Characterization of Autographa californica Nucleopolyhedrovirus Infection in Cell Lines from Bombyx mori

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To characterize Autographa californica nucleopolyhedrovirus (AcMNPV) infection of the cells derived from Bombyx mori, cytopathology, cellular viability and metabolic activity, viral DNA replication, viral polypeptide synthesis, and polyhedrin gene expression were examined. AcMNPV-infected Bombyx cells displayed cytopathology and were impaired in their ability of proliferation. Immunoblot analysis showed that viral capsid polypeptides were produced in AcMNPV-infected Bombyx cells in a large quantity that was comparable to that in conventional permissive Sf21 cells, while there was a striking decrease in the production of envelope fusion protein GP64 and viral genomic DNA in Bombyx cells as compared to that in Sf21 cells. Bombyx cells supported the production of budded virions (BVs) into culture medium, although BV yield in AcMNPV-infected Bombyx cells was lower by one to two orders of magnitude than that in AcMNPV-infected Sf21 cells. No detectable amount of polyhedrin protein was produced in AcMNPV-infected Bombyx cells, and northern blot analysis demonstrated that the defect in polyhedrin production in AcMNPV-infected Bombyx cells was due to the transcriptional restriction of polyhedrin gene expression. These results indicate that in AcMNPV-infected Bombyx cells, virus replication cycle proceeds to yield a limited number of progeny BVs into culture medium, without an optimal expression of polyhedrin gene. The present study thus demonstrates that AcMNPV-infected Bombyx cells provide a unique model system to define cellular factors responsible for host-dependent viral gene expression.

Key words: Nucleopolyhedroviruses, Autographa californica NPV, Bombyx mori cells, polyhedrin, budded virion.

INTRODUCTION

Nucleopolyhedroviruses (NPVs), members of the family Baculoviridae, are large entomoviruses that are characterized by enveloped, rod-shaped nucleocapsids containing a double-stranded, covalently closed circular DNA genome of approximately 90-160 kbp (Volkman et al., 1995). NPVs have been used as a biopesticide in agriculture and forestry (Black et al., 1997; Moscardi, 1999) and as an eukaryotic expression vector for the production of functional foreign proteins (Jarvis, 1997). Recently, NPVs have also been the subject of intensive study to exploit their potential as a gene delivery vector for human gene therapy (Hofmann et al., 1995; Boyce et al., 1996; Palombo et al., 1998) and for the functional analysis of genes that play essential roles during insect development (Hajos et al., 1999; Oppenheimer et al., 1999). NPVs may also be feasible as a vector for the gene targeting in insects (Yamao et al., 1999). Successful application of NPVs to any of such approaches relies on extensive characterization of the biological properties of the viruses, especially those relevant to the expression of pathogenicity and host specificity.

NPVs have been isolated from more than 520 insect species (Adams and McClintock, 1991). NPVs from the alfalfa looper, Autographa californica, (AcMNPV) and the silkworm, Bombyx mori, (BmNPV) are among the most extensively studied NPVs and complete nucleotide sequences of their genomic DNA are available (Ayres et al., 1994; Gomi et al., 1999). Comparative analysis of the genomic DNA has demonstrated that BmNPV and AcMNPV are closely related in their genome organization, gene content, and amino acid sequence of homologous genes (Gomi et al., 1999), and recombinants between AcMNPV and BmNPV are readily obtained in the coinfected cell culture (Kondo and Maeda, 1991). Despite such genetical similarities, AcMNPV and BmNPV exhibit distinct host range properties, in such a way that AcMNPV has a broad host range while BmNPV has a narrow host range (Groner, 1986), and no unequivocal overlapping has been reported in the host range of AcMNPV and BmNPV. Comparative biological characterization of AcMNPV and BmNPV in different cell lines will thus provide insights into viral and cellular factors responsible for the determination of NPV host specificity.

