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Processing of 60,000-dalton *sarc* gene protein synthesized by cell-free translation

(signal sequence/transformation-specific protein/messenger-dependent reticulocyte lysate/Rous sarcoma virus/wheat germ extract)

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ABSTRACT **In this report we show that antiserum prepared against the M_r 60,000 transformation-specific antigen of Rous sarcoma virus immunoprecipitates both the M_r 60,000 and M_r 25,000 transformation-specific proteins that are synthesized by cell-free translation of virion RNA; however, in the cell-free system the M_r 60,000 protein appears to be synthesized as a precursor that is approximately M_r 2000 larger than the [^{35}S]-methionine-labeled protein immunoprecipitated from Rous sarcoma virus-infected cells. Peptide mapping of the cell-free translation product and of this cellular protein has confirmed that they are structurally related to one another. The addition of membrane vesicles to the reticulocyte lysate system during translation specifically cleaves a M_r 2000 segment from the M_r 60,000 protein so that it comigrates with the cellular species. Secretory proteins and probably at least some integral membrane proteins are synthesized with short hydrophobic signal sequences at their NH_2 terminus. Two facts suggest that the segment lost from the M_r 60,000 transformation-specific protein is a signal sequence: (*i*) the membrane vesicles process the M_r 60,000 protein only during translation, and (*ii*) the processed protein is sequestered by the vesicles.**

The 38S genomic RNA of Rous sarcoma virus (RSV) is believed to code for four genes: 5′-*gag-polymerase-envelope glycoprotein-sarc*-poly(A)-3′ (1, 2). Genetic evidence has indicated that the transformation-specific gene of RSV (*src* or *sarc* gene) codes for a transformation-specific function that is unrelated to the replicative functions of the virus (3). The existence of temperature-sensitive mutants for transformation, all of which map in the *sarc* gene, suggests that *sarc* gene codes for a transformation-specific protein of unknown function (4) which is responsible for the generation of the transformed phenotype. We have previously used virion RNAs from nondefective RSV and transformation-defective RSV to synthesize proteins in the reticulocyte lysate system and have been able to identify proteins of M_r 60,000, 25,000, and 18,000 unique to the translation products of nondefective RSV RNA. We have proposed that they are the protein products of the *sarc* gene (5, 6). A report of the identification of the M_r 25,000 and 17,000 *sarc* proteins has also been published recently by Beemon and Hunter (7). From the peptide maps of the M_r 60,000 and 25,000 proteins we also concluded that these two proteins are structurally related to one another (6). Recently, Brugge and Erikson (8) and Jay *et al.* (9) have reported that an antibody to a transformation-specific protein of from M_r 56,000–60,000 is produced in tumor-bearing mammals injected with RSV. The antibody reacts with the M_r 60,000 antigen isolated from RSV infected cells or synthesized *in vitro* (10).

In the present report we show that both the M_r 60,000 and the M_r 25,000 *sarc* proteins that are synthesized in the reticulocyte lysate translation system are immunoprecipitated by antiserum directed against the transformation-specific antigen. However, the *in vitro* M_r 60,000 product is approximately M_r 2000 larger than the protein immunoprecipitated by this antiserum from RSV-infected cells. This small segment can be specifically cleaved from the translation product *in vitro*, if membrane vesicles are present during synthesis. Evidence is presented to show that the segment lost from the M_r 60,000 *sarc* protein is a signal sequence for the insertion of the M_r 60,000 protein into cellular membranes.

MATERIALS AND METHODS

Antiserum and Immunoprecipitation. Antiserum against RSV-induced tumors was prepared by injecting newborn New Zealand White rabbits with 10^9 focus-forming units of purified Schmidt–Ruppin strain, subgroup D, of RSV (SRD-RSV) as described by Brugge and Erikson (8). Rabbits were bled for sera 4–6 weeks after birth. [^{35}S]Methionine-labeled cell protein was prepared by infecting secondary cultures of chicken embryo fibroblasts with SRD-RSV (1 focus-forming unit per cell) and incubating the transformed cells on day 3 with 100 μCi of [^{35}S]methionine per ml of methionine-free Dulbecco's modified Eagle's medium containing 5% dialyzed calf serum. After a 3-hr labeling period, the medium was removed and the cells were lysed in 0.15 M NaCl/1% sodium deoxycholate/1% Triton X-100/0.1% sodium dodecyl sulfate ($NaDodSO_4$). Labeled cell protein or [^{35}S]methionine-labeled cell-free translation products were incubated with the indicated serum for 20 min at 4° and the antigen-antibody complex was collected by means of the *Staphylococcus aureus* adsorbent described by Kessler (11).

