly homologous DNA-binding motif, referred to as the POU domain (for the Pit-1, Oct-1 and -2 and unc-86 proteins in which it was first discovered; 12). The POU domain is composed of two distinct DNA-binding structures. The carboxy terminal POU-homeodomain belongs to the family of factors containing the homeodomain, while the amino terminal POU-specific domain is characteristic of the POU proteins (reviewed in Verrijzer and van der Vliet, 13). Recent NMR studies have demonstrated that the POU-specific domain contains a helix-turn-helix motif, structurally similar to the DNA-binding domain of the λ phage repressor (14,15), although in the POU domain the turn region is longer. While POU domain proteins recognize similar binding sites, they elicit distinct physiological and tissue-specific effects. The selectivity probably arises in part from specific protein–protein interactions which modulate activity. For instance, while the POU domain of Oct-1 (Oct-1/POU) can interact with VP16 and C1, the affinity of Oct-2/POU for the additional proteins is at least two orders of magnitude weaker (16). Interaction with specific amino acids of the Oct-1 POU homeodomain allow VP16 to discriminate between Oct-1 and Oct-2 (17–20). The interaction of Oct-1 with VP16 is therefore a model system for discriminatory protein–protein associations that give rise to the functional diversity of the POU class of proteins.

Although Oct-1 contains transcriptional activation domains and can regulate certain classes of promoters (21,22), binding of Oct-1 by itself does not appear to activate HSV IE transcription (2,23). Oct-1 appears to function by directing VP16 to the promoter of IE genes, where the viral protein activates transcription \textit{in vivo} via its acidic C terminus (24,25). When fused to DNA-binding proteins, this domain imparts a strong transcriptional activating capability (26,27).

Considerable effort has been made to characterize two aspects of activation by VP16: First, how specificity is achieved through protein–protein and protein–DNA interactions, and second, how VP16 interacts with the basal transcription apparatus (Figure 1b). Previous tests of VP16 specificity involved measuring only physical interactions \textit{in vitro}. Transcriptional activation by VP16 has been studied \textit{in vivo}, but \textit{in vitro} analyses have concentrated on activation mediated by chimeric proteins containing the acidic C terminal region of VP16 linked to the DNA binding region.

* To whom correspondence should be addressed

¹ Present address: University of California, San Diego, Department of Biology, 0322, 9500 Gilman Drive, La Jolla, CA 92093-0322, USA
of the yeast GAL4 protein. This chimeric activator apparently interacts with the basal transcriptional apparatus by binding to TFIIID and/or to TFIIIB (27–29). In order to examine the basis of VP16 specificity at a functional level, we have used in vitro transcription systems with purified full length VP16 and POU domain proteins derived from Oct-1 and Oct-2. The results show the octamer/TAATGARAT segment. For comparison, the consensus octamer element is aligned to the homologous region of the ICPO site. The oct element has substitutions in the ICPO site, and the cut element has substitutions in the corresponding ICPO region. The hepta+octa- and hepta+/octa+ elements are derived from the mouse 104-2 IgH promoter, and the KAP1 element is from the 5' x light chain promoter. The sequences are underlined where they differ from the ICPO site. Elements were inserted 5' to the ICPO element. As the results of this study suggest, this unique HindIII site of pET-8c was removed by end-filling with Klenow enzyme and re-ligation, and the annealed oligonucleotide pair 5'-CATGCCGCAAAGCTTCCG-3' and 5'-GATCCCCCGAGCTTCCAGC-3' was inserted between the NcoI and BamHI sites of the progeny, thereby introducing a HindIII site in the required reading frame. The resulting plasmid was named pET-8c1. Plasmid pMC17, containing the VP16 coding sequences (31), was partially digested with Sall, re-digested with HindIII, end-filled and re-ligated. A plasmid with the Sall site proximal to the initiator ATG fused to the upstream HindIII site was isolated. The VP16 coding sequences were excised from this new plasmid as a HindIII/BamHI fragment and cloned between the HindIII and BamHI sites of pET-8c, to yield the VP16 expression vector pETVP16. The modifications create a N-terminal 5 amino acid sequence of Met-Gly-Gly-Ser-Phe, compared with Met-Asp-Leu-Leu-Val in the native protein.

