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Addition of High-Mannose Sugars Must Precede Disulfide Bond Formation for Proper Folding of Sendai Virus Glycoproteins

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The role of glycosylation and of disulfide bonds in the formation of the native structure of the Sendai virus hemagglutinin-neuraminidase (HN) and fusion (F_0) glycoproteins was studied. In contrast to the HN and F_0 synthesized in vivo, the proteins made from pSP6 transcripts in reticulocyte lysates, whether glycosylated or not, were not recognized by monoclonal antibodies or polyclonal rabbit sera raised against the native proteins; they efficiently reacted only with rabbit antisera raised against the reduced sodium dodecyl sulfate-denatured proteins. These in vitro-made proteins, however, did not contain disulfide bonds. The proteins made in vivo in the presence of tunicamycin, which were also not recognized by the anti-native protein antibodies, did contain disulfide bonds, but they were mainly incorrect interchain disulfide bonds. Moreover, while F_0 acquired proper disulfide bonds as soon as it was synthesized under normal conditions in vivo, the disulfides were formed in HN only after a lag of 10 to 30 min. This lag coincides with the delay observed in HN native structure formation. We therefore conclude that the maturation of the HN and F_0 proteins depends on the formation of proper intramolecular disulfide bonds, which in turn depends on the previous addition of high-mannose sugars.

Sendai virus, a member of the *Paramyxoviridae*, is an enveloped virus with six major structural proteins. NP, P, and L proteins are associated with the nucleocapsid, and hemagglutinin-neuraminidase (HN), fusion F_0, and M proteins constitute, along with host-derived lipids, the envelope. The M protein is thought to form the inner layer of the membrane, while HN and F_0 are transmembrane glycoproteins that protrude to the outside and constitute the spikes present at the surfaces of virions and infected cells (for a review, see reference 4). HN and F_0 are anchored in the membrane by their N and C termini, respectively, and are expressed at the cell surface at different rates (1, 2). Both proteins appear to contain only conformation-dependent epitopes. Polyclonal and monoclonal antibodies raised against the native proteins (anti-native antibodies) do not react with the β-mercaptoethanol (β-ME)- and sodium dodecyl sulfate (SDS)-denatured proteins, whereas polyclonal antibodies raised against the denatured proteins (anti-denatured antibodies) react well with only the denatured proteins (27). These antibodies therefore allow us to follow the maturation of F_0 and HN to their native immunoreactivities or conformations.

In attempts to define the pathways in the formation of the native immunoreactivity of these proteins, the following results were obtained (27). The addition of high-mannose sugars appeared to be necessary for the process, based on lack of immunoreactivity of the unglycosylated proteins (i.e., made in presence of tunicamycin [TM]) to anti-native antibodies, while these proteins did react with anti-denatured antibodies. On the other hand, sugar residues were not required to maintain the native immunoreactivities once formed, as digestion with endoglycosidase F, which cleaves N-linked glycans at any degree of processing, failed to abolish this reactivity for both proteins. However, a difference was observed between F_0 and HN during maturation. While F_0 gained full immunoreactivity as soon as high-mannose sugars were added (i.e., when still totally sensitive to endo-β-*N*-acetylglucosaminidase H), HN appeared to require a further maturation step, as there was a lag of about 30 min after sugar addition before HN became fully immunoreactive (27, 34, 36). This delay did not appear to be due to the processing of the sugars from high-mannose to complex types, since blocking sugar processing did not prevent immunoreactivity maturation (27). The precise role of the sugars in the modeling of HN and F_0 native immunoreactivities thus remains unclear.

Sendai virus and influenza virus surface glycoproteins share many properties, and the influenza virus hemagglutinin (HA) protein has been intensively studied as a model for transport of integral membrane proteins to the cell surface. HA is also first detected after pulse-labeling in a nonnative conformation, but it acquires its native structure relatively quickly (within 7 to 10 min [5, 10]). HA assumes its native conformation first as a monomer (43), but quickly forms a trimer before the protein moves beyond the endoplasmic reticulum (5, 10). Trimerization creates new epitopes not found on monomers as monoclonal antibodies exist which are specific for the oligomer (5, 10). In the case of Sendai virus HN, no monoclonal antibodies specific for the oligomer forms have been described to date. Oligomer-dependent epitopes are therefore either absent on HN or more rare on this protein. As the anti-native HN antibodies recognize the monomer and oligomer forms equally (27), these antibodies follow the maturation of the native structure independent of oligomerization. Therefore, the 30-min lag for HN immunoreactivity maturation that is detected by these anti-native antibodies cannot be due to oligomerization.

Besides sugar addition, the HN and F_0 proteins are also modified by intramolecular disulfide bonds, like the majority of transmembrane and secretory proteins (9). In this report, we examine the contribution of disulfide bond formation in creating the native HN and F_0 conformations. Our results showed a close correlation between proper folding of the proteins and formation of correct disulfide bonds. Correct disulfide bond formation appears to be essential to forming and maintaining the native conformations; sugar addition

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appears to be important in allowing the correct disulfide bonds to be formed.

MATERIALS AND METHODS

Virus and cells. Sendai virus strain Harris was grown in 9-day-old embryonated chicken eggs infected with 1/10th the yield of a virus plaque. After 3 days of incubation at 33°C, the allantoic fluid was collected, clarified (3,000 × *g*, 1 h), and frozen in aliquots at −70°C. BHK-21 cells from the American Type Culture Collection (Rockville, Md.) were routinely grown in Eagle minimal essential medium supplemented with 5% heat-inactivated fetal bovine serum under 5% CO_2 atmosphere.

Sendai virus rabbit antisera and monoclonal antibodies. Rabbit sera against the whole purified Sendai virus (RAb-vir) and against denatured HN and F_0 proteins (RAb-HN_{SDS}, RAb-$F_{0_{SDS}}$) were obtained as described before (27, 35). Monoclonal antibodies derived from mouse hybridoma cell lines secreting anti-HN or anti-F_0 antibodies were prepared as described previously (32). Anti-HN S-16, M-11, M-9, and M-21 and anti-F_0 M-16, M-33, and M-38 monoclonal antibodies were shown previously to specifically precipitate HN and F_0, respectively (34). S-16, M-9, and M-11 can neutralize viral infectivity, and all the monoclonal antibodies were shown to be directed against nonoverlapping epitopes by competitive binding assays (33).

Labeling of infected cells with [^{35}S]methionine. In vivo-labeled viral proteins were obtained by labeling infected BHK-21 cells with 20 μCi of [^{35}S]methionine (700 to 1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per ml in a medium containing 1/10th the normal concentration of methionine. For pulse-labeling experiments, the infected cells were first incubated for 30 min in a methionine-free medium, labeled for 5 min with 100 μCi of [^{35}S]methionine per ml, and then chased in the presence of 10 mM cold methionine.