Cell-Free Translation. Reticulocyte lysates were prepared as described by Villa-Komaroff *et al.* (12) and then treated with micrococcal nuclease as described by Pelham and Jackson (13). The procedure for isolation of virion RNA from RSV and for cell-free translation has been described (5). Membrane vesicles prepared from canine pancreas were generously supplied by James Rothman (14). Cell-free translation with wheat germ extracts was performed according to Roberts and Paterson (15).

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. [^{35}S]-Methionine-labeled cell-free translation products or [^{35}S]methionine-labeled protein of immunoprecipitates was analyzed as described by Laemmli (16) with a 5–15% $NaDodSO_4$/polyacrylamide separation gel. Autoradiography of dried gels was done with Kodak X-Omat R film. Gels were prepared for fluorography as described (17).

Peptide Mapping. Bands of [^{35}S]methionine-labeled protein were identified by autoradiography of dried $NaDodSO_4$/polyacrylamide gels. Bands were then cut from the dried gels, rehydrated, placed in the sample wells of another $NaDodSO_4$/polyacrylamide gel, and then overlayed with *S. aureus* protease V8 (Miles Laboratories) as described by Cleveland *et*

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Abbreviations: RSV, Rous sarcoma virus; SRD-RSV, Schmidt–Ruppin strain, subgroup D, of RSV; *sarc*, transformation-specific gene of RSV (also *src*); $NaDodSO_4$, sodium dodecyl sulfate.

al. (18). Dried gels were subjected to fluorography for 10 days.

RESULTS

We previously identified proteins of M_r 60,000, 25,000, and 18,000 as the products of the *sarc* gene of RSV by cell-free translation in the reticulocyte lysate system (5, 6). These proteins are synthesized from subgenomic RNA of transforming virions of RSV but are absent from the translation products of RNA of nontransforming deletion mutants of RSV which lack *sarc*. Peptide mapping has also shown that the M_r 60,000 and 25,000 *sarc* proteins are structurally related to one another (6).

Recently, Brugge and Erikson (8) and Jay *et al.* (9) reported that newborn mammals injected with SRD-RSV produce antibodies against a transformation-specific antigen of RSV-transformed cells. We prepared serum from RSV-induced tumor-bearing rabbits as described (8). This serum immunoprecipitated both M_r 60,000 and M_r 25,000 proteins synthesized by cell-free translation of virion RNA (Fig. 1, track a). Neither of these proteins is immunoprecipitated by antiserum against the structural proteins of RSV (6, 8). The major band of protein immunoprecipitated in track a of Fig. 1 is the M_r 76,000 precursor of the viral *gs* antigens or *gag* gene proteins. In addition to antibodies against the transformation-specific antigen of RSV, the antitumor serum has been shown to contain antibodies against the viral structural proteins, which can be preadsorbed by incubation with disrupted RSV virions (8). Upon incubation of the antitumor serum with [^{35}S]methionine-labeled protein from SRD-RSV-infected cells (Fig. 1, track b), several virus-specific proteins were immunoprecipitated that were not immunoprecipitated by control rabbit serum (Fig. 1, track c). One was M_r 25,000 and the other was slightly smaller than M_r 60,000. Peptide mapping of NaDodSO$_4$/polyacrylamide gels indicated that the M_r 60,000 translation product and the M_r 60,000 cellular protein are closely related structurally (Fig. 2). The M_r 25,000 protein has not been identified as yet. This protein is probably the M_r 27,000 *gs* antigen of the virion, which is known to migrate as M_r 25,000 on NaDodSO$_4$/polyacrylamide gels. However, it is possible that the M_r 25,000 *sarc* protein is also present as a comigrating species.

