Antigen and Polypeptide Synthesis by Temperature-sensitive Mutants of Respiratory Syncytial Virus

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SUMMARY

A revised nomenclature for the polypeptides of respiratory syncytial (RS) virus has been devised on the basis of comparison of the Long, A2 and RSN-2 strains by slab-gel electrophoresis. Seven polypeptides, now designated VP200, VGP48, VPN41, VPP32, VPM27, VP25 and VP10, were observed in preparations of all three strains of RS virus, irrespective of the host cell of origin. In addition, a slowly migrating glycopolypeptide GP1 was prominent in partially purified RS virus of the Long and A2 strains obtained from Hep-2 cells, and to a lesser extent from BS-C-1 cells. In the case of the RSN-2 strain, this polypeptide was only resolved clearly in virus obtained from Hep-2 cells. GP1 was an atypical glycopolypeptide in that $^{35}$S-methionine incorporation was poor relative to $^3$H-glucosamine incorporation.

The ts mutants of RS virus exhibited four distinct phenotypes with respect to intracellular polypeptide synthesis and antigen production at 39 °C. Mutants ts 17 (complementation group B') and ts 19 (group E) were almost completely restricted, suggesting defective early functions. Mutants ts A1 (group A), ts A7 (group C) and ts 1 (group D) synthesized antigen and polypeptides normally, but the amount of antigen at the cell surface was reduced, suggesting maturation defects. In addition, the VPP32 of ts 1 (group D) exhibited an aberrant mobility, confirming its viral specificity. The remaining mutants, representing groups B, F and G exhibited generally impaired synthesis at 39 °C.

Absence of surface filaments in ts mutant-infected cells at 39 °C confirmed their virus-specific nature.

INTRODUCTION

Temperature-sensitive (ts) mutants of the RSN-2 strain of human respiratory syncytial (RS) virus have been classified into six complementation groups, two of which are homologous with two of the three complementation groups of the A2 strain described by Wright et al. (1973). Thus, a minimum of seven groups have been identified, designated A, B and C for the A2 strain, and A, B, C, D, E, F and G for the RSN-2 strain (Gimenez & Pringle, 1978). The conditions for obtaining complementation between mutants of different strains were stringent and more recent work has indicated that complementation can occur between mutant ts A2 (A2 strain) and mutant ts 17 (RSN-2 strain), both previously classified in group B (Richardson & Chanock, personal communication; C. R. Pringle, unpublished data). Consequently, a minimum of eight complementation groups may exist. Since several of

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the groups (C, D, E, F and G) contain a single mutant only, it is not possible to determine whether there are eight independent gene functions, or whether some of the complementation represents intragenic complementation. To analyse this problem, we have initiated a study of the properties of these complementing mutants to define the defective functions.

Representative mutants of the eight groups (now designated A, B, B', C, D, E, F and G) have been characterized in terms of antigen and polypeptide synthesis at restrictive temperature. Between six and nine viral polypeptides have been described (Wunner et al., 1975; Wunner & Pringle, 1976; Levine, 1977; Cash et al., 1977, 1979; Peeples & Levine, 1979). Immunoprecipitation (Pringle & Cross, 1978) has verified the viral specificity of some of these polypeptides and the function of a few has been established (Wunner & Pringle, 1976; Peeples & Levine, 1979), but there remains some confusion and doubt about the identity and mol. wt. of others. The characterization of the polypeptides synthesized in ts mutant-infected cells has confirmed the viral specificity of most of these polypeptides and helped to resolve some of the confusion. We have been particularly interested in examining the nature of the high mol. wt. glycopolypeptide consistently observed in Hep-2 cells infected with the Long strain of RS virus (Levine, 1977; Peeples & Levine, 1979).

**METHODS**

**Cells.** BS-C-1 and Hep-2 cells were maintained in Eagle's medium (Glasgow modification) supplemented with 5% foetal calf serum (Flow Laboratories; mycoplasma and virus screened).