In vitro synthesis of viral mRNAs. A typical in vitro transcription reaction mixture contained 10 mM dithiothreitol (DTT), 0.5 mM ATP, CTP, and UTP, 0.05 mM GTP, 0.5 mM ^{7m}GpppG (Pharmacia, Uppsala, Sweden), 1 U of RNasin (Genofit), about 1 μg of linearized SP64 plasmid containing either gene, 1 U of SP6 RNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in a final concentration of 40 mM Tris hydrochloride (pH 7.5), 6 mM $MgCl_2$, and 2 mM spermidine. Incubation was performed at 40°C for 60 min.

In vitro synthesis of viral proteins. About 1/10th to 1/15th of the result of an in vitro transcription reaction was used in a typical in vitro translation reaction with either a rabbit reticulocyte lysate or a wheat germ extract. The rabbit reticulocyte lysate (micrococcal nuclease treated; Promega Biotec, Madison, Wis.), used at a 70% concentration and supplemented with 15 μCi of [^{35}S]methionine per 10 μl, was incubated for 1 h at 30°C. When glycosylation was required, 1 μl of a dog pancreas rough microsomal membrane (DPM) (prepared by the method of Walter et al. [41]) was added per 10-μl reaction mixture. The wheat germ extract (Amersham) was used at a 50% concentration. It was supplemented with 15 μCi of [^{35}S]methionine per 15-μl reaction mixture and with potassium acetate and a methionine-free amino acid mix (final concentrations of 66 mM and 66 μM, respectively) and incubated at 25°C for 1 h.

Synthesis of viral proteins in frog oocytes. Microinjection of mRNAs into oocytes was modified from the method of Giglioni et al. (14) as follows. Oocytes were defolliculated by digesting pieces of ovary with 0.2% collagenase (type I) in calcium-free OR2 medium (8). All further incubations were in complete OR2 medium. Fully grown oocytes were selected manually, and batches of 25 were injected into the cytoplasm with 50 ng of the various in vitro-synthesized viral mRNAs (see above) suspended in 50 nl of 88 mM NaCl–1 mM KCl–15 mM HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid) (pH 7.0). The injection volume was controlled with an automatic pressure generator (Inject + Matic; Gabay, Geneva, Switzerland). One hour after injection, 500 μCi of [^{35}S]methionine per ml was added to the medium for 24 h. After that time, the radioactive supernatants were replaced by regular nonradioactive medium for a further 24-h incubation. The frog oocytes were then collected, and the cellular extracts were prepared for immunoprecipitation as described below.

Immunoprecipitation. [^{35}S]methionine-injected frog oocytes were washed twice with cold phosphate-buffered saline and then disrupted in Triton-SDS buffer (150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM Tris hydrochloride [pH 7.8], 2 mM phenylmethylsulfonyl fluoride), sonicated at 40 W for 15 s (Branson Sonic Power Co., Danbury, Conn.), and centrifuged for 15 min in an Eppendorf centrifuge. The supernatants (cellular extracts) were used in immunoprecipitations. The in vitro-made proteins were diluted with Triton-SDS buffer in the proportions described in Results before immunoprecipitation. The frog oocyte cellular extracts and the diluted in vitro translation reaction mixtures were incubated for 1 h at 4°C with the different antibodies (see Results) and then incubated for 2 more hours at 4°C with protein A-Sepharose CL-4B (Pharmacia), a 50% suspension used at 5 times the volume of the rabbit sera and at 10 times that of the monoclonal antibody preparations. Immunoprecipitates were then treated as described before (36) and analyzed by polyacrylamide gel electrophoresis (PAGE) (18). When analyzed under nonreducing conditions, the immunoprecipitates were suspended in a sample buffer devoid of β-ME, but were otherwise treated as the reduced samples.

Preparation of cDNA clones from Sendai virus genome. The molecular cloning of the Sendai virus genome was performed under two particular conditions. First, in vitro-made viral mRNAs (20) were used as templates for first-strand synthesis by the reverse transcriptase. Second, the primer for this first-strand synthesis was a synthetic oligonucleotide, DK-21, complementary to the 3′ end of the viral messengers and flanked at its 5′ end with six nucleotides (underlined) representing the *Kpn*I restriction site: 5′-d (C<u>GGTACC</u>$(T)_{10}$CTTA-3′) (supplied by André Cholet, Biogen). The first-strand cDNA was made by mixing 70 μg of in vitro-made mRNAs with 500 pmol of the synthetic oligonucleotide DK-21 with reverse transcriptase. For the second-strand synthesis, up to 2 μg of RNA-DNA hybrid made with reverse transcriptase was used under conditions described by Gubler and Hoffman (17). The resulting cDNA product was digested with *Kpn*I and ligated to pUC19 digested with *Kpn*I-*Hin*cII and phosphatase treated. After transformation of *Escherichia coli* HB101 and screening by the method of Grunstein and Hogness (16), 50% of the colonies were found to be virus specific. Identification of the clones representing the various Sendai virus genes was performed by rescreening the virus-specific colonies with specific nick-translated DNA probes already described (1–3, 7). The HN and F_0 genes were then constructed by shortening two respective clones containing the exact 3′ end of the messengers flanked by the *Kpn*I site, but extending over the adjacent gene in

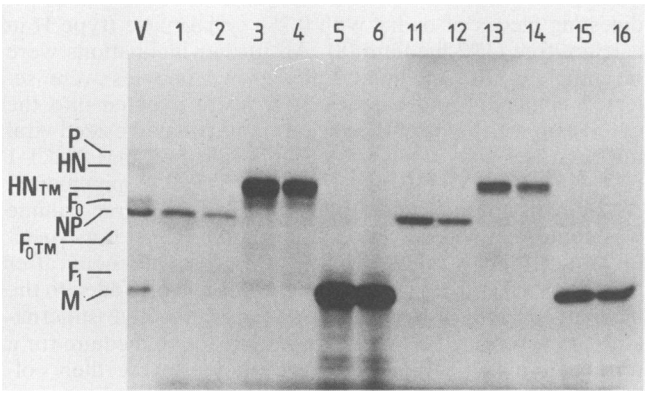

FIG. 1. In vitro synthesis of proteins from in vitro-made messengers. mRNAs synthesized in vitro from the pSP64-SVM, -F_0, and -HN plasmids (see Materials and Methods) were used to prime reticulocyte lysate (lanes 1 to 6) and wheat germ extract (lanes 11 and 16) systems in reaction volumes of 10 and 30 μl, respectively, under conditions described in Materials and Methods. One-half of the reaction volume was dissolved in PAGE sample buffer and analyzed by gel electrophoresis. Lanes: 1, 2, 11, 12, pSP64-SVF_0; 3, 4, 13, and 14, pSP64-SVHN; 5, 6, 15, and 16, pSP64-SVM. Odd-numbered lanes, Reactions supplemented with 2 mM phenylmethylsulfonyl fluoride and 1% aprotinin. Lane V, Viral protein markers: P, polymerase-associated protein; NP, nucleocapsid protein. F_1, C-terminus portion of the activated F_0 protein; M, matrix protein; HN_{TM} and $F_{0_{TM}}$, apparent molecular weight of HN and F_0 made in vivo in the presence of TM (from reference 27).