The fact that the M_r 60,000 translation product is approximately M_r 2000 larger than the cellular species suggests that a precursor/product relationship between these two forms may exist inside the cell. A M_r 2000 segment is in the size range (M_r 1500–3000) of the signal sequence that is cleaved from the primary translation products of secretory proteins in the first step in their intracellular processing. Secretory proteins such as the polypeptide hormones and IgG are synthesized in a cell-free translation system as precursors with a short hydrophobic signal sequence at their NH_2 terminus. This segment of the polypeptide is cleaved from the nascent chain if membrane vesicles are added during translation. The primary translation products of proteins such as globin, which are not secreted by the cell, do not have signal sequences and are not processed by the addition of membrane vesicles during translation (19, 20).

Fig. 3 shows the effect of adding membrane vesicles from canine pancreas during cell-free translation of *sarc* mRNA in the reticulocyte lysate system. As the concentration of vesicles was increased, the M_r 60,000 *sarc* protein was processed to a size M_r 2000 smaller. After processing, the M_r 60,000 protein appeared to comigrate with the protein immunoprecipitated from SRD-RSV infected cells (Fig. 4). The presence of these membrane vesicles during cell-free translation tends to inhibit translation, presumably by nonspecific binding of mRNA with resulting inhibition of polypeptide chain initiation (James Rothman, personal communication). As shown in Fig. 3, the amounts of the M_r 25,000 and 18,000 *sarc* proteins decreased as the concentration of membrane vesicles was increased in these assays.

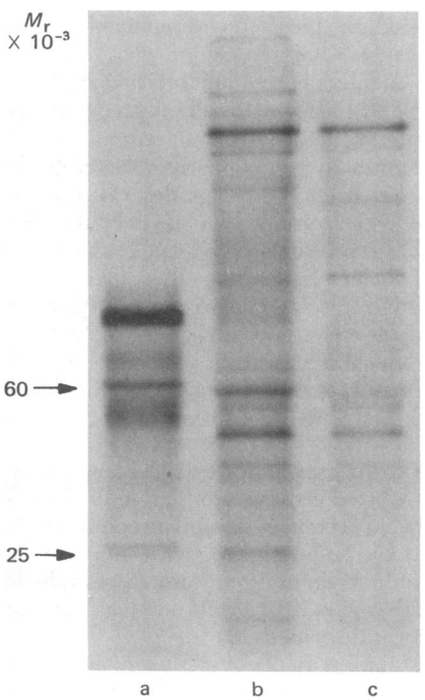

FIG. 1. Fluorograph of NaDodSO$_4$/polyacrylamide gel of [^{35}S]-methionine-labeled protein immunoprecipitated with serum from tumor-bearing rabbits or control rabbit serum. Tracks: a, reticulocyte lysate cell-free translation products from 20S virion mRNA of SRD-RSV immunoprecipitated with antitumor serum; b, [^{35}S]methionine-labeled protein from SRD-RSV-transformed chicken embryo fibroblasts immunoprecipitated with antitumor serum; c, as b but with control rabbit serum.

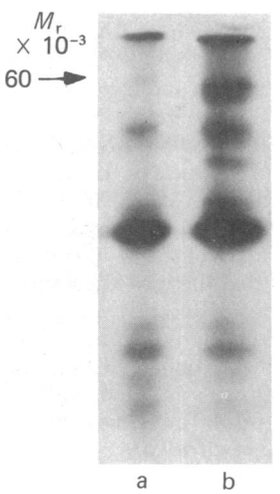

FIG. 2. Fluorograph of NaDodSO$_4$/polyacrylamide gel of polypeptide fragments. [^{35}S]Methionine-labeled proteins were localized on the gel by autoradiography. The bands were then cut from the gels and peptide mapping was done as described by Cleveland *et al.* (18). Tracks: a, M_r 60,000 *sarc* protein synthesized in reticulocyte lysate translation system after digestion with *S. aureus* protease V8; b, presumptive cellular M_r 60,000 *sarc* protein digested with *S. aureus* V8 protease.