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AcMNPV has been shown to produce viral structural polypeptides as well as viral DNA in Bombyx cells but there is no agreement whether budded virions (BV) are yielded in AcMNPV infected Bombyx cells (Summers et al., 1978; Volkman and Goldsmith, 1982; Maeda et al., 1990, 1993; Morris and Miller, 1993; Crozier et al., 1994). Summers et al. (1978) detected infectious BVs in the medium of B. mori 5 cell cultures infected with AcMNPV while no significant BV production was observed in either of BmN-4 and BM-N cells infected with L1 and OT2 variants of AcMNPV, respectively (Maeda et al., 1990, 1993; Morris and Miller, 1993; Sakurai et al., 1998). In a previous paper, we suggested the production of BVs in AcMNPV-infected BmN-4 cells (Shirata et al., 1999). In the present study, we characterize extensively the AcMNPV infection of Bombyx cells and demonstrate that not only viral DNA and viral structural polypeptides but also BVs are produced in AcMNPV-infected Bombyx cells. Our data also indicate that in Bombyx cells, AcMNPV polyhedrin gene expression is restricted strikingly at the level of transcription.

**MATERIALS AND METHODS**

**Viruses, cells and virus infection**

Viruses used were BmNPV N9 (Nagamine et al., 1989), AcMNPV E2 (Smith and Summers, 1978) and AcMNPV L1 (Lee and Miller, 1978). BM-N (Volkman and Goldsmith, 1982) and BmN-4 cells (Maeda, 1989) derived from the silkworm, B. mori, and Sf21 cells from the fall armyworm, Spodoptera frugiperda, (Vaughn et al., 1977) were grown at 28°C in TC 100 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum. These cells were passaged every 3 days.

Each 25-cm² culture flask (Nunc, 63371) was seeded with 2.5 × 10⁶ cells to prepare a semiconfluent monolayer culture. These cultures were infected with 1 ml of virus inoculum and rocked gently on a seesaw shaker for 60 min at room temperature to allow the viruses to be adsorbed to the cells. The cultures were washed twice with TC100 medium and incubated at 28°C in 5 ml of fresh TC100 medium. The input MOI (multiplicity of infection) was 10 PFU (plaque forming units) per cell unless otherwise noted.

**Immunoblot analysis**

Polypeptides were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) and were electroblotted onto either a nitrocellulose membrane (Advantec Toyo, Osaka, Japan) or a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA) at 200 mA for 70 min in blotting buffer (100 mM Tris, 192 mM glycine, 20% (v/v) methanol) using Horizblot (Atto, Tokyo, Japan). Polypeptides on the nitrocellulose membranes were reacted with antiserum against BmNPV occluded virions, BmNPV polyhedrin, or AcMNPV BVs as previously described (Choi et al., 1989). Antibodies reacted with corresponding antigens were then reacted with horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG or anti-mouse IgG antibody (Zymed Laboratories, San Francisco, CA), and visualized by Konica immunostain HRP-1000 (Konica, Tokyo, Japan). For GP64 protein detection, the PVDF membrane was blocked with 5% skim milk in TBS (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl) and polypeptides on the PVDF membrane were reacted with anti-GP64 monoclonal antibody in TBS containing 0.05% Tween 20 and 2% skim milk. Monoclonal antibody reacted with GP64 was then reacted with goat anti-mouse IgG antibody conjugated with HRP. The membrane was reacted with ECL western blotting detection reagents (Amersham Pharmacia Biotec, Buckinghamshire, England) and contacted to a Hyperfilm (Amersham Pharmacia Biotec) to detect GP64 protein.

Antisera against BmNPV occluded virions and polyhedrin were produced in rabbits as previously described (Kobayashi et al., 1990), whereas antiserum against AcMNPV E2 BVs was prepared in mice according to the method described previously (Nagamine et al., 1991). AcMNPV E2 BVs for immunization were purified from the medium of AcMNPV E2-infected Sf21 cells as described previously (Nagamine et al., 1991). Monoclonal antibody against AcMNPV GP64 protein was purchased from Clontech Laboratories (Palo Alto, CA).

