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In Vitro Synthesis and Processing of Herpes Simplex Virus Type 2 gG-2, Using Cell-Free Transcription and Translation Systems

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Translation of in vitro-synthesized herpes simplex virus type 2 (HSV-2) gG-2 mRNA in a reticulocyte lysate system was used to study the processing of HSV-2 gG-2. In the presence of canine pancreatic microsomal membranes, a single species that is protected from trypsin digestion was detected. This product comigrates with the 104,000-M_r (104K) high mannose intermediate seen in HSV-2-infected-cell lysates. Endo-β-*N*-acetylglucosaminidase H treatment of the in vitro-synthesized 104K protein yielded a single product migrating at 100 K. The 72K and 31K cleavage products of gG-2 were not observed in the in vitro system. These data show that the molecular weight of the nonglycosylated form of the gG-2 protein is 100,000 and that the cotranslational processing of this protein in the endoplasmic reticulum yields the 104K high-mannose intermediate.

Herpes simplex viruses (HSV) are reported to encode seven antigenically distinct glycoproteins (gB, gC, gD, gE, gG, gH, and gI) which are incorporated into the viral envelope and the plasma membranes of infected cells (4, 8). Of these, the HSV type 2 (HSV-2) glycoprotein, gG-2, is the only known HSV glycoprotein that is cleaved to yield a secreted product (2, 10). The synthesis of gG-2 involves the formation of several intermediate products. Our studies have indicated that gG-2 is cotranslationally glycosylated to yield a 104,000-M_r (104K) high-mannose intermediate, which is subsequently cleaved to give 72K and 31K proteins. Further glycosylation of the 72K and 31K high-mannose intermediates yields a 108K mature glycoprotein and a 34K secreted product, respectively (10). Although the nature and location of this cleavage event are currently unknown, other experiments from our laboratory have shown that the processing and cleavage of gG-2 can occur in the absence of other viral gene products (9). In the present study, in vitro transcription and translation systems were used to examine the initial events that occur during gG-2 biosynthesis.

Our initial studies used tunicamycin, a specific inhibitor of N-linked glycosylation (11), and endo-β-*N*-acetylglucosaminidase H (endo H) to examine the processing of gG-2 in infected cells. HEp-2 cells infected with HSV-2 186 at a multiplicity of infection of approximately 20 were incubated with either 0, 1, or 10 μg of tunicamycin per ml. The cells were harvested 12 h postinfection and were treated or mock treated with endo H before analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to nitrocellulose and analyzed by radioimmunoblotting with anti-gG-2 serum (10). In the absence of tunicamycin, two bands were recognized by the gG-2 antiserum: a high-molecular-weight diffuse species consisting of the 108K mature glycoprotein and the 104K high-mannose intermediate, and a second, 72K band corresponding to one of the gG-2 cleavage products (Fig. 1). Endo H-mediated removal of N-linked oligosaccharides from the 104K and 72K high-mannose intermediates resulted in the appearance of 100K and 69K bands, respectively (Fig. 1). Detection of the gG-2 31K high-mannose cleavage product (10) was not possible, since it is not recognized by the anti-gG-2 serum. Glycoprotein gG-2 synthesized in the presence of either 1 or 10 μg of tunicamycin per ml comigrated with the 100K product of the endo H digestion (Fig. 1). These data suggested that the molecular weight of the nonglycosylated precursor protein is 100K and that the gG-2 synthesized in the presence of tunicamycin is not cleaved. Furthermore, the sensitivity of one of the cleavage products (72K) to endo H digestion suggests that cleavage of gG-2 may occur in either the endoplasmic reticulum or a *cis*-Golgi compartment before the sugars are converted to complex oligosaccharides. To identify the primary translation product of gG-2 and to determine if cleavage of the 104K product occurs within the endoplasmic reticulum, the synthesis and processing of gG-2 were examined with in vitro transcription and translation systems.

The region encoding HSV-2 gG-2 (7, 9) was inserted into the Riboprobe SP64 vector (Promega Biotec, Madison, Wis.) under the transcriptional control of the SP6 promoter. This plasmid, designated pSPG, was then linearized and transcribed by the procedure described by the supplier with the following exceptions. (i) The RNA cap structure analog, m7G(5′)ppp(5′)G, was included in the reaction mixture at a concentration of 50 μM and the GTP concentration was reduced to 5 μM. (ii) After the reaction mixture was incubated for 60 min at 40°C, 10 U of SP6 polymerase was added to the reaction mixture, and incubation was continued for an additional 60 min. The resulting mRNAs were translated in a rabbit reticulocyte lysate system (Du Pont, NEN Research Products, Boston, Mass.) in the presence of [^{35}S]methionine under conditions recommended by the supplier, except that the magnesium acetate concentration in the reaction mixtures was reduced to 250 μM to achieve optimal translational efficiency. Canine pancreatic microsomal membranes were added to the translation reaction mixtures when indicated. After incubation for 90 min at 30°C, SDS was added to give a final concentration of 4%. Immunoprecipitations were performed by the method of Anderson and Blobel (1) with the anti-gG-2 serum which recognized the 108K, 104K, and 72K components. In addition, radiolabeled lysates from HSV-2-infected HEp-2 cells were immunoprecipitated with anti-pgG-2 serum (9), which recognizes the 104K, 72K, and 31K intermediates. The immunoprecipitates were solubi-