Cells harbouring VP16 expressing plasmids were grown in 2 X YT media, induced at mid-logarithmic growth by addition of IPTG to 0.5 mM and then harvested two hours later. Cell pellets were frozen then thawed after suspension in 50 mM Tris-Cl pH 8.2, 1 M NaCl, 2 mM dithiothreitol, 2 mM PMSF, 5 mM EDTA, 0.37 mg/ml CHAPS and 0.13 mg/ml hen egg white lysozyme. MgCl2 was added to 10 mM final concentration and then DNase I was mixed into the extract to a net concentration of approximately 50 µg/ml. The cell pellets were centrifuged at 20,000 g for 30 minutes, and polymerin P was added to the supernatant at 0.5%, to precipitate nucleic acids. After centrifugation, ammonium sulfate was added to the supernatant to 35% saturation and the precipitate, which was enriched for VP16, was collected by centrifugation. The pellet was resuspended in 50 mM Tris-Cl pH 7.5, 200 mM NaCl, 0.1% CHAPS and applied to a FAST-Q column equilibrated with the same buffer. The protein was eluted with a 200 to 600 mM linear NaCl gradient. The fractions were pooled and dialyzed against 50 mM HEPES pH 7.0, 50 mM NaCl and applied to a FAST-Q column. The proteins were eluted with a linear gradient of 50 to 350 mM NaCl. The protein was approximately 95% pure at this stage. Identification of VP16 was corroborated by reaction with monoclonal antibody LP-1, which is specific for the protein (32). Extinction coefficients were derived by quantitative amino acid analyses and were used to measure protein concentrations optically. 12 µM VP16 was calculated to absorb 1.0 OD unit/cm light path at 280 nm.

Expression and purification of Oct-1/POU domain

The pET system described above was used for expression of Oct-1/POU. To construct a plasmid for expression of Oct-1/POU,
the annealed oligonucleotide pair 5'-AATTCCCATGGGTCG-ACGATATCCAAGCTTGGAGATTATGATGC-3' and 5'-GATCCGA-
TCATAATCCACTGCTTGGATATCGTAGCCCCATGG-3' was cloned between the EcoRI and BamHI sites in a
derivative of pUC9 from which the HincII site had been removed. The resultant plasmid was cleaved at the HincII and PstI sites introduced by the oligonucleotides, and a 515 base pair HincII/ PstI fragment from pBsoct1 (33), containing the Oct-1 POU domain, was cloned into the modified pUC9 vector, to yield pUC9OP. The Oct-1/POU domain was excised from pUC9OP as a Ncol/BamHI fragment and cloned between the Ncol and BamHI sites of pEVTP16, replacing the VP16 coding sequences and yielding pETOP.

Cells harboring pETOP were grown, induced, harvested and lysed as described above for the VP16 preparation, without the addition of lysozyme, which was found to co-purify with the Oct-1/POU. To the polymerin P precipitate, ammonium sulfate was added to 35% saturation, and the material was applied to a phenyl-
sepharose column equilibrated with 50 mM Tris—Cl pH 7.5, 35% saturated ammonium sulfate. A reverse salt gradient was applied from 35% to 10% saturated ammonium sulfate. Oct-1/ POU typically eluted from this column at roughly 80% purity. The fractions enriched with Oct-1/POU were pooled and dialyzed against 50 mM HEPES pH 7.0 and applied to a FAST-S column. The protein was eluted with a linear gradient 50 to 350 mM NaCl, at which point it was roughly 95% pure.