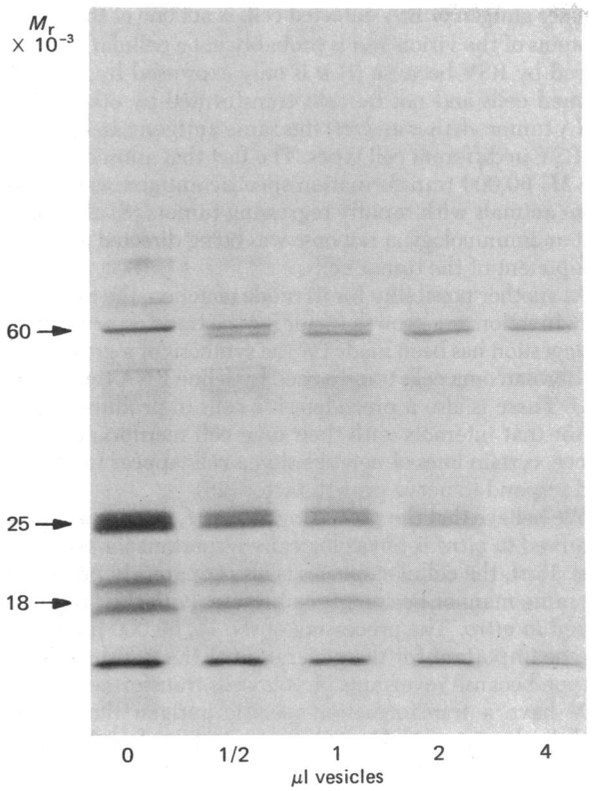

FIG. 3. Processing of M_r 60,000 *sarc* cell-free translation product by membrane vesicles from canine pancreas. Autoradiogram of $NaDodSO_4$/polyacrylamide gel of [^{35}S]methionine-labeled translation products made from 20S virion mRNA of SRD-RSV. All translation assays contained 200 μM N^7-methyl-GTP, which selectively decreases translation of non-*sarc* mRNA. Either 0, 0.5, 1, 2, or 4 μl of membrane vesicles (50 OD_{280} units/ml) was included in each 25-μl assay during translation.

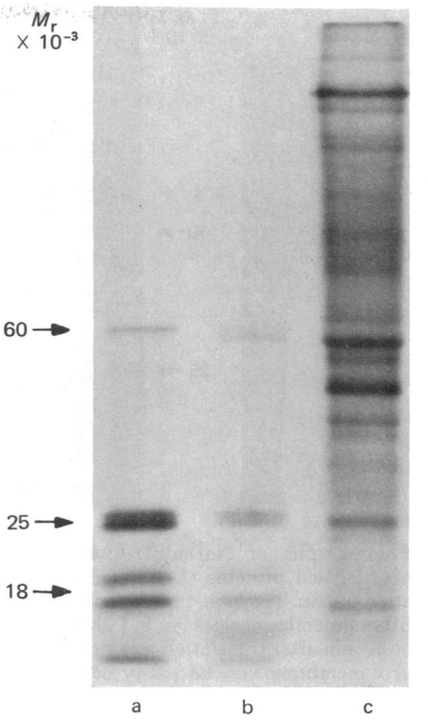

FIG. 4. Comigration of processed M_r 60,000 translation product and cellular species. Fluorograph of $NaDodSO_4$/polyacrylamide gel of [^{35}S]methionine-labeled protein from reticulocyte lysate translation system. Tracks: a, in the absence of membrane vesicles; b, with 1 μl of membrane vesicles (50 OD_{280} units/ml) added during translation. Track c, [^{35}S]methionine-labeled protein immunoprecipitated from SRD-RSV-transformed chicken embryo fibroblasts with serum from tumor-bearing rabbits. In a and b, translation assays contained 200 μM N^7-methyl-GTP.

If the segment lost from the M_r 60,000 protein is a signal sequence, several predictions about the processing can be made. First, the signal sequence should only be removed from the nascent chain; if membranes are added after translation, no processing should occur. In the experiment shown in Fig. 5 *left*, membrane vesicles were added to the reticulocyte cell-free translation assays either during translation (track c) or after translation had been stopped by the addition of RNase (track b). Processing of the M_r 60,000 *sarc* protein occurred only if the membranes were present during translation.