**Virus.** The wild-type and mutants of the A2 strain isolated by Gharpure et al. (1969) were obtained from Dr R. M. Chanock, National Institutes of Health, Bethesda, Md., U.S.A. For clarity in this paper these mutants have been given the prefix A, i.e. ts A1 (group A), ts A2 (group B) and ts A7 (group C). The origin of the RSN-2 strain and its mutants has been described previously (Faulkner et al., 1976). The Long strain was a plaque-purified isolate propagated in HeLa cells (Levine, 1977).

**Virus concentration and purification.** Virus was grown in roller bottle cultures of BS-C-1 or Hep-2 cells and the medium was harvested when c.p.e. was extensive, but before cells had detached from the glass surface. The medium was clarified by centrifugation in an MSE Coolspin centrifuge for 15 min at 2000 rev/min. This clarified material was then centrifuged through 20% sucrose in Dulbecco's phosphate-buffered saline (PBS) at 4 °C for 4 h at 25000 rev/min (Sorvall rotor AH627) on to a 60% sucrose cushion. A virus-containing band was collected from the interface between the 20 and 60% sucrose.

This material was subjected to a second cycle of centrifugation at 4 °C through a 30% sucrose layer on to a 60% sucrose cushion (4 h at 25000 rev/min, Sorvall rotor AH627). The virus-containing band at the interface was collected and stored at −70 °C. The partially purified virus from the 30 to 60% sucrose interface was diluted with Dulbecco's PBS and then pelleted by centrifugation at 45000 rev/min for 2 h at 4 °C in a Sorvall AH650 rotor. The pellet was resuspended in a small volume of electrophoresis boiling mix.

**Radiolabelling, preparation of protein samples and SDS–polyacrylamide gel electrophoresis.** These procedures were identical to those described by Cash et al. (1977). L-35S-methionine (5 μCi/ml, 200 to 300 Ci/mmol) and D-1-3H-glucosamine (10 μCi/ml, 1 Ci/mmol) were obtained from The Radiochemical Centre, Amersham. 35S-methionine-containing gels were autoradiographed and 3H-glucosamine-containing gels were fluorographed as described by Cash et al. (1977).

**Immunofluorescent staining.** Indirect immunofluorescent staining of live and acetone-fixed infected cells were carried out as described previously (Faulkner et al., 1976; Pringle et al., 1978). The bovine anti-RS virus serum was a gift from Dr J. Stott, Agricultural Research Council Animal Diseases Research Institute, Compton, U.K. Fluorescein isothiocyanate
Table 1. Surface and intracellular antigen in mutant-infected cells at restrictive temperature

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Strain</th>
<th>Complementation group</th>
<th>Antigen Ratio 39/31 °C (%)</th>
<th>Heat Stability* (2 h at 39 °C)</th>
<th>Intra-cellular protein synthesis†</th>
</tr>
</thead>
<tbody>
<tr>
<td>ts A1</td>
<td>A2</td>
<td>A</td>
<td>77.3</td>
<td>4.6</td>
<td>Sensitive</td>
</tr>
<tr>
<td>ts 26</td>
<td>RSN-2</td>
<td>A</td>
<td>95.0</td>
<td>21.9</td>
<td>Resistant</td>
</tr>
<tr>
<td>ts A2</td>
<td>B</td>
<td>A</td>
<td>15.4</td>
<td>34.6</td>
<td>Resistant (+)</td>
</tr>
<tr>
<td>ts 17</td>
<td>RSN-2</td>
<td>B*</td>
<td>11.6</td>
<td>&lt;3.0</td>
<td>Resistant</td>
</tr>
<tr>
<td>ts A7</td>
<td>B</td>
<td>C</td>
<td>96.3</td>
<td>13.8</td>
<td>Resistant</td>
</tr>
<tr>
<td>ts 1</td>
<td>RSN-2</td>
<td>D</td>
<td>250.0</td>
<td>50.0</td>
<td>Resistant</td>
</tr>
<tr>
<td>ts 19</td>
<td>RSN-2</td>
<td>E</td>
<td>7.9</td>
<td>1.4</td>
<td>Resistant</td>
</tr>
<tr>
<td>ts 20</td>
<td>RSN-2</td>
<td>F</td>
<td>19.7</td>
<td>20.1</td>
<td>Resistant</td>
</tr>
<tr>
<td>ts 6</td>
<td>RSN-2</td>
<td>G</td>
<td>22.7</td>
<td>29.9</td>
<td>Resistant</td>
</tr>
<tr>
<td>Wild-type</td>
<td>RSN-2</td>
<td>—</td>
<td>88.9</td>
<td>ND‡</td>
<td>Resistant</td>
</tr>
<tr>
<td>Wild-type</td>
<td>RSN-2</td>
<td>—</td>
<td>132.0</td>
<td>68.3</td>
<td>Resistant</td>
</tr>
</tbody>
</table>