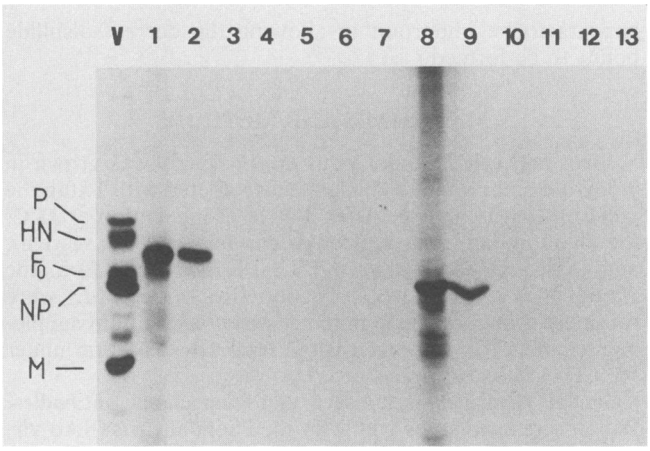

FIG. 2. Immunoreactivity of the in vitro-made nonglycosylated HN and F_0 proteins. The remaining one-half of the reaction mixtures (reticulocyte lysate and wheat germ extract) described in Fig. 1, primed with pSP64-SVHN or pSP64-SVF_0 mRNAs, was mixed and divided in, respectively, seven (lanes 1 to 7) and six (lanes 8 to 13) aliquots. One part of each mixture (lanes 1 and 8) was directly analyzed by PAGE. The others parts were diluted to 100 μl with the Triton-SDS buffer and immunoprecipitated with 5 μl of RAb-HN_{SDS} serum (lane 2), 5 μl of RAb-vir serum (lanes 3 and 10), 3 μl of the anti-HN monoclonal antibodies S-16, M-9, M-11, and M-21 (lanes 4 to 7), 5 μl of RAb-$F_{0_{SDS}}$ serum (lane 9), and 3 μl of the anti-F_0 monoclonal antibodies M-16, M-33, and M-38 (lanes 11 to 13). The immunoprecipitates were then analyzed by PAGE. Lane V, Viral protein markers as described in the legend to Fig. 1.

their 5′ end portion. These trimmed clones containing the whole coding sequences of the HN and F_0 proteins were finally subcloned into pSP64 to generate pSP64-SVHN and pSP64-SVF_0.

RESULTS

Immunoreactivity of in vitro-synthesized HN and F_0. Many transmembrane proteins can be made in in vitro systems, and the addition of DPMs allows for their correct glycosylation. Disulfide bond formation in vivo is also localized to the microsomal pathway, but it is unclear whether the preparations used are competent to form these bonds. It was therefore of interest to examine the immunoreactivities of the proteins made in vitro and to correlate their immunoreactivities or both glycosylation and disulfide bond formation.

Transcripts made in vitro from pSP64-SVHN and pSP64-SVF_0 were first translated in a reticulocyte lysate system and a wheat germ extract without added microsomes. The results were directly analyzed by PAGE (Fig. 1). Transcripts from pSP64-SVHN and pSP64-SVF_0 coded for proteins with apparent molecular weights smaller than those of the viral proteins (respectively, lanes 3, 4, 13, and 14 and lanes 1, 2, 11, and 12) but corresponding to those made in vivo in the presence of TM (27). These proteins represent the nonglycosylated forms of HN and F_0. For comparison, the Sendai virus M protein was also synthesized from pSP64-SVM (lanes 5, 6, 15, and 16). As M is not modified by glycosylation, its in vitro molecular weight corresponded to that of its in vivo counterpart. Both the wheat germ extract and the reticulocyte lysate gave similar results, and the presence of protease inhibitors (odd-numbered lanes) had no beneficial effect on the yields of the translation reactions. By comparison with the nonglycosylated proteins made in vivo in the presence of TM, we would expect these in vitro proteins to be in nonnative conformations and to react efficiently only with anti-denatured antibodies. Figure 2 shows that this was indeed so.

The addition of microsomes (Fig. 3, lane 1) resulted in the glycosylation of 50 to 80% of the HN and F_0 synthesized, as evidenced by their increased apparent molecular weights. Most of the glycosylated proteins migrated as a homogeneous band with a molecular weight slightly larger than that of the viral glycoproteins, and they were found to be totally sensitive to endo-β-*N*-acetylglucosaminidase digestion (data not shown) (38). This indicated that glycosylation took place on all the possible sites but that processing of high-mannose sugars did not. The immunoreactivity of these proteins, however, was not significantly different from that of their unglycosylated counterparts (Fig. 3). The glycosylated proteins still failed to react with their respective anti-native antibodies (Fig. 3A, lanes 4 to 7, and Fig. 3B, lanes 4 to 6). Only F_0 appeared to react partially with RAb-vir (Fig. 3B, lane 3). Both HN and F_0 proteins were again recognized well by anti-denatured antisera. Addition of high-mannose sugars in vitro was therefore insufficient to generate the antigenic properties of the native proteins.