The Oct-1/POU sequence was corroborated by amino terminal sequencing, amino acid composition analysis and mass spectrometry. The protein ran as a single species on isolectric focusing and native gels. By amino acid quantitation, 58 µM Oct-1/POU absorbs 1.0 OD unit/cm light-path at 280 nm.

Preparation of Oct-2 and Oct-2/POU

Full length Oct-2 and the Oct-2/POU domain were prepared as described (34,35). Briefly, proteins were overexpressed in E.coli using the T7 RNA polymerase system, recovered and renatured from inclusion bodies, and purified using a combination of ion-
exchange and DNA affinity chromatography to greater than 95% homogeneity.

Transcription templates and in vitro transcription assay

The assay conditions used were as described (34) with HeLa cell nuclear extracts. 14 to 30 µg of HeLa nuclear extract was incubated with 400 ng each of a test plasmid with a rabbit β globin gene reporter gene and the reference plasmid (OVEC-REF) containing the rabbit β-globin gene with a small deletion just 3' of the transcription initiation site (36). The reference plasmid generates a shorter S1 nuclease resistant product. To prepare templates, oligonucleotides harboring the ICP0 site or its derivatives were inserted between the SacI and SalI sites of the rabbit β globin gene reporter plasmid (OVEC; 36). The sequence of the ICP0 insert was 5'-CGAGCCGTGCATGCTAATGATA-
TTCCTTTGGG-3'. For the ICP0 octamer construct, the underlined oligonucleotide 5'-CTCAGAGCTTTAATAATTTGCAATCCC-TGAAGGCAAGGAG'3'. Oligonucleotides introduced into the OVEC plasmid are listed in figure 1c.

Transcribed RNA from the assay was purified, hybridized with 32P-labelled antisense DNA, and the mixture was digested with S1 single strand nuclease and analyzed on denaturing gels as described (34). Transcription was quantified by phosphor imaging. Transcription analyses with recombinant andchromatographically enriched basal transcription factors, and single-rounds of transcription were performed as described earlier (34).

RESULTS

Characterization of recombinant VP16 and Oct-1/POU

The Oct-1/POU domain and VP16 were overexpressed in E.coli and purified to approximately 95% homogeneity (Figure 2). Both Oct-1/POU and VP16 were stable in solution and soluble to 5 mg/ml or higher. On molecular sizing columns, the proteins had apparent molecular masses of 35 and 65 kD, respectively (results not shown), compared with expected sizes of 20 and 56 kD. It is possible that Oct-1/POU is dimeric, although the observed result may arise from non-spherical protein shape.

The purified Oct-1/POU formed a complex detectable by electrophoretic mobility shift assay (EMSA) when incubated with a radiolabelled probe containing the ICP0 TAATGARAT. VP16 formed the multiprotein complex designated IEC (10) when added to HeLa cell nuclear extract and radiolabelled ICP0 TAATG-
ARAT probe (results not shown). VP16 alone did not form complexes with the probe that were detectable by EMSA, although weak binding of this protein to TAATGARAT has been reported by others (6,38).

VP16, Oct-1/POU and target DNA are required together for in vitro activation of transcription

The purified recombinant VP16 and Oct-1/POU were tested for the ability to activate transcription in an in vitro assay. Test templates containing the HSV ICP0 promoter element inserted in OVEC (8,36) were transcribed in vitro with HeLa nuclear extracts in the presence of VP16 and Oct-1 POU. Purified Oct-2 protein was added to the cell-free system as a positive control. As reported previously, Oct-2 stimulates transcription from OVEC plasmids containing an octamer element (34) and, as expected, Oct-2 activated transcription from the template containing the ICP0 TAATGARAT by approximately 15 fold (Figure 3, lanes 2 and 3). Individually, Oct-1/POU and VP16 had minimal effects on transcription levels (Figure 3, lanes 4—7). However, in combination, the two proteins significantly enhanced transcription, to a maximal extent (Figure 3, lanes 12—15) after correction for the signals from the reference plasmid. The levels of endogenous Oct-1 in the nuclear extract were apparently not great enough to affect transcription significantly, even in combination with VP16 (Figure 3, compare lane 2 with lanes 8—11). A weak activation of the reference plasmid occurs at the highest concentrations of Oct-1/POU (200 to 400 ng of protein; see Figure 3, lanes 6 and 7) and probably results from non-specific binding. However, there is little effect on the reference plasmid when less Oct-1/POU is used (30 to 100 ng; see Figures 4 and 5).