**Slot-blot hybridization analysis of viral DNA**

Slot-blot hybridization analysis of viral genomic DNA was performed as described previously (Ikeda and Kobayashi, 1999). Briefly, viral DNA was extracted from the virus-infected cells into heated supersaturated-NaI (Bresser and Gillespie, 1983) and blotted onto Hybond N+ nylon membranes (Amer- sham Pharmacia Biote). The membranes were processed for hybridization with fluorescein-labeled DNA probe according to the protocols of Gene Images Random-Prime Labeling Module and CDP-Star Detection Module (Amer- sham Pharmacia Biote). The DNA probe used for the detection of AcMNPV genomic DNA was HindIII-I fragment (approximately 4.2 kbp) of AcMNPV genome (Shirata et al., 1999).
Northern blot analysis

Total RNA was isolated from infected cultures (1 × 10^7 cells/80-cm² flask) with TRIzol (Gibco BRL). Infected cultures were rinsed with PBS (8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl) and lysed in 6 ml of TRIzol. After a 5-min incubation at room temperature, lysates were mixed with 1.2 ml of chloroform and the mixtures were shaken gently for 3 min, followed by centrifugation at 12,000 g for 15 min at 4°C. The aqueous phase was transferred into a new tube, to which 3 ml of 2-propanol was added to precipitate RNA. The RNA precipitate was collected by centrifugation at 12,000 g for 10 min at 4°C, washed with 75% ethanol, dissolved in 0.5% SDS, and heated at 60°C for 10 min. The RNA samples were stored at -80°C until used.

Total RNAs (25 μg/lane) were electrophoresed on formaldehyde-denaturing agarose gels [1.2% SeaKem GTG agarose (FMC BioProducts, Rockland, ME), 0.02 M morpholinopropanesulfonic acid (MOPS), 2.2 M formaldehyde] in 0.02 M MOPS buffer. Following the electrophoresis, RNAs were blotted onto a Hybond N+ nylon membrane (Amersham Pharmacia Biotec) in 0.05 M NaOH. The DNA probes were denatured in boiling water and hybridized to RNAs on the membrane at 42°C for 16 h. The hybridized membranes were successively washed twice in 2 X SSPE (SSPE: 150 mM NaCl, 10 mM Na₂HPO₄, 1 mM EDTA, pH 7.4) at room temperature and twice in 0.2 X SSPE-0.1% SDS at 65°C. Then the membranes were been in contact with an imaging plate or an X-ray film. The DNA probe used was a 736-bp DNA fragment amplified by PCR with AcMNPV E2 DNA as the template and two synthetic oligonucleotides, 5'-GCCGGATTTACATACGGTC-3' and 5'-TTAATACGCCGGACCAGTG-3', as the primers. These two paired primers are based on AcMNPV polyhedron gene sequences that are mapped to nucleotides 4,522 to 4,541 and 5,238 to 5,257, respectively (Ayres et al., 1994).

Cellular viability assay

Monolayer cultures of Sf21 or BM-N cells (2.5 × 10⁴ cells/well) were prepared in 96-well tissue culture plates (Falcon 3072) and infected with AcMNPV E2 or BmNPV at an input MOI of 2 or 20 PFU per cell. The infected cultures were incubated at 28°C in 100 μl of TC100 medium, and at 24 h intervals until 120 h postinfection (pi), infected cells were assayed with MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]-based cell proliferation kit I (Boehringer Mannheim, Indianapolis, IN) according to the protocol supplied by the manufacturer.

Plaque assay

Plaque assay was performed in BM-N cells and Sf21 cells for BmNPV and AcMNPV, respectively, as described previously (Shirata et al., 1999). Semicontiguous monolayer cultures consisting of 8 × 10⁶ cells were prepared in 35-mm tissue culture dishes (Falcon 3001), and a 200 μl of virus suspension to be assayed was applied to each culture after removing the medium. After a 60-min adsorption period at room temperature, the inoculum was replaced by 3 ml of TC100 medium containing 0.75% SeaPlaque GTG agarose (FMC BioProducts) and the cultures were incubated at 28°C for 5-6 days until plaques became clearly observed.