To eliminate the possibility that accidental modifications of the genes during cloning and subcloning were responsible for this unexpected result, we injected the SP6 mRNAs into the cytoplasm of frog oocytes. The two proteins recovered from the oocytes reacted well with the anti-native antibodies (Fig. 4). Unexpectedly, the reactivity with the anti-denatured antibodies (lanes 1 and 6), which react very inefficiently with the native proteins made in BHK-21 cells (27), was significant. This could reflect a less complete folding of HN and F_0 in frog oocytes, leading to the exposure of epitopes that are not seen in the native proteins made in BHK-21 cells. Nevertheless, the immunoreactivity of HN and F_0 made in oocytes demonstrated that these mRNAs

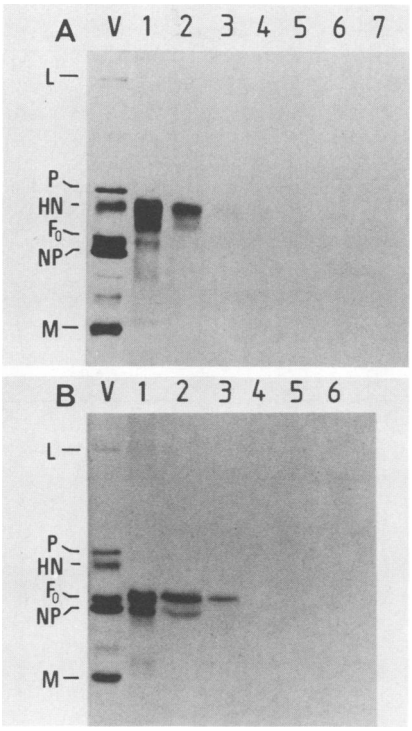

FIG. 3. Immunoreactivity of the in vitro-made glycosylated HN and F_0 proteins. HN (A) and F_0 (B) were synthesized in 20 μl of reticulocyte lysate reaction mixture supplemented with 1 μl of DPMs as described in Materials and Methods. The reaction mixtures were divided in seven and six aliquots, respectively, for HN and F_0. One part of each reaction mixture was directly analyzed by PAGE (lanes 1). The remaining part was immunoprecipitated under the conditions described in the legend to Fig. 2. Panel A, lane 2, RAb-HN_{SDS} serum. Lanes 3, RAb-vir serum. Panel A, lanes 4 to 7, Anti-HN monoclonal antibodies S-16, M-9, M-11, and M-21. Panel B, lane 2, RAb-$F_{0_{SDS}}$ serum. Panel B, lanes 4 to 6, Anti-F_0 monoclonal antibodies M-16, M-33, and M-38. The immunoprecipitates were then analyzed by PAGE. Lane V, Viral protein markers as described in the legend to Fig. 1. L, Large polymerase viral protein.

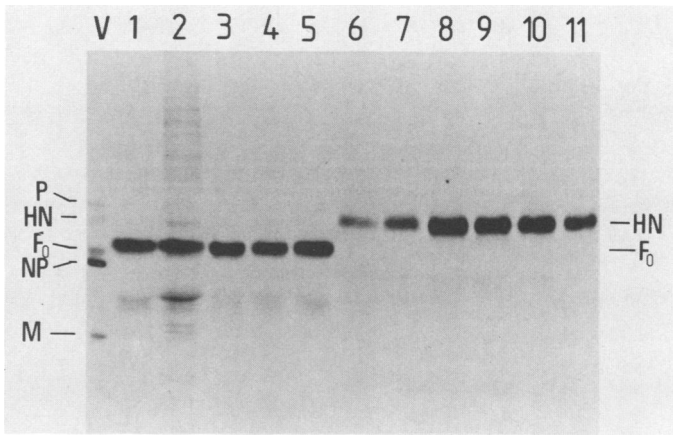

FIG. 4. Viral proteins synthesized in frog oocytes. Frog oocytes injected with in vitro-made HN, M, and F_0 mRNAs (or mock injected) and labeled with [^{35}S]methionine as described in Materials and Methods were disrupted in 800 μl of Triton-SDS buffer. A 400-μl sample of the F_0 and HN mRNA oocyte extracts was diluted to 1.0 and 1.2 ml, respectively, with Triton-SDS buffer. Aliquots of 200 μl were reacted with the different antibody preparations, and the immunoprecipitates were analyzed by PAGE. Lanes: 2 and 7, 10 μl of RAb-vir; 1, 10 μl of RAb-$F_{0_{SDS}}$ serum; 3 to 5, 3 μl of anti-F_0 monoclonal antibodies M-16, M-33, and M-38; 6, 10 μl of RAb-HN_{SDS} serum; 8 to 11, 3 μl of the anti-HN monoclonal antibodies S-16, M-9, M-11, and M-21; V, viral protein markers as described in the legend to Fig. 1.

could direct the synthesis of proteins with native immunoreactivity.

The presence of intramolecular disulfide bonds in HN and F_0 is indicated by the difference in their migration rates on SDS-PAGE under reducing or nonreducing conditions. Boiling HN in SDS in the absence of β-ME not only retained its homodimer form which depends on disulfides (23), but also caused the monomeric form of HN to migrate faster [Fig. 5, compare HN, lane 1, with $(HN)_1$, lanes 2 and 3]. The same result applied to F_0 (Fig. 5, compare F_0, lane 1, with F_0, lanes 4 and 5). This faster migration under nonreducing conditions presumably reflects the more compact globular mass of the proteins.

When the in vitro-made proteins were electrophoresed under reducing and nonreducing conditions (Fig. 6), their migration rates did not differ. This was true for both the nonglycosylated (Fig. 6A and B, compare lanes 2 and 3 with lanes 7 and 8) and the glycosylated proteins (Fig. 6A and B, compare lanes 4 and 5 with lanes 9 and 10). Further, the in vitro-glycosylated proteins, whether reduced or not, comigrated with the reduced in vivo proteins. The glycosylated proteins made in vitro therefore did not contain any disulfide bonds detectable by changes in PAGE migration, including those involved in dimerization. We do not know whether non-disulfide-linked oligomers were formed in vitro. However, if so, such oligomers did not contain the properly folded proteins, since no reactivity for anti-native antibodies was observed.

Attempts to generate disulfide bonds in vitro by incubation of the translation products with different concentrations of reduced-oxidized glutathione (42) in the presence or absence of urea proved unsuccessful in that no evidence of PAGE migration shifts or increased immunoreactivity for anti-native antibodies was observed (data not shown).

Intramolecular disulfide bonds and native HN and F_0 immunoreactivity. The results obtained so far suggest that intramolecular disulfide bond formation rather than glycosylation constitutes the determining step in HN and F_0 maturation. Since our in vitro system failed to generate disulfides, the in vivo-made proteins were examined further. If correct disulfides are essential for the formation of native protein structures, we would predict that (i) the reduction of disulfide bonds would abolish the native immunoreactivities, (ii) the proteins made in the presence of TM that do not react with the anti-native antibodies would not contain intramolecular disulfides, or at least not the correct ones, and (iii) the correct disulfide bonds should be found in F_0 right after its synthesis (since F_0 is fully reactive right after its synthesis) but in HN only after a delay.