A second characteristic of the processing of a signal sequence from a secretory protein is that the protein should be sequestered by the vesicles—that is, it should now be resistant to exogenous proteases. We failed to demonstrate protection of processed M_r 60,000 protein in the reticulocyte lysate system when trypsin was added after translation (Fig. 5 *left*, track d). The addition of trypsin degraded all of the labeled protein, regardless of whether membranes were present or not. This effect might result from the vesicles being permeable to trypsin in the reticulocyte lysate system. Published experiments of protection of proteins processed by membranes have usually utilized either a reconstructed translation system (19, 20) or the wheat germ extract system (14). Recent unpublished work indicates that protection can often not be demonstrated in the reticulocyte lysate system (see *Note Added in Proof*). For instance, parathyroid hormone is synthesized as a precursor with a signal sequence in both the reticulocyte lysate and wheat germ extract translation systems and is accurately processed in both. If membrane vesicles are added during translation, however, the processed protein is only protected from proteolysis in the wheat germ system (Joseph Majzoub, personal communication). We therefore translated SRD-RSV 20S virion RNA in the wheat germ cell-free system.

The major translation products from this RNA in the wheat germ system are the M_r 60,000 and 25,000 *sarc* proteins. When translation was performed in the absence of membrane vesicles, the addition of trypsin resulted in the disappearance of all [^{35}S]methionine-labeled protein as had been shown with the reticulocyte lysate system (data not shown); however, when translation was performed in the presence of membrane vesicles, the subsequent addition of trypsin degraded all of the [^{35}S]methionine-labeled protein except the M_r 60,000 protein. These results indicate that the processed protein is sequestered by the vesicles (Fig. 5 *right*). We conclude, therefore, that the M_r 60,000 *sarc* protein is synthesized as a precursor with a signal sequence that functions either to insert or to transport the processed protein into or through cellular membranes.

DISCUSSION

In the present study, we prepared antiserum from tumor-bearing rabbits as described (8) and immunoprecipitated [^{35}S]methionine-labeled cell-free translation products made from RSV-RNA and [^{35}S]methionine-labeled protein from RSV-transformed cells. We find that both the M_r 60,000 and M_r 25,000 *sarc* proteins synthesized by cell-free translation are immunoprecipitated by the antitumor serum; however, the M_r 60,000 translation product appears to be approximately M_r 2000 larger than the [^{35}S]methionine-labeled protein immu-

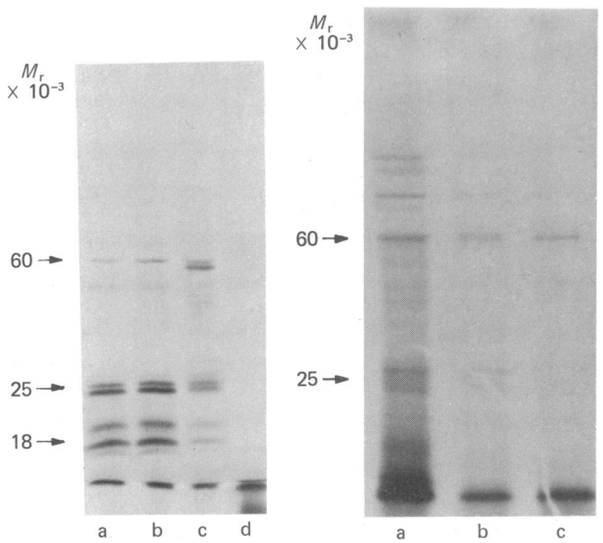

FIG. 5. Fluorographs of NaDodSO$_4$/polyacrylamide gels of [^{35}S]methionine-labeled proteins (*Left*) made in the reticulocyte lysate translation system. Tracks: a, translation assays were incubated for 60 min without membrane vesicles; b, 1 μl of membrane vesicles was added for 60 min after translation was stopped with RNase (10 μg/ml); c, 1 μl of membrane vesicles was present during translation; d, trypsin (1 mg/ml) was added to assay c for 30 min at 25° after translation was completed [the activity of the trypsin (Worthington) was 269 units/mg]. All translation assays contained 200 μM N^7-methyl-GTP. (*Right*) Translation products synthesized in the wheat germ cell-free translation system. Tracks: a, products synthesized in the presence of 1 μl of membrane vesicles; b and c, after translation for 60 min, either 0.1 mg or 1 mg of trypsin, respectively, was added for 30 min at 25°. All translation assays contained 200 μM N^7-methyl-GTP.

noprecipitated from transformed cells. These experiments suggest that the primary translation product is processed intracellularly.