The in vitro activation effect of Oct-1/POU and VP16 required the ICP0 ATGCTAATGATAT motif. Mutations in either the octamer or the GATAT portion of the ICP0 promoter reduced.
Figure 3. VP16 activates transcription in vitro in the presence of Oct-1/POU. The HSV derived ICP0 promoter construct was transcribed in vitro with HeLa nuclear extract after addition of protein preparations. Lane 1, S1 probe. Lane 2, HeLa cell nuclear extract alone. Lane 3, 400 ng of Oct-2, Lanes 4—7, 50, 100, 200 and 400 ng of Oct-1/POU, respectively. Lanes 8—11, 50, 100, 200 and 400 ng of VP16, respectively. Lane 12, 100 ng each of VP16 and Oct-1/POU. Lane 13, 400 ng Oct-1/POU and 100 ng VP16. Lane 14, 100 ng Oct-1/POU and 400 ng VP16. Lane 15, 400 ng each Oct-1/POU and VP16. In vitro transcription reactions were performed as described in Materials and Methods and RNA was quantified by S1 nuclease protection assays. Relative transcription levels were normalized to the level obtained with HeLa cell extract alone (lane 2). The faint band which migrates below the S1 probe is a read-through transcript. Bands appearing below the reference gene represent breakdown from the test transcript.

Figure 4. VP16 plus Oct-1/POU efficiently activate only promoters containing TAATGATAT sequence. Templates possessing test promoters were transcribed in vitro with HeLa nuclear extract. In lanes 1, 4, 7, 10 and 13 no proteins were added. In lanes 2, 5, 8, 11, 14 and 17, 200 ng of Oct-2 were added to the reaction. In lanes 3, 6, 9, 12, 15 and 18, 100 ng each of VP16 and Oct-1/POU were included. 400 ng of DNA with the indicated promoter sequence were used in each experiment. Relative transcription levels were normalized for each promoter to the signal obtained without Oct-2 or VP16-Oct-1/POU.

VP16 activation considerably, while Oct-2 mediated activation was only affected by mutations in the octamer site, as expected, but not in GATAT (see Figure 1c and Figure 4, lanes 13—18). The hepta/oct- and hepta/oct+ elements derived from the murine 104-2 IgH promoter lack homology to GATAT. The former did not respond to VP16 plus Oct-1/POU. Only a weak response was observed from the hepta/oct+ element in the presence of VP16 and Oct-1/POU whereas, as reported previously (37), this element mediated activation by Oct-2 (Figure 4, lanes 1—6). The weak response of hepta/oct+ to VP16 and Oct-1/POU is due to the 2—3 fold activation caused by the POU domain; a similar weak effect has been noted previously (34). A promoter possessing a x light-chain octamer element gave similar results: VP16 plus Oct-1/POU was relatively inefficient compared to Oct-2 at stimulating transcription (Figure 4, lanes 7—9).

Functional specificity for Oct-1/POU by VP16

While numerous studies, using EMSA, have identified the domains critical for VP16's discrimination between Oct-1 and Oct-2 and for forming high-affinity complexes, selectivity at a functional level has not been shown. However, we found a good correlation between previously demonstrated selectivity of complex formation and transcriptional activation in vitro. When tested in the in vitro system, VP16 activated transcription in the presence of the Oct-1 POU domain but not in the presence of
Figure 5. VP16 activation is specific for Oct-1/POU. A template possessing the ICPO promoter was transcribed with HeLa nuclear extract. Lane 1, SI probe. Lane 2, nuclear extract alone. Lanes 3–5, 20, 40 and 80 ng of full length Oct-2. Lanes 6–11, 30 or 100 ng of Oct-1/POU. Lanes 8–9, 30 ng VP16. Lanes 10–11, 100 ng VP16. Lanes 12–17: the same titration range as shown in lanes 6–11 but with Oct-2/POU instead of Oct-1/POU. Relative transcription (numbers below lanes) was normalized to that of lane 2.