RESULTS

Cytopathology and cellular proliferation

Light microscopic observations demonstrated that the growth of BM-N cells was arrested upon infection with AcMNPV E2 and AcMNPV E2-infected BM-N cells manifested marked morphological alterations. The morphological alterations in AcMNPV E2-infected BM-N cells could be detected by 12 h postinfection (pi), and were more clearly observed as the times pi increased (Fig. 1). The AcMNPV E2-infected BM-N cells became spherical with swollen nuclei which contained virogenic stroma-like structure. These features in AcMNPV E2-infected BM-N cells differed from those in either AcMNPV E2-infected Sf21 cells or BmNPV-infected BM-N cells.

To examine if BM-N cells survived the AcMNPV E2 infection, MTT assay was performed. Monolayer cultures prepared in a 96-well tissue culture plate were infected with AcMNPV E2 or BmNPV, and assayed for viable cells at intervals up to 120 h pi (Fig. 2A). The results showed that MTT activity (cellular reduction activity for MTT) in AcMNPV E2-infected BM-N cells declined progressively throughout the experiment, while that in mock-infected cells increased continuously and reached a plateau at 120 h pi. These features in AcMNPV E2-infected BM-N cells differed from those in either AcMNPV E2-infected Sf21 cells or BmNPV-infected BM-N cells.

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Experiments with Sf21 cell line that is permissive for AcMNPV E2 infection showed results similar to those in AcMNPV E2-infected BM-N cells, in which MTT activity in AcMNPV E2-infected Sf21 cells decreased continuously as the infection proceeded (Fig. 2B). In BmNPV-infected Sf21 cells, on the other hand, MTT activity continued to increase as in mock-infected cells, except for the presence of a 24-h lag period and the ultimate MTT activity that was significantly lower than that in mock-infected cells (Fig. 2B).

### Viral DNA replication

Monolayer cultures of BM-N and Sf21 cells were infected with AcMNPV E2, and at 0, 6, 12, 24, 48 and 72 h pi, viral DNA accumulation in the infected cells was examined by slot-blot hybridization analysis (Fig. 3). Under the conditions used, viral DNA accumulation in Sf21 cells increased abruptly and linearly from 12 h pi and reached a plateau at 48 h pi. Viral DNA was also produced unequivocally in AcMNPV-infected BM-N cells, but an ultimate yield of viral DNA in AcMNPV-infected BM-N cells was about one-fourth of that in AcMNPV-infected Sf21 cells.

### Accumulation of viral structural polypeptides

To examine whether viral polypeptides are accumulated in AcMNPV E2-infected BM-N cells, monolayer cultures of BM-N cells were infected with AcMNPV E2, and at 48 h pi, polypeptides were resolved by SDS-PAGE and viral polypeptides were identified by immunoblot analysis using antisera against BmNPV occluded virions or against AcMNPV E2 BVs. The immunoblots showed that viral polypeptides of AcMNPV E2 and BmNPV were reactive not only with homologous but also with heterologous antisera, and minimum 8 polypeptides were clearly detected in BM-N cells infected with AcMNPV (Fig. 4). On the basis of its amount and MW, strong band with approximate MWs of 40K consisted of VP39.
major capsid protein. Another major polypeptide in Figure 4B with approximate MW of 66K that was specifically detected with anti-AcMNPV BV antiserum probably corresponded to GP64 envelope fusion protein. The amount of 66K polypeptide (arrowhead) as relative to that of putative VP39 protein (arrow) was higher in Sf21 cells than in BM-N cells (Fig. 4B, lane 5 vs 6).

To examine kinetics of viral polypeptide accumulation, BM-N and Sf21 cells were infected with AcMNPV E2, and polypeptides from infected cells at 0, 12, 24, 48, and 72 h pi were analyzed by immunoblotting with anti-BmNPV occluded virion antiserum as a probe (Fig. 5A). The immunoblots showed that kinetics of viral polypeptides accumulation following AcMNPV E2 infection were similar between BM-N and Sf21 cells.