The signal sequence hypothesis developed by Blobel and Dobberstein (19, 20) from work on cell-free synthesis of IgG and polypeptide hormones states that secretory proteins are synthesized as precursors with a hydrophobic sequence of 15–25 amino acids at their NH_2 terminus. This sequence allows the nascent chain of the protein to attach to the membranes of the endoplasmic reticulum. Before the nascent chain is completed, the signal sequence is cleaved from the protein by a membrane-bound peptidase, followed, upon completion of synthesis, by discharge of the protein through the membrane. The processing of the M_r 60,000 protein has several features that suggest that the 20-amino acid segment removed is a signal sequence. First, the processing only occurs if vesicles are present during translation, presumably because after synthesis the protein adopts a native conformation that conceals the hydrophobic signal sequence.

The second feature is that the processed protein is sequestered by the vesicles. The fact that the M_r 60,000 protein is synthesized as a precursor with a signal sequence indicates that it probably passes through or is inserted into a cellular membrane *in vivo*. Although it has been proposed that integral membrane proteins are also inserted into cellular membranes by virtue of their possessing a signal sequence (21), there is no *a priori* reason to expect that the signal sequence must necessarily be removed from such a protein. Many experiments have suggested that the *sarc* product may be located on the plasma membrane of transformed cells in the form of a tumor-specific surface antigen (22, 23). A recent study (24) has shown that the tumor-specific surface antigen of RSV-infected cells is not one of the structural proteins of the virion and is probably not a cellular protein induced by RSV because (*i*) it is only expressed by RSV-transformed cells and not by cells transformed by other RNA or DNA tumor viruses and (*ii*) this same antigen can be induced by RSV in different cell types. The fact that antiserum against the M_r 60,000 transformation-specific antigen was obtained from animals with rapidly regressing tumors (8) also suggests that an immunological response was being directed at a surface component of the tumor cells.

As another possibility for its mode of action, the *sarc* protein may function as a growth factor for the transformed cell. Such a suggestion has been made for the synthesis of a growth factor by fibrosarcoma cells transformed by feline RNA tumor viruses (25). There is also a precedent for cells to produce a growth factor that interacts with their own cell membranes. For instance, certain lines of neuroblastoma cells appear to synthesize and respond to nerve growth factor (26).

We believe that the processing of the M_r 60,000 *sarc* protein observed *in vitro* is physiologically important for several reasons. First, the cellular species is also apparently processed in the same manner because it comigrates with the protein processed *in vitro*. The processing of the M_r 60,000 protein may also be important for the generation of the transformed phenotype because revertants of vole cells transformed by SRD-RSV have a transformation-specific antigen that migrates slightly slower on NaDodSO$_4$/polyacrylamide gels than that of the parental cells. The exact nature of this electrophoretic difference is not yet known; however, it is quite possible that it represents an alteration in primary structure (A. Lau, personal communication). This difference in size suggests that a defect in processing of the M_r 60,000 protein results in a concomitant loss of the transformed phenotype. Proteins possessing signal sequences usually undergo extensive intracellular and extracellular modifications that are essential for their physiological functioning. A recent report (27) attributes protein kinase activity to the M_r 60,000 transformation-specific antigen immunoprecipitated from infected cells. The processing we have observed might also be necessary to render it enzymatically active. In fact, preliminary data from our laboratory indicate that the processing reported here for the M_r 60,000 protein results in at least a 5-fold increase in its kinase activity.

If the M_r 60,000 protein does undergo additional processing after the signal sequence is removed, a change in its immunospecificity could result. In fact, the M_r 25,000 *sarc* protein made *in vitro* is immunoprecipitated much less efficiently than the M_r 60,000, and the M_r 18,000 is not immunoprecipitated at all from cell-free translation assays. Processing and change of immunospecificity could also explain why the *sarc* antigen of Jay *et al.* (9) appears to have only a 20-min half-life in transformed cells and why the M_r 25,000 and 18,000 *sarc* proteins have not been found in cells as yet.

Note Added in Proof. Recently it has been shown (28) that protection of processed presecretory proteins can be demonstrated in the reticulocyte lysate system in the presence of supplemented microsomal membranes. The demonstration of protection seems to require that post-translational proteolysis be carried out at 4° rather than at 25°.

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