Figure 6. VP16 and Oct-1/POU facilitate formation of the pre-initiation complex. HeLa cell nuclear extracts, reporter and reference plasmids, were pre-incubated for the indicated times in transcription buffer before addition of Sarkosyl and NTPs for the indicated times. In lanes 2 and 5, 100 ng each of VP16 and Oct-1/POU were added with the other components of the mixture, while in lanes 3 and 6, they were added just prior to the NTPs and detergent In lanes 1 and 4, single round transcription was carried out without addition of proteins In all cases, the transcription reactions were stopped 20 minutes after addition of NTPs. A sixty-minute pre-incubation yielded roughly the same level of transcription as the forty-minute pre-incubation (results not shown).

Figure 7. Activation by VP16 in a fractionated system. Multiple-round transcriptions of the ICPO template were carried out with purified basal transcription factors. 100 ng Oct-1/POU (lanes 2 and 4) and 100 ng VP16 (lanes 3 and 4) were added to reactions. The highest band is the read-through transcript; below this is the authentic test gene transcript. Relative transcription was normalized to that without added proteins (lane 1). The reference plasmid promoter is not transcribed efficiently in this purified system, therefore there was no signal in this portion of the gel.

The Oct-2 POU domain (Figure 5). A mutant Oct-2/POU stabilized against oxidation by a Cys to Ser substitution also failed to activate with VP16 (results not shown). Therefore, although both POU domains bind to the target sequence, activation of transcription in vitro requires the specific features of Oct-1/POU shown to be crucial for binding (16,17,34,39).

VP16 and Oct-1/POU facilitate pre-initiation complex formation

Accurate initiation of transcription at polymerase II promoters requires a number of basal factors (including TFIIA, B, D, E, F and H) which form transcriptional pre-initiation complexes with RNA polymerase II (reviewed in 40; see Figure 1b). In vitro studies suggest that the components of the pre-initiation complex assemble in a specific order, with binding of TFIID (a multiprotein complex containing the TATA binding protein) to the TATA element nucleating the association of the other factors. Several transcription factors, including the chimeric GAL4-VP16 activator, have been shown to affect pre-initiation complex assembly (27–29). The effect of VP16 and Oct-1/POU proteins on the assembly of the pre-initiation complex was examined by use of single-round transcription assays, whereby pre-initiation complexes are formed in the absence of nucleoside triphosphates (NTPs) and re-initiation is prevented by addition of the detergent Sarkosyl (41) after the first round of initiation. Transcript levels
are therefore proportional to pre-initiation complex formation. Addition of VP16 and Oct-1/POU to the incubation mixture of template with nuclear extract significantly increased the level of pre-initiation complexes formed (Figure 6, lanes 2 and 5). In ‘late’ addition experiments, VP16 and Oct-1/POU were added just prior to NTPs and detergent at the end of the pre-incubation period with nuclear extracts. Under these conditions the proteins only weakly affected template commitment (Figure 6, lanes 3 and 6). The results suggest that VP16 and Oct-1/POU augment pre-initiation complex formation and act before template commitment to the transcription process.

We also examined whether purified basal transcription factors are sufficient to support the activation effect of VP16 with Oct-1/POU. Full length Oct-2 activates transcription in vitro approximately 10-fold with these purified basal factors, similar to its activity seen with crude nuclear extracts (34). In contrast, VP16 together with Oct-1/POU only modestly increased levels of transcription above the basal level (about 5 fold), with Oct-1/POU accounting for approximately half of this effect (Figure 7). It is possible that one or more factors are required in addition for VP16 and Oct-1/POU activity and that these factors may be lost or inactivated during the purification of the basal factors.