* $V_0 - V_1$; resistance = $\leq 1.0 \log_{10}$ units, sensitivity = $1.8 \log_{10}$ units.
† See Fig. 3 and 4.
‡ ND, Not done.

(FITC)-conjugated rabbit anti-bovine globulin was obtained from Nordic Diagnostics, London, U.K. The values in Table 1 are based on average counts of 384 cells for unfixed preparations and 439 cells for fixed preparations.

**Thermal inactivation.** Virus-containing culture supernatant was clarified by centrifugation at 3000 rev/min for 10 min in an MSE Minor centrifuge, and diluted 1/5 in Eagle's medium with 5% foetal calf serum. Aliquots in screw-capped vials were totally immersed in a water bath at 39 °C. Tubes were removed at 30-min intervals and assayed for residual infectivity.

**RESULTS**

**Antigen production in mutant-infected cells at 39 °C**

BS-C-1 cell monolayers infected with wild-type or *ts* mutants were stained by indirect immunofluorescence and the number of cells exhibiting viral antigen counted. Staining of unfixed cells gave the number of cells expressing viral antigen at the cell surface and staining of fixed cells gave the number of cells with intracellular antigen. Table 1 (column 5) shows that the number of cells with surface fluorescence at 39 °C was reduced in all cultures infected with *ts* mutants of all groups. The number of cells exhibiting intracellular fluorescence at 39 °C (Table 1, column 4) was also reduced except in cultures infected with *ts* 26 (group A), *ts* A7 (group C) and *ts* 1 (group D).

Qualitative differences in immunofluorescent staining were observed in addition to these quantitative differences. Fig. 1 illustrates the types of membrane fluorescence observed. In wild-type (RSN-2 strain)-infected cells the surface processes characteristic of RS virus infection were clearly visible in syncytial cells at both permissive (Fig. 1 a) and restrictive (Fig. 1 b) temperatures. Polarization of antigen was more marked at 39 °C, but otherwise there was no difference in the pattern of staining. In cultures infected by mutant *ts* A1 (group A) there was a marked reduction of membrane antigen at 39 °C in the few cells which exhibited fluorescence, and processes were absent (Fig. 1 d). In cells infected with mutant *ts* 20 (group F) the amount of antigen was markedly reduced at the restrictive temperature and the staining was more linear (Fig. 1 f). The distribution of antigen in fixed cells is shown in Fig. 2. In wild-type-infected cells filaments were observed at 31 and 39 °C (Fig. 2 a, b), and in mutant-infected cells at 31 °C (not shown). However, filamentous processes were not
Fig. 1. Indirect immunofluorescence staining of unfixed cells showing antigen at the membrane. The cells were stained with bovine anti-RS virus serum and FITC-conjugated anti-bovine globulin. (a) A syncytium of BS-C-1 cells 48 h after infection with wild-type (RSN-2) virus at 31 °C, showing surface filaments. (b) The same at 39 °C. (c) BS-C-1 cells infected with mutant ts A1 at 31 °C, showing surface filaments. (d) The same at 39 °C, showing reduction of antigen and absence of filaments. (e) A BS-C-1 cell infected with mutant ts 20 at 31 °C, showing surface filaments. (f) The same at 39 °C, showing reduction and linearization of antigen and absence of filaments.