To examine the first prediction, infected cell extracts were treated with increasing concentrations of DTT, diluted, and then reacted with anti-native antibodies. HN immunoreactivity was found to be very sensitive to DTT treatment (Fig. 7A), suggesting that disulfide bonds play a major role in maintaining its native structure. F_0 immunoreactivity, on the other hand, was not affected by DTT treatment (Fig. 7B, part R). The extent of F_0 reduction was therefore controlled by electrophoresis under nonreducing conditions. F_0 was much more resistant to reduction (Fig. 7B, part NR; see the presence of a major form of nonreduced F_0, $F_{0_{NR}}$, after

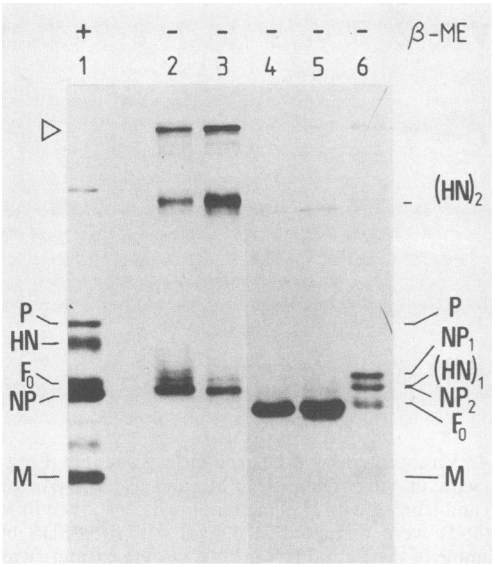

FIG. 5. PAGE migration of the in vivo-made F_0 and HN under nonreducing conditions. About 10^6 Sendai virus-infected BHK-21 cells were labeled with [^{35}S]methionine for 30 min (lanes 2 and 4) at 18 h postinfection or for 6 h (lanes 3 and 5) from 16 h postinfection. The cells were then disrupted in 200 μl of Triton-SDS buffer, and the cellular extracts were reacted with 3 μl of anti-HN monoclonal antibody S-16 or anti-F_0 monoclonal antibody M-38. The immunoprecipitates were then suspended and boiled in Laemmli (18) PAGE sample buffer lacking β-ME before electrophoresis. Lane 1 and 6, Viral protein markers from viral particles electrophoresed after boiling in the sample buffer with or without β-ME, respectively. Viral protein markers are described in the legend to Fig. 1. $(HN)_2$ and $(HN)_1$, Dimer and monomer forms, respectively, of the HN protein. NP_1 and NP_2, Two different forms of the nucleocapsid protein separated by electrophoresis under nonreducing conditions. ▷, Beginning of separating gel.

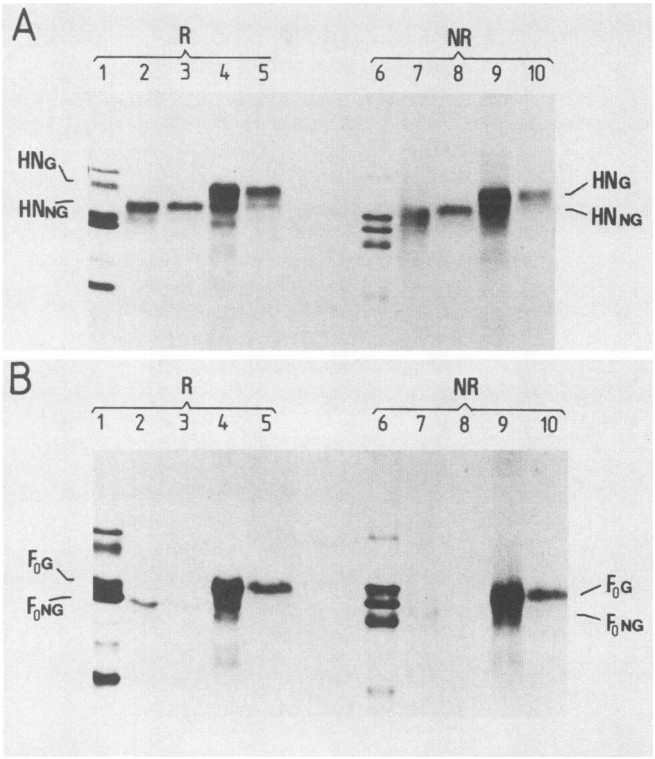

FIG. 6. Comparison of the PAGE migration properties under reducing and nonreducing conditions of the in vitro-made HN and F_0. HN (A) and F_0 (B) were synthesized in the reticulocyte lysate system in the absence (lanes 2, 3, 7, and 8) or presence (lanes 4, 5, 9, and 10) of DPMs as described in the legends to Fig. 2 and 4 and Materials and Methods. The proteins were either directly analyzed by PAGE (lanes 2, 4, 7, and 9) or immunoprecipitated with RAb-HN_{SDS} or RAb-$F_{0_{SDS}}$ (lanes 3, 5, 8, and 10 of panels A and B, respectively). The proteins were boiled in Laemmli (18) PAGE sample buffer under reducing (R, +β-ME) or nonreducing (NR, −β-ME) conditions before electrophoresis. HN_G and F_{0_G}, Glycosylated proteins; HN_{NG} and $F_{0_{NG}}$, nonglycosylated proteins.

treatment with 80 mM DTT). However, some partial or full reduction (respectively, $F_{0_{PR}}$ and F_{0_R} forms) did take place, and interestingly, these reduced forms conserved their native immunoreactivity. The difference in sensitivity to DTT reduction and the effect of reduction on native immunoreactivity between HN and F_0 may reflect differences in structure as indicated by their primary sequences. Unlike HN (2), virtually the entire F_0 polypeptide chain is predicted to be hydrophobic, and seven of its nine cysteines are clustered within 80 amino acids of the central portion of the F_1 moiety (1). This clustering of disulfides in a protein which is uniformly hydrophobic may render reduction by DTT difficult, and strong hydrophobic interactions may help retain the native structure in the absence of disulfides, even in the presence of SDS. This indeed appears to be so, as F_0 native immunoreactivity, unlike that of HN, was found to be very resistant to disruption by SDS alone (data not shown).