**DISCUSSION**

We have utilized an in vitro transcription system to study the functional properties of the full-length VP16 molecule, and determined that VP16 can activate transcription in conjunction with the DNA-binding POU domain from Oct-1 (Figure 3). Thus, we conclude that the portions of Oct-1 outside of the POU domain, including the glutamine-rich activation domains (Figure 1a), are not required for VP16 activity. VP16 preferentially activates promoters in vivo containing a TAATGARAT element; this specificity was reproduced in vitro (Figure 4). Mutations in the GAT AT portion of the ICPO promoter element severely weakened VP16-mediated activation but not Oct-2 activation. As expected, mutations affecting the octamer site decreased activation by both Oct-2 and the mixture of Oct-1/POU with VP16. These observations corroborate in vivo findings (19) and confirm the predictions (based on EMSAs) concerning the activational properties of VP16, Oct-1/POU, and its complex with nuclear factors (4,7). Previous in vitro transcription studies have relied on chimeric proteins which fuse the DNA binding domain of the yeast GAL4 protein and the acidic carboxy-terminal portion of the VP16, but it has not been clear that the mechanism of activation by VP16 and the chimera were the same. In crude nuclear extracts, VP16 facilitates the assembly of functional pre-initiation complexes (Figure 6), indicating that it acts at an early step in the transcription cycle. Recent work has demonstrated that GAL4/VP16 chimera also acts at an early step as well, facilitating the association of TFIID and/or TFIIB with the nascent transcriptional complex (27–29). White et al. (42) have also suggested that GAL4/VP16 acts on the pre-initiation complex early, although after association of TFIID with the promoter.

Transcriptional activators appear to interact with the basal transcription machinery through components of TFIID termed TAFs, for TATA-binding protein associated factors (27,43). We noted that VP16 was less effective in activating transcription in a partially purified system than it was in a crude nuclear extract (Figure 7), even though full length Oct-2 was equally effective in either system (34). The activation domain of Oct-2 is glutamine-rich, unlike that of VP16, and these activation domains appear to have different functional properties in vivo (44) as well as in vitro (45). Therefore, it is possible that a VP16-specific TAF was lost during the purification of basal factors. It is also possible that the decrease in VP16 activity in the purified system is due to loss of C1, or conceivably other factors, which promote association of VP16 and Oct-1/POU.

Unlike the Oct-1/POU protein, the Oct-2 factor POU domain was not capable of supporting VP16 activation (Figure 5), demonstrating that the selectivity in VP16-POU domain binding identified in earlier studies (6,20,38) is indeed responsible for the functional differences of the two octamer proteins. The principal amino acid differences between the two POU domain proteins map to a surface in the homeobox domain which would be exposed in the DNA complex (17,19,20). The possible orientation of Oct-1/POU on the ICP0 element may be inferred from recent studies dissecting its recognition of the octamer sequence. The POU-homeo domain has been shown to contact the TAAT portion of the octamer target site, while the POU-specific domain is believed to contact the ATGC portion (46). If VP16 were to interact with the homeobox portion of the Oct-1/POU, this would place its region of contact over the GATAT portion of the ICP0 promoter. Footprinting experiments suggest that the VP16 induced complex covers the major groove in this portion of the sequence (36). Because of the helical geometry, this might require that VP16 or C1 cellular factor continue to wrap around the DNA to also interact with the homeobox portion of Oct-1/POU. Perhaps to satisfy this geometry, the exposed carboxy terminal region of VP16 may lie on the opposite surface of the complex from the Oct-1 homeobox domain. Better understanding of the basis of the selectivity and activity of VP16 will come about through structural analysis of VP16 and the cellular proteins with which it interacts.

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