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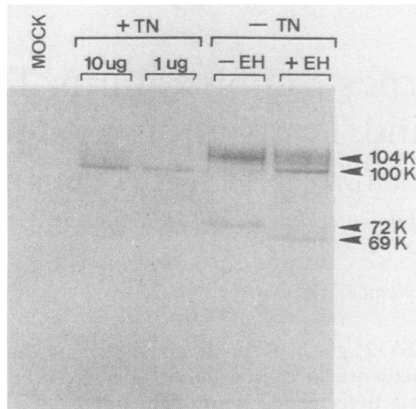

FIG. 1. Autoradiogram of HSV-2-infected cell lysates grown in the presence or absence of tunicamycin. Western blot (immunoblot) analysis was performed with anti-gG-2 serum. Infected cell lysates digested with endo H were boiled for 2 min in the presence of 0.8% SDS before the addition of sodium citrate (50 mM), phenylmethylsulfonylfluoride (1 mM), and endo H (0.6 μg/ml). After an overnight incubation at 37°C, the proteins were acetone precipitated, pelleted, and loaded on a 7% polyacrylamide gel. Abbreviations: TN, tunicamycin; EH, endo H; MOCK, mock treatment.

lized in sample buffer containing 3% SDS, subjected to SDS-PAGE, and visualized by autoradiography.

The major translation products from pSPG mRNA migrated as a doublet slightly higher than the 97K marker (Fig. 2A, lane 2). Although the precise initiation codon for gG-2 is unknown, the predicted amino acid sequence contains two methionine residues, either of which could serve as the initiator, separated by 21 amino acids. Since both of these AUGs are present in the in vitro-synthesized mRNA, the doublet may be due to initiation at the two different methionine codons. Interestingly, when canine pancreatic microsomal membranes were included in the reaction, both bands were processed to yield a single product migrating with an apparent molecular weight of 104K (Fig. 2A, lanes 2 and 3). If the primary translation products are due to initiations at two different methionines, this result suggests that both products are capable of translocating across microsomal membranes. It should be noted that cleavage of the signal sequence would yield a common product, irrespective of which methionine is used to initiate translation.

To determine if the in vitro-synthesized gG-2 peptides were indeed translocated into the microsomal vesicles, the accessibility of the peptides to trypsin digestion was examined. The in vitro translation reactions containing microsomal membranes were first incubated for 90 min and then treated with either trypsin (500 μg/ml) alone or trypsin plus 0.5% Nonidet P-40 for 1 h at 0°C. The trypsin digestion reaction was terminated by the addition of soybean trypsin inhibitor (1,500 μg/ml) and phenylmethylsulfonyl fluoride (400 μg/ml), and the protected products were immunoprecipitated with the anti-gG-2 serum and analyzed by SDS-PAGE. The 104K protein was protected from trypsin digestion in the presence of microsomal membranes only in the absence of Nonidet P-40 (Fig. 2B, lanes 6 and 7). This result is consistent with the 104K protein being sequestered in the microsomal vesicles.

To determine if the in vitro translation products were glycosylated, samples from in vitro translation reactions containing microsomal membranes were immunoprecipitated and either treated or mock treated with endo H before SDS-PAGE. HSV-2-infected-cell lysates were included as controls. As expected, the 104K and 72K intermediates in the HSV-2-infected-cell lysates were reduced to 100K and 69K, respectively, upon digestion with endo H (Fig. 2B; compare lanes 4 and 5). The 104K component synthesized in vitro showed an identical drop in molecular weight to yield a single band also migrating at 100K (Fig. 2B, lanes 2 and 3). These results indicate that the 104K product synthesized in