observed at 39 °C in cells infected with mutants of any complementation group (Fig. 2 c to f), which established conclusively the virus specificity of these modifications of the cell membrane and their association with release of infectious virus. Distinct nuclear antigen was detected occasionally in mutant-infected cells at 39 °C (Fig. 2 h) and frequently this antigen was distributed as a ring around the nucleolus (Fig. 2 e). Nucleolar rings have been described...
Fig. 2. Indirect immunofluorescence staining of acetone-fixed cells showing intracellular antigen. The cells were stained with bovine anti-RS virus serum and FITC-conjugated anti-bovine globulin. (a, b) BS-C-1 cells infected with wild-type (RSN-2) virus after 48 h at (a) 31 °C and (b) 39 °C respectively, showing filaments. (c to j) Pattern of immunofluorescence observed in mutant-infected cells at 39 °C: (c) ts A1, (d) ts 26, (e) ts 17, (f) ts A7, (g) ts 1, (h) ts 19, (i) ts 20 and (j) ts 6. Filaments are absent and faint nuclear staining can be observed in (e) (mutant ts 17) and (h) (mutant ts 19); in (e) the staining is located as a ring around the nucleolus.
Previously in persistently infected BS-C-1 cells (Pringle et al., 1978), and it is likely that these can only be observed under conditions where cytoplasmic fluorescence is reduced.

These data suggest that mutants ts17 (group B') and ts19 (group E) may be defective in early functions, since intracellular and surface antigen production were almost totally restricted at 39 °C, whereas the other mutants are defective in late functions. The difference in immunofluorescence phenotype (Table 1, column 5) of ts A2 (group B) and ts17 (group B') also suggests that these mutants represent lesions in different genes, and hence that groups B and B' should rank as distinct complementation groups.

**Thermolability of released virus**

The thermolability of the ts mutants was examined by incubating released virus for varying periods at the restrictive temperature of 39 °C. Only one mutant, ts A1 (group A), as previously reported by Kalica et al. (1973), was heat-sensitive (Table 1). Since mutant ts 26...
of group A was heat-resistant, however, it was not possible to conclude unequivocally that complementation group A identified a structural gene.

**Polypeptide synthesis in infected BS-C-1 cells at 39 °C**

In general, the pattern of intracellular polypeptide synthesis was consistent with the pattern of intracellular antigen (Fig. 3, 4; Table 1). Mutants *ts* A1 (group A), *ts* A2 (group B) and *ts* A7 (group C) of the A2 strain, and mutants *ts* 17 (group B'), *ts* 1 (group D), *ts* 19 (group E), *ts* 20 (group F) and *ts* 6 (group G) of the RSN-2 strain were grown at 31 °C in the presence of 35S-methionine until c.p.e. was extensive. Released virus was concentrated and purified as described and the radiolabelled polypeptides compared by polyacrylamide gel electrophoresis in gradient slab gels. Minor differences were observed in the mobility of VGP48, VPP32 and VPM27 of the A2 and RSN-2 strains as reported previously (Cash et al., 1977) but significant differences were not detected between the mutants of each strain and their wild-type parent except for the VPP32 of *ts* 1 (group D) which ran slower (data not shown).

Monolayers of BS-C-1 cells were infected and incubated at 31 or 39 °C for 48 h, and then incubated in medium containing 5 μCi/ml 35S-methionine at both 31 and 39 °C for a further 24 h. Protein was extracted as described and the radiolabelled intracellular polypeptides compared by electrophoresis in polyacrylamide gels. Fig. 3(b) shows the profile of polypeptides observed in cells infected with RSN-2 wild-type at both temperatures (tracks 3 and 4). There was no marked shut-off of host protein synthesis, but five viral polypeptides were clearly resolved at both temperatures. These were VP200 (a high mol. wt. core protein), VPN41 (the nucleocapsid protein), VPN38 (a cleavage product of VPN41), VPP32 (a phosphoprotein) and VPM27 (a non-glycosylated membrane protein). The glycosylated membrane protein VGP48 was resolved at 31 °C, but less well at 39 °C.

The phosphorylated VPP32 of mutant *ts* 1 (group D) migrated more slowly than the corresponding polypeptide extracted from cells infected with the wild-type or other mutants (Fig. 3b). This mutational effect on VPP32 indicates that this polypeptide is a viral gene product, confirmation of which had been lacking by its absence in immunoprecipitates (Pringle & Cross, 1978). Cash et al. (1979) observed that the unphosphorylated VPP32 migrated slightly more rapidly than the phosphorylated form, and a change in phosphorylation could account for the mobility change of VPP32 of *ts* 1 (group D).