Since data in Fig. 4B indicated that less GP64 protein was produced in BM-N cells than in Sf21 cells following the infection with AcMNPV, kinetics of GP64 protein accumulation were examined in AcMNPV E2-infected BM-N and Sf21 cells with anti-AcMNPV GP64 monoclonal antibody (Fig. 5B). Staying consistent with the data in Fig. 4B, GP64 band was detected earlier in Sf21 cells and a yield of GP64 protein on infected-cell number basis was significantly higher in S21 cells than in BM-N cells.
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To exclude the possibility that BV production is confined to BM-N cells infected with AcMNPV E2, BV production was also examined in both BM-N and BmN-4 cells infected with AcMNPV L1 (Fig. 6B). The results showed that both BM-N cells and BmN-4 cells supported replication of AcMNPV L1 to produce progeny virions into culture medium. Under these conditions, AcMNPV L1 BV titre increased to about 200-fold and 600-fold in BmN-4 and BM-N cells, respectively, during 48 h of infection. BmNPV BV titre, on the other hand, increased more than 7,000-fold in BM-N cells during the same period of infection (Fig.

**DISCUSSION**

In the present study, we have characterized the interaction between Autographa californica NPV (AcMNPV) and cells derived from Bombyx mori (BM-N and BmN-4 cells), by examining various parameters relevant to virus infection that include cytopathology, cellular viability and metabolic activity, viral DNA replication, viral polypeptide synthesis, budded virion (BV) production, and polyhedrin gene expression. Our data demonstrate that Bombyx cells infected with AcMNPV synthesize viral DNA and viral structural polypeptides, and produce unequivocal amount of progeny BVs into the culture medium. It is also demonstrated that polyhedrin does not accumulate significantly in AcMNPV-infected Bombyx cells and that this restriction of polyhedrin accumulation is due to the transcriptional restriction of polyhedrin gene expression. Our data in the present study thus corroborate and extend earlier finding that infectious progeny BVs are produced without detectable production of polyhedrin in AcMNPV-infected Bombyx cells (Summers et al., 1978; Shirata et al., 1999).

Our finding that BVs are produced significantly in AcMNPV-infected Bombyx cells agrees with that in AcMNPV-infected Bm5 cells (Summers et al., 1978), but is in contrast to the results obtained in BmN-4 and BM-N cells infected with AcMNPV L1 and OT2.
respectively (Maeda et al., 1990; Morris and Miller, 1993; Sakurai et al., 1998). The basis that explains such a discrepancy in the ability of Bombyx cells to support AcMNPV BV production into culture medium is not known. However, it seems unlikely that the discrepancy is attributed to the differences in the Bombyx cell lines and/or AcMNPV variants used in the experiments since our data in the present study together with those in the previous study (Shirata et al., 1999) showed that BV production occurred in any of the AcMNPV-Bombyx cell combinations that consisted of two AcMNPV variants, E2 and L1, and two Bombyx cell lines, BM-N and BmN-4. Our data also indicated that AcMNPV BV yield on the cell number basis was higher in BM-N cells than in BmN-4 cells (Fig. 4B). The higher BV productivity in BM-N cells compared with that in BmN-4 cells was also observed following the infection with BmNPV (data not shown)

Fig. 6. Growth curves of AcMNPV budded virion in Bombyx cells. Monolayer cultures (2.5 x 10^6 cells/25-cm² flask) of BM-N cells were infected with AcMNPV E2 (A), and monolayer cultures of BM-N (○) and BmN-4 cells (△) were infected with AcMNPV L1 (B). After the adsorption period of 60 min, infected cultures were washed three times with 2 ml of TC100 medium, and incubated at 28°C in 5 ml of fresh TC100 medium. AcMNPV budded virion yield into culture medium was titred by plaque assay on Sf21 cells. Growth curve of BmNPV budded virion in BM-N cells (○) was also determined by plaque assay on BM-N cells and incorporated into panel B.

Fig. 7. Immunoblot analysis of AcMNPV polyhedrin production in BM-N cells. BM-N cells were infected with AcMNPV E2 (AcMNPV/BM) and at 48 h postinfection, polypeptides from infected cells were examined for polyhedrin by immunoblot analysis with anti-BmNPV polyhedrin antiserum. Polyhedrin production in AcMNPV E2-infected Sf21 cells (AcMNPV/SF), BmNPV-infected Sf21 cells (BmNPV/SF), and BmNPV-infected BM-N cells (BmNPV/BM) was also examined.