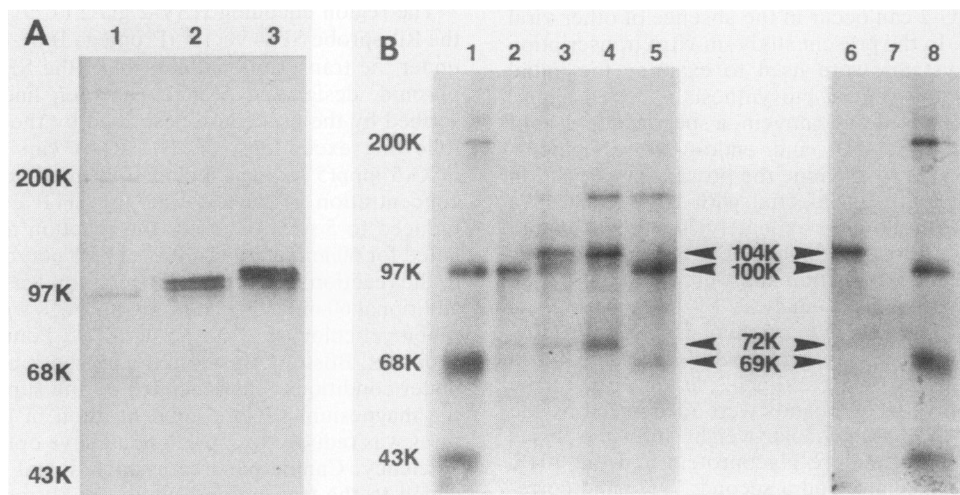

FIG. 2. Comparison of in vitro-synthesized gG-2 with gG-2 synthesized in HSV-2-infected cells. HSV-2-infected-cell lysates and products from the in vitro translations were immunoprecipitated with anti-pgG-2 and anti-gG-2 sera, respectively. (A) Autoradiogram of in vitro translation products synthesized in the presence or absence of microsomal membranes. Lane 1, ^{14}C-labeled molecular weight standards; lane 2, products from in vitro-translated pSPG mRNA; lane 3, products from pSPG mRNA translated in vitro in the presence of microsomal vesicles. (B) Autoradiogram of immunoprecipitated in vitro translation products digested with endo H and trypsin. Lanes 1 and 8, ^{14}C-labeled molecular weight standards; lanes 2 and 3, products of pSPG mRNA translated in the presence of microsomal membranes, digested or mock digested, respectively, with endo H; lanes 4 and 5, HSV-2-infected HEp-2 cell lysates mock digested or digested, respectively, with endo H; lanes 6 and 7, products of pSPG mRNA translated in the presence of microsomal membranes and digested with trypsin or trypsin plus Nonidet P-40, respectively.

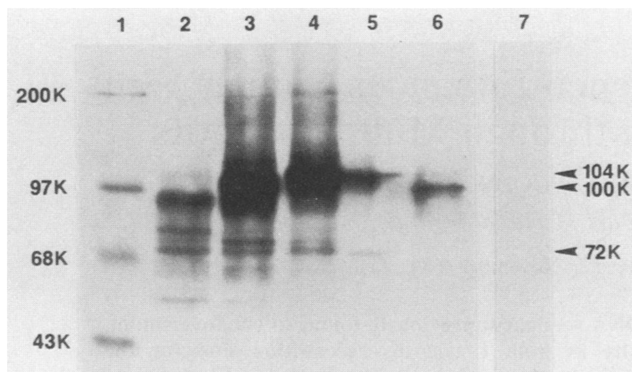

FIG. 3. Autoradiogram of in vitro translation products synthesized from pSPG, pSPG.SSP I, and poly(A)$^+$ mRNA. The HSV-2-infected-cell lysates and products from in vitro translations were immunoprecipitated with anti-pgG-2 and anti-gG-2 sera, respectively. Lane 1, ^{14}C-labeled molecular weight standards; lane 2, products of in vitro-translated pSPG.SSP I mRNA; lanes 3 and 4, products of pSPG mRNA translated in vitro in the absence or presence of microsomal membranes, respectively; lane 5, HSV-2-infected HEp-2 cell lysate; lanes 6 and 7, products of in vitro-translated poly(A)$^+$ mRNA from HSV-2-infected or mock-infected NIH 3T3 cells, respectively.

vitro in the presence of canine microsomal membranes contains N-linked high-mannose oligosaccharides. The doublet produced by translation of pSPG mRNA in the absence of microsomal membranes was not sensitive to endo H digestion (data not shown).