Disulfide bonds appear to be critical for both the creation and maintenance of the native structure, at least that of HN. The presence of disulfides in the nonglycosylated HN and F_0 made in vivo was therefore examined next. Proteins synthesized in the presence of TM (+TM) were recovered by immunoprecipitation with anti-denatured antibodies and then electrophoresed under reduced and nonreduced conditions (Fig. 8). A comparison with the proteins made under normal conditions (−TM) was also made. When glycosylated and analyzed under reducing conditions, HN and F_0 were, as expected, each recognized quite specifically by their respective antibodies (see −TM, +β-ME lanes, Fig. 8A and B). However, when the same antibodies were used to select the unglycosylated proteins, RAb-HN_{SDS} was found to precipitate considerable amounts of $F_{0_{TM}}$ and vice versa (+TM, +β-ME lanes, Fig. 8A and B). Moreover, when analyzed under nonreducing conditions, the unglycosylated proteins appeared not to enter the gel, as evidenced by the absence of discrete bands above background in the separatory gel and by the large amount of radioactivity at the origin of migration (closed arrow) and at the beginning of the separatory gel (open arrow) (see +TM, −β-ME, −iodoacetamide [−IAA] lanes, Fig. 8A and B). Treatment with 4 M urea did not improve their penetration into the gel (data not shown). HN_{TM} and $F_{0_{TM}}$ therefore appeared to be aggregated with each other (and possibly with other unlabeled proteins) in a form which was resolved by β-ME but not by 4 M urea, suggesting that interchain disulfides were responsible for this aggregation. To determine whether these disulfides were created intracellularly or formed upon cell lysis and extract preparation, we added the sulfhydryl reagent IAA to the buffers. IAA did not resolve the aggregation of HN_{TM} (+TM, −β-ME, + and −IAA lanes Fig. 8A), although it appeared to release a minor amount of a polypeptide whose migration is consistent with that of $F_{0_{TMNR}}$

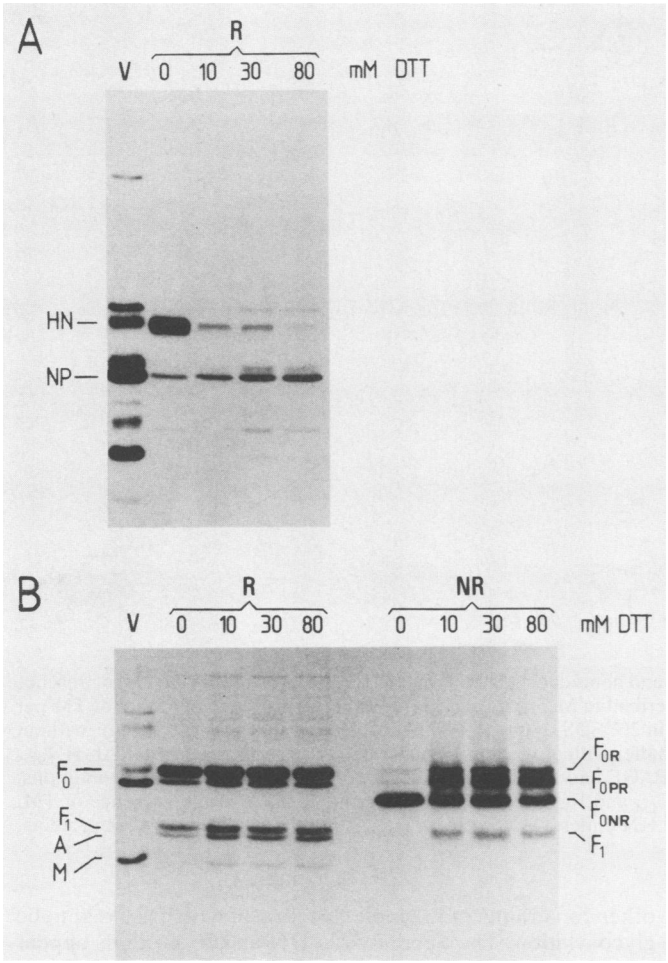

FIG. 7. Effect of disulfide bond reduction on the in vivo-made HN and F_0 immunoreactivities. Infected BHK-21 cells were labeled with [^{35}S]methionine as described in Materials and Methods. The cellular extracts, prepared as for immunoprecipitations, were incubated for 2.5 h at 22°C in presence of increasing concentrations of DTT. Immunoprecipitations were then performed at 4°C, after dilution of DTT to 5 mM with Triton-SDS buffer, with anti-HN monoclonal antibody M-11 (A) or anti-F_0 monoclonal antibody M-33 (B). The immunoprecipitates were then analyzed by PAGE under reducing (R) or nonreducing (NR) conditions. Lane V, Viral markers as described in the legend to Fig. 1. F_{0_R}, $F_{0_{PR}}$, $F_{0_{NR}}$, F_0 protein reduced, partially reduced, or nonreduced, respectively.

($F_{0_{TMNR}}$?, +TM, −β-ME, + and −IAA lanes, Fig. 8B). Interestingly, IAA allowed a better resolution of glycosylated HN dimers in particular and some monomers as well (−TM, −β-ME, +IAA lanes, Fig. 8A), which must have aggregated during extract preparation as shown by the material which did not enter the gel at all (solid and open arrows, −TM, −β-ME, −IAA lanes, Fig. 8A). This result serves as a control for the inability of IAA to resolve the aggregation of the nonglycosylated proteins and supports the intracellular formation of HN_{TM} and $F_{0_{TM}}$ aggregates. Such disulfide-linked aggregates of nonglycosylated proteins have been observed before (13, 22, 25). It was therefore impossible to determine whether the in vivo nonglycosylated proteins contained intramolecular disulfides. However, aberrant interchain disulfides were clearly formed in the absence of glycosylation. The absence of native immunoreactivity of HN_{TM} and $F_{0_{TM}}$ therefore correlates with the formation of aberrant disulfide bonds.

Finally, the time course of intrachain disulfide bond formation was analyzed. This was done by pulse-chase experiments in which the proteins were again recovered with anti-denatured antibodies after SDS denaturation of the cytoplasmic extracts to ensure recovery of any form of the proteins. Figure 9B demonstrates that correct formation of disulfides, as evidenced by the shift in mobility under nonreduced conditions (−β-ME lanes), took place in the majority of F_0, right at the end of the 5-min pulse (lane 0′). Only a minor amount of a protein migrating with the mobility of the reduced protein (marked F_{0_R}?) was present just after the pulse, and most of this appeared to be chased to $F_{0_{NR}}$. This was not the case for HN (Fig. 9A). For at least 10 min following its synthesis, the migration of the protein was identical under reduced and nonreduced conditions (Fig. 9A, compare lanes 0′ and 10′, +β-ME and −β-ME), demonstrating that no disulfides were formed during this period. The material at the beginning of the separatory gel (open arrow) probably represents an aggregated form of HN consistently observed when HN is electrophoresed under nonreducing conditions, whether HN is glycosylated or not (Fig. 5 and 8). Therefore, the inability of HN to react with anti-native antibodies soon after its synthesis correlates with lack of disulfide bond formation. By 30 min of pulse, however, disulfides were obviously formed since HN was found as a disulfide-linked dimer. Why the monomer form of HN with the proper disulfides was not found during the chase remains unclear. It was easily observed with longer pulse labeling times and anti-native antibodies (Fig. 5) (27). One possibility is that once the proper intrachain disulfides were formed, dimerization took place shortly afterward, and this could have been missed in our timing protocol. Nevertheless, the above data demonstrate a strong correlation between the lack of formation of the correct disulfides and the absence of the native structures.