Fig. 8. Northern blot analysis of polyhedrin gene expression in AcMNPV E2-infected BM-N cells. Monolayer cultures of BM-N cells (A) and Sf21 cells (B) were infected with AcMNPV E2 and at 0, 12, 24, 48, and 72 h postinfection, total RNA was isolated from infected cells with TRizol, resolved on an agarose gel, blotted onto a Hybond N+ nylon membrane, and hybridized with 32P-labeled polyhedrin gene. M, Mock-infected cells.
BV yield in AcMNPV-infected *Bombbyx* cells was lower by one to two orders of magnitude than that in AcMNPV-infected Sf21 cells, in which BV titres by plaque assay usually attain to a level of $1 \times 10^7$ PFU per ml of culture medium or higher. Kinetic studies showed that viral capsid polypeptides accumulated in AcMNPV-infected BM-N cells in a large quantity that was comparable to that in conventional permissive Sf21 cells infected with AcMNPV (Fig. 5A), while both viral DNA and GP64 envelope protein were produced to a lesser extent in AcMNPV-infected BM-N cells compared to AcMNPV-infected Sf21 cells (Figs. 3, 5B). On the basis of the fact that a GP64-null mutant of AcMNPV not only produces little infectious BVs but also is a defect in cell-to-cell movement in cell culture (Monsma et al., 1996; Oomens and Blissard, 1999), it is possible that reduced BV production in AcMNPV-infected *Bombbyx* cells is a direct consequence of restricted GP64 expression. It is also possible that restricted production of viral genomic DNA is responsible for the reduced production of BVs in AcMNPV-infected *Bombbyx* cells.

Northern blot analysis revealed that low but significant levels of polyhedrin gene transcript were detected in the AcMNPV E2-infected BM-N cells (Fig. 8), indicating that AcMNPV polyhedrin gene was transcriptionally expressed in the AcMNPV-infected *Bombbyx* cells. Further experiment with BacPAK6 recombinant virus (Clontech Laboratories), a derivative of AcMNPV C6 that carried lacZ gene driven by the polyhedrin promoter, suggested that transcripts from AcMNPV polyhedrin gene were translated in *Bombbyx* cells, showing that BacPAK6-infected BmN-4 cells were stained blue with X-gal (Yamada, unpublished). Taking into account the fact that polyhedrin gene is categorized into a very late gene and its expression depends upon prior expression of certain early viral genes, these results imply that viral proteins involved in transactivation of AcMNPV polyhedrin gene expression are produced in AcMNPV-infected BmN-4 cells. It is possible that in AcMNPV-infected *Bombbyx* cells, an AcMNPV-specific gene product, such as AcMNPV VLF-1, is specifically responsible for the burst expression of very late gene in permissive Sf21 cells (Mclachlin and Miller, 1994; Yang and Miller, 1998a, b, 1999), is produced insufficiently to trigger the optimal expression of polyhedrin gene. Alternatively, it is possible that such product is much less functional in *Bombbyx* cells in the transactivation of AcMNPV polyhedrin promoter.

Visual examination under a microscope showed that growth of BM-N cells was arrested following the infection with AcMNPV E2, and the infected BM-N cells exhibited a general cytopathology that was characteristic of NPV infection except for polyhedron formation (Fig. 1). Data from MTT assay further showed that cytopathology in AcMNPV E2-infected BM-N cells accompanied suppression of cellular metabolic activity, and patterns of the virus-induced metabolic suppression was similar between BM-N and Sf21 cells (Fig. 2). These results are in accord with the fact that shut down of cellular protein synthesis occurs in *Bombbyx* cells upon infection not only with BmNPV but also with AcMNPV (Shirata, unpublished), and indicate that after viral entry into the cells, AcMNPV interacts with *Bombbyx* cells in a similar manner as with Sf21 cells, without being able to produce polyhedrin and to yield optimal amount of BVs. These facts suggest that AcMNPV-infected *Bombbyx* cells provide a unique system to define possible cellular and viral factors that are responsible for polyhedrin production and progeny BV yield into culture medium. Experiments are currently underway in our laboratory to examine if VLF-1 and the observed defect in polyhedrin production in AcMNPV-infected *Bombbyx* cells are related.

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