In addition to the major 104K translation product, several minor lower-molecular-weight products, including a band migrating at approximately 72K, were precipitated by the anti-gG-2 antibody (Fig. 2B, lane 3). This 72K band does not represent a cleavage product of the 104K component, since it is resistant to endo H (lane 2) and is sensitive to trypsin digestion (lane 6). Aberrant translation initiation can occur in the reticulocyte lysate system (5) and may be responsible for these additional lower-molecular-weight products detected in the in vitro translation reactions. To determine whether the 72K product synthesized in vitro was a result of aberrant translation initiation or perhaps of further processing of the 104K intermediate, a second plasmid, designated pSPG.SSP I, was constructed. The first 254 base pairs of the cloned gG-2 gene (9) are missing from pSPG.SSP I; therefore, transcription of pSPG.SSP I should produce mRNAs lacking the first two AUG codons, and one would expect translation to initiate at internal AUG codons. Translation of mRNA derived from the pSPG.SSP I vector produced several proteins, all of which, including the product migrating at approximately 72K, were present in the translations performed with pSPG mRNA (Fig. 3; compare lanes 2, 3, and 4). These results indicate that the minor translation products are a result of internal initiation and are not due to premature termination or proteolytic processing of in vitro-synthesized gG-2.

To determine if similar products are synthesized by the in vitro translation of viral gG-2 mRNA and in vitro-synthesized gG-2 mRNA, poly(A)$^+$ mRNA was isolated from HSV-2-infected cells and translated. Confluent NIH 3T3 cell monolayers were infected or mock infected with HSV-2 186; at 18 h postinfection, the cells were scraped into guanidinium isothiocyanate (6) and the total cellular RNA was pelleted through cesium chloride (3). The poly(A)$^+$ mRNA was selected on oligo(dT) columns and translated in vitro as previously described with 5 μg of either HSV-2-infected or mock-infected poly(A)$^+$ mRNA. The translation products were immunoprecipitated before analysis by SDS-PAGE and autoradiography.

The major product of translation with the poly(A)$^+$ mRNA from HSV-2-infected cells was a single band (Fig. 3, lane 6) that comigrated with the lower band of the doublet seen in translations with pSPG mRNA (Fig. 2A, lane 2). These data suggest that the doublet resulting from translation of the in vitro-synthesized gG-2 mRNA may be due to separate initiations at the first two AUG codons in the gG-2 gene. Placing the gene under the control of the SP6 promoter could have resulted in the inclusion in the in vitro mRNA transcripts of an in-frame upstream AUG which may not be present in the in vivo mRNA. The origin of the faint higher-molecular-weight band also seen in Fig. 3 (lane 6) is not known.

The data presented in this report show that the nonglycosylated gG-2 precursor protein has a molecular weight of 100K. Cotranslational processing of the 100K product to yield the 104K high-mannose intermediate does occur in vitro, involving the addition of N-linked oligosaccharides and probably the removal of the signal sequence. The absence of the 72K and 31K cleavage products in the in vitro translation reaction suggests that the cleavage processing events do not occur in the endoplasmic reticulum. However, we cannot exclude the possibility that conditions in the in vitro system are not conducive to the processing of gG-2.

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LITERATURE CITED

1. **Anderson, D. J., and G. Blobel.** 1983. Immunoprecipitation of proteins from cell-free translations. Methods Enzymol. **96:** 111–120.
2. **Balachandran, N., and L. M. Hutt-Fletcher.** 1985. Synthesis and processing of glycoprotein gG of herpes simplex virus type 2. J. Virol. **54:**825–832.
3. **Glisin, V., R. Crkvenjakov, and C. Byrus.** 1974. Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry **13:**2633–2637.
4. **Hodgman, T. C., and A. C. Minson.** 1986. The herpes simplex virus type 2 equivalent of the herpes simplex type 1 US7 gene and its flanking sequences. Virology **153:**1–11.
5. **Kozak, M.** 1988. A profusion of controls. J. Cell Biol. **107:**1–7.
6. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
7. **McGeoch, D. J., H. W. Moss, D. McNab, and M. C. Frame.** 1987. DNA sequence and genetic content of the HindIII L region in the short unique component of the herpes simplex virus type 2 genome: identification of the gene encoding glycoprotein g, and evolutionary comparisons. J. Gen. Virol. **68:** 19–38.
8. **Spear, P. G.** 1985. Glycoproteins specified by herpes simplex viruses, p. 315–356. *In* B. Roizman (ed.), The herpesviruses, vol. 3. Plenum Publishing Corp., New York.
9. **Su, H. K., and R. J. Courtney.** 1988. Inducible expression of herpes simplex virus type 2 glycoprotein gene gG-2 in a mammalian cell line. J. Virol. **62:**3668–3674.
10. **Su, H. K., R. Eberle, and R. J. Courtney.** 1987. Processing of the herpes simplex virus type 2 glycoprotein gG-2 results in secretion of a 34,000-M_r cleavage product. J. Virol. **61:**1735–1737.
11. **Takasuki, A., and G. Tamura.** 1971. Effect of tunicamycin on the synthesis of macromolecules in culture of chick embryo fibroblasts infected with Newcastle disease virus. J. Antibiot. **24:**785–794.