DISCUSSION

The Sendai virus HN and F_0 proteins appear to contain only conformation-dependent epitopes. Polyclonal and monoclonal antibodies raised against the native proteins do not react with SDS- and β-ME-treated proteins (27). The in vitro-made proteins, whether glycosylated or not, are also unreactive with anti-native antibodies, but react well with antibodies to the denatured proteins. Thus, high-mannose sugar addition alone is not sufficient to form the native immunoreactivity. These in vitro-made proteins, however, did not contain disulfide bonds. This suggested that disulfides were critical in forming the native structures. DTT reduction of HN largely eliminated its native immunoreactivity, showing that disulfides were also important in maintaining its native structure. Fully reduced F_0, however, continued to react with anti-native antibodies; thus, disulfides are not required to maintain its native structure. Once formed, the structure of F_0 appears to be maintained by interactions which also make this protein extremely difficult to reduce—presumably hydrophobic interactions, as this protein is predicted to be hydrophobic throughout. Another example of resistance to reduction by DTT is that of the influenza virus HA protein in which the disulfide bond linking HA_1 to HA_2 could be reduced only after a conformational change induced by acid treatment (15).

The nonglycosylated HN and F_0 made in vivo (+TM) were found mainly as aberrant disulfide-linked aggregates.

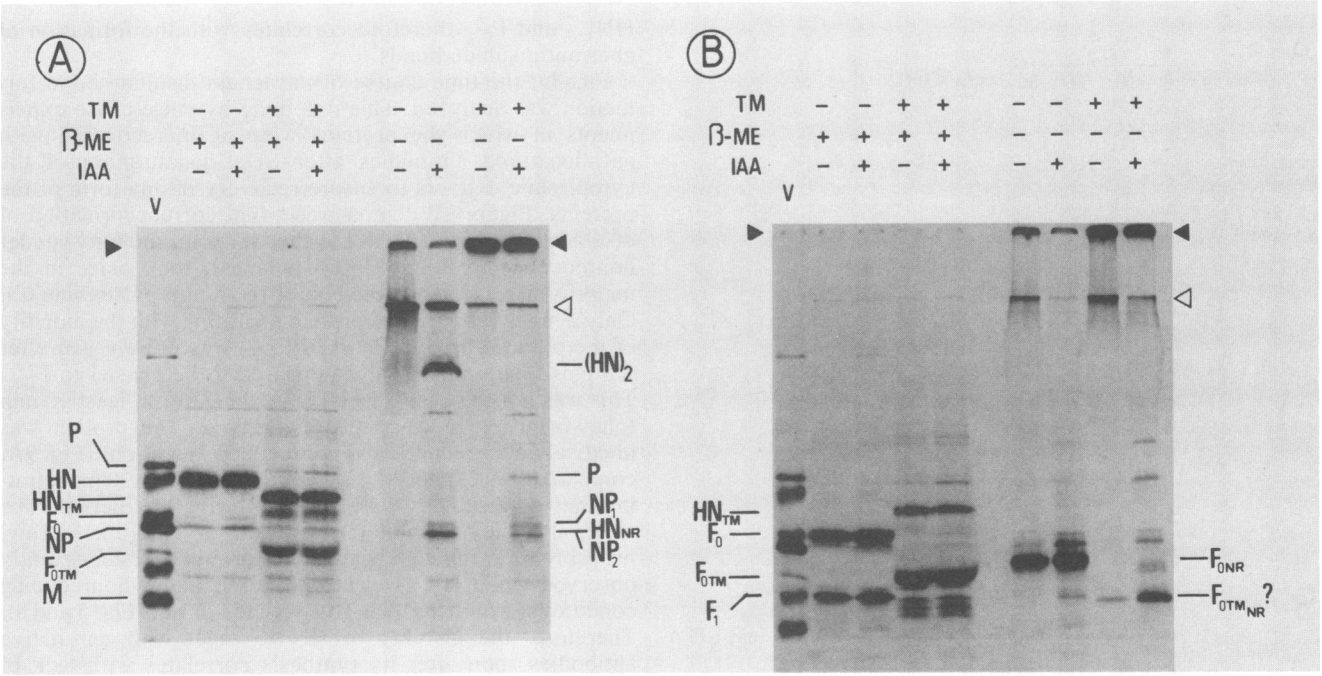

FIG. 8. Comparison of the PAGE migration properties under reducing and nonreducing conditions of HN and F_0 made in vivo in the presence of TM. Infected BHK-21 cells were labeled with [^{35}S]methionine as described in Materials and Methods in the presence of 0.5 μg of TM per ml from 5 to 24 h postinfection. The cells were collected and disrupted in 2% SDS–100 mM Tris hydrochloride (pH 7.5) with (+) or without (−) 10 mM IAA. Cellular extracts were diluted 20-fold with Triton-SDS buffer with or without IAA and immunoprecipitated with RAb-HN_{SDS} (A) or RAb-$F_{0_{SDS}}$ (B). The immunoprecipitates were finally analyzed by PAGE under reducing (+β-ME) or nonreducing (−β-ME) conditions. Lane V, Viral protein markers as described in the legend to Fig. 1. HN_{TM} and $F_{0_{TM}}$, Unglycosylated proteins made in the presence of TM. NP_1 and NP_2 are defined in the legend to Fig. 5. $(HN)_2$, Disulfide-linked HN dimers; HN_{NR}, $F_{0_{NR}}$, $F_{0_{TMNR}}$, nonreduced forms of the proteins. ▶, Origin of gel migration; ▷, beginning of separatory gel.

Moreover, absence of the correct disulfides correlated with the absence of native structure on the glycosylated proteins made in vivo. The native structures of HN and F_0 thus require the formation of correct intrachain disulfides, and glycosylation is required to allow the correct disulfides to form, presumably by preventing incorrect interchain disulfides from forming. These results corroborate and extend the observations made previously on the role of sugars in the folding of vesicular stomatitis virus G protein (13, 21, 22). Machamer and Rose (21, 22) have recently shown that improperly glycosylated G protein of vesicular stomatitis virus was very inefficiently transported to the plasma membrane, if at all, and that the nonglycosylated protein was also subject to aberrant intermolecular disulfide bonding. These authors conclude that the sugars play an indirect role in the intracellular transport of G protein. We can presently make this indirect role more explicit by saying that carbohydrate addition is required for proper intramolecular disulfide bonding. This then allows proper folding, which in turn is presumably required for efficient transport to the surface.