Viral polypeptide synthesis was markedly reduced at 39 °C in cells infected with mutants *ts* 17 (group B') and *ts* 19 (group E) (Fig. 4a), but similar to wild-type synthesis in cells infected with mutants *ts* A1 (group A), *ts* A7 (group C), *ts* 1 (group D), *ts* 20 (group F) and *ts* 6 (group G) (Fig. 3, 4).

**Glycoprotein synthesis in RS virus-infected cells**

Comparison of the Long, A2 and RSN-2 strains in the same gel system has established that the polypeptides designated VP0, VP2, VP3, VP4 and VP5 by Peeples & Levine (1979) correspond to the VP200, VGP48, VP41, VPP32, VP27 and VP25 described by Wunner & Pringle (1976) and Cash et al. (1977). However, no polypeptide corresponding to the strongly labelled VP1 glycopolypeptide described by Levine (1977) and Peeples & Levine (1979) with the Long strain of RS virus grown in Hep-2 cells was ever detected either intracellularly in BS-C-1 cells (Fig. 3, 4), or in partially purified virus of the RSN-2 strain (Wunner & Pringle, 1976; Cash et al., 1977, 1979; Pringle & Cross, 1978). To resolve this discrepancy, we compared the polypeptide profiles of partially purified virus obtained when the Long, RSN-2 and A2 strains were grown in both Hep-2 and BS-C-1 cells, labelled with both 3H-glucosamine and 35S-methionine.

The GP1 band (previously designated VP1; see Table 2) was clearly resolved only when 3H-glucosamine was used as the radiolabel, and was scarcely detectable in 35S-labelled virus.
GP1 was present both in infected cell extracts and partially purified virus. Hep-2 cell cultures were infected with either mutant ts A1 or mutant ts A7 and incubated for 48 h in the presence of $^3$H-glucosamine. The isotope-containing medium was replaced with normal medium and the culture fluids collected after 4 h incubation at 31 °C. The proteins of the virus released during the chase period (Fig. 5, tracks 1 to 3) were compared with $^{35}$S-methionyl proteins (Fig. 5, track 4) labelled under similar conditions. A prominent GP1 band was observed in virus released from Hep-2 cells infected at 31 °C with either ts A1 (track 3) or ts A7 (track 1). No corresponding $^3$H-glucosamine-labelled band was observed when ts A1-infected cells were incubated at restrictive temperature before the 4 h chase at 31 °C (track 2), and no virus particles were released. Thus, there appeared to be a specific association between GP1 and productive RS virus infection.

The GP1 band was prominent in preparations of the Long and A2 strains grown in Hep-2 cells. GP1 was also detectable in RSN-2 strain virus from Hep-2 cells, but less so in RSN-2
**RS virus ts mutants**

Table 2. *A revised nomenclature for the polypeptides of RS virus*

<table>
<thead>
<tr>
<th>New nomenclature</th>
<th>Previous designation</th>
<th>Evidence for virus specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP200</td>
<td>VP0</td>
<td>?</td>
</tr>
<tr>
<td>GP1</td>
<td>VP1</td>
<td>?</td>
</tr>
<tr>
<td>VGP48</td>
<td>VP2</td>
<td>Immunoprecipitation</td>
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<td>VPN41</td>
<td>VP3</td>
<td>Immunoprecipitation</td>
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<tr>
<td>VPP32</td>
<td>VP4</td>
<td>Mutation</td>
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<td>VPM27</td>
<td>VP5</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>VP25</td>
<td>VP6</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>VP10</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

† Cash et al., 1977.
‡ Pringle & Cross, 1978; and this paper.