Disulfide bond formation has long been recognized as important in the folding of transmembrane and secretory proteins in particular (for example, see reference 31). Disulfide bond formation in vivo is thought to be restricted to the secretory membrane pathway, both because the cytoplasm is a highly reducing environment and because the enzyme disulfide isomerase is localized in the endoplasmic reticulum. This enzyme, as its name implies, is thought not to directly catalyze bond formation, which would take place spontaneously in the less-reducing environment of the endoplasmic reticulum, but to allow rearrangement of incorrect disulfides (9). The correct disulfides would be determined by other constraints of the molecule, one of which is likely to be glycosylation. The Sendai virus HN and F_0 proteins appear to be examples in which, in absence of sugar addition, conformations which form incorrect intrachain disulfides are favored and in which the addition of sugars disfavors these conformations and allows rearrangement into correct ones, a prerequisite for the native conformation. An example of rearrangement of disulfide bonds during glycoprotein maturation has previously been documented (24, 26). This scheme appears to account for the maturation of both Sendai virus HN and F_0. For HN, however, the formation of disulfides is delayed, and this may account for the lag of 20 to 30 min observed in HN maturation. The reason for such a delay is not clear. Possible explanations include slow lipid addition, influence of the N-terminus membrane anchorage, or association with another protein during retention in the rough endoplasmic reticulum (10), or transport to the membrane (28), as suggested for influenza virus HA.

The experiment presented above in Fig. 9, in which disulfide bond formation in HN coincided with the appearance of dimers, could lead to the conclusion that the HN native structure formation is tied to dimerization and therefore to intermolecular disulfide bonding. However, previous experiments (27) showed (i) that the HN monomer was recognized efficiently by anti-native antibodies (Fig. 5) and (ii) that the kinetics of dimer formation does not correlate with that of HN maturation. These data support the conclusions that HN monomers do possess the native structure after intramolecular disulfide bond formation. In this respect, HN would not differ from F_0. F_0 is also known to self-associate on the surfaces of virions (23, 37), yet its native structure is formed as a monomer, since it is present

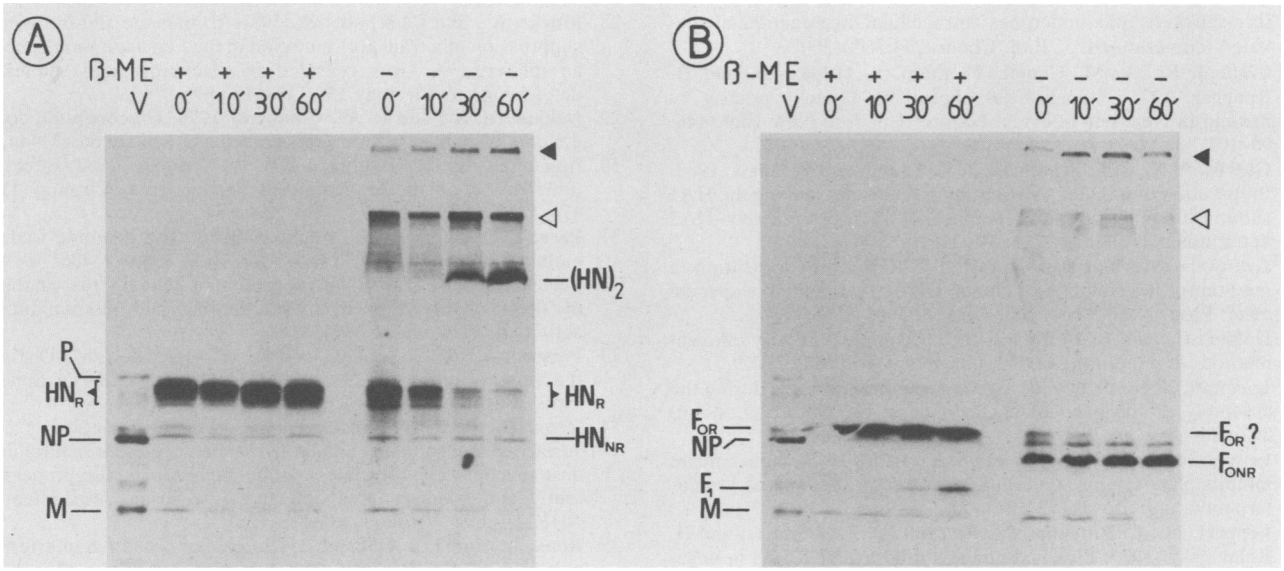

FIG. 9. Time course analysis of disulfide bond formation in HN and F_0 made in vivo under normal conditions. At 18 h postinfection, infected BHK-21 cells were pulse-labeled with [^{35}S]methionine for 5 min and then chased for 0, 10, 30, and 60 min. Cellular extracts were then prepared as described in the legend to Fig. 8 in the presence of 10 mM IAA and immunoreacted with RAb-HN_{SDS} (A) or RAb-$F_{0_{SDS}}$ (B). The immunoprecipitates were then analyzed by PAGE under reducing (+β-ME) or nonreducing (−β-ME) conditions. Lane V, Viral protein markers as defined in the legend to Fig. 1. $(HN)_2$, Disulfide-linked HN dimers; HN_R and F_{0_R}, reduced forms of the proteins lacking disulfides; HN_{NR}, $F_{0_{NR}}$, $F_{0_{TMNR}}$, nonreduced forms of the proteins. ◀, Origin of gel migration; ◁, beginning of the separatory gel.

as soon as the protein is made. Finally, this situation is similar to that observed with the influenza virus HA and the vesicular stomatitis virus G proteins, in which folding of the monomer is thought to take place before trimerization (6, 43).

In view of their aberrant interchain disulfides when HN and F_0 are made in the presence of TM, it is not surprising that these proteins never reach the cell surface and are unstable as well (27, 30). There are now many examples of this in glycoproteins of other viruses (19, 21, 22, 39, 40), although the proteins may be more or less stable. On the other hand, several examples have also been reported of nonglycosylated proteins which are apparently of normal stability and do reach the cell surface (12, 25, 29, 39). In one case (vesicular stomatitis virus Indiana), the G protein was functional as well (12), even though it was more sensitive to high temperature and refolded less easily after guanidine denaturation (11). It is possible that the reason that different proteins are affected differently in their stability and progress to the surface in the absence of glycosylation depends on the nature of the disulfides that form under these conditions.

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