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Fig. 5. Comparison of ³H-glucosamine- (tracks 1 to 3) and ³S-methionine- (track 4) labelled polypeptides of the A2 strain of RS virus released from Hep-2 cells. Track 1, ³H-glucosamine-labelled polypeptides of virus released from *ts* A7-infected Hep-2 cells during a 4 h chase period after incubation for 48 h at 31 °C in isotope containing medium; track 3, similarly labelled polypeptides from *ts* A1-infected Hep-2 cells; track 2, the same but with incubation at 39 °C during the labelling period; and track 4, ³S-methionine labelled marker polypeptides of wild-type RS virus (A2 strain).

Fig. 6. Comparison of ³H-glucosamine- (tracks 2 and 3) and ³S-methionine- (tracks 4 and 5) labelled polypeptides of RS virus released from BS-C-1 and Hep-2 cells. Track 1, ³H-glucosamine-labelled mock-infected BS-C-1 cells; track 2, wild-type (RSN-2) virus from BS-C-1 cells; track 3, wild-type (RSN-2) virus from Hep-2 cells; track 4, wild-type (Long) virus from BS-C-1 cells; and track 5, wild-type (RSN-2) virus from BS-C-1 cells.
virus from BS-C-1 cells (Fig. 6). Comparison of the GP1 of Long and RSN-2 strain virus suggested that the latter had a slightly faster mobility (not shown).

It is clear the GP1 was associated with RS virus infection, but it has not been established whether GP1 was a virus-specified glycoprotein or a host-specified glycoprotein which accumulated in the cell membrane as a consequence of RS virus infection. Immuno-precipitation of $^3$H-glucosamine-labelled protein with specific anti-RS virus serum would confirm the virus specificity of GP1, but so far it has not been obtained and the question remains unresolved.

**DISCUSSION**

One practical consequence of this study has been agreement on the identity of RS virus polypeptides. The polypeptides designated VP0, VP2, VP3, VP4, VP5 and VP6 in Detroit are synonymous with the polypeptides previously designated VP200, VP48, VP41, VP32, VP27 and VP25 in Glasgow. To avoid further confusion we suggest a new nomenclature for these polypeptides (Table 2). The virus specificity of GP1 (VP1) remains to be established unequivocally. It is clear, however, that this band is specifically associated with RS virus infection. Its abundance was correlated with virus titre, since GP1 was most prominent with the more highly culture-adapted A2 and Long strains grown in Hep-2 cells, the most permissive system. GP1 is an unusual polypeptide, however, in that its incorporation of $^{35}$S-methionine was poor relative to the abundant incorporation of $^3$H-glucosamine (Fig. 5, 6). This was also a characteristic of the 125000 mol. wt. glycoprotein (VP1) of Ebola virus (Kiley et al., 1980). It is possible that the V161 polypeptide synthesized *in vitro* (Cash et al., 1979) may represent a non-glycosylated form of GP1.

The mutants representing the three complementation groups from the A2 strain and the six groups of the RSN-2 strain exhibited four distinct phenotypes in terms of antigen and intracellular polypeptide synthesis (Table 1). This indicates that the RS virus genome codes for at least four and possibly as many as eight separate functions. The uncertainty exists because six (B, C, D, E, F and G) of the complementation groups contain one mutant only and inter- and intragenic complementation cannot be discriminated.

Mutant *ts* 17 (group B') and *ts* 19 (group E) were almost completely restricted at 39 °C suggesting an early function defect. Mutant *ts* 1 (group D) was indistinguishable from wild-type, except that no infectious virus was released, suggesting a defective maturation function. The VPP32 of this mutant exhibited an aberrant mobility. It has not been established, however, that this lesion is associated with the *ts* mutation, since it has not been possible to isolate and characterize non-*ts* revertants. Mutants *ts* A1 and *ts* 26 (group A), and *ts* A7 (group C) synthesized antigen and polypeptides normally at 39 °C, but were defective in a maturation function affecting the insertion of viral antigen into the plasma membrane. The remaining mutants belonging to groups B, F and G exhibited reduced synthesis at 39 °C.

The distinct phenotypes of the complementing mutants *ts* A2 (group B) and *ts* 17 (group B'), previously assigned to the same group, suggests that these mutants are indicative of lesions in different genes, and that the type of complementation observed may have been intergenic rather than intragenic.

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**REFERENCES**


RS virus ts mutants


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