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Structural Requirements for *O*-Glycosylation of the Mouse Hepatitis Virus Membrane Protein*

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The mouse hepatitis virus (MHV) membrane (M) protein contains only *O*-linked oligosaccharides. We have used this protein as a model to study the structural requirements for *O*-glycosylation. We show that MHV M is modified by the addition of a single oligosaccharide side chain at the cluster of 4 hydroxylamino acids present at its extreme amino terminus and identified Thr at position 5 as the functional acceptor site. The hydroxylamino acid cluster, which is quite conserved among *O*-glycosylated coronavirus M proteins, is not in itself sufficient for *O*-glycosylation. Downstream amino acids are required to introduce a functional *O*-glycosylation site into a foreign protein. In a mutagenic analysis *O*-glycosylation was found to be sensitive to some particular changes but no unique sequence motif for *O*-glycosylation could be identified. Expression of mutant M proteins in cells revealed that substitution of any 1 residue was tolerated, conceivably due to the occurrence of multiple UDP-*N*-acetylgalactosamine:polypeptide *N*-acetylgalactosaminyltransferases (GalNAc transferases). Indeed, MHV M served as a substrate for GalNac-T1, -T2, and -T3, as was demonstrated using an *in situ* glycosylation assay based on the co-expression of endoplasmic reticulum-retained forms of the GalNAc transferases with endoplasmic reticulum-resident MHV M mutants. The GalNAc transferases were found to have largely overlapping, but distinct substrate specificities. The requirement for a threonine as acceptor rather than a serine residue and the requirement for a proline residue three positions downstream of the acceptor site were found to be distinctive features.

Proteins become glycosylated either at asparagine residues (*N*-linked glycosylation) or at the hydroxyl groups of serine and threonine residues (*O*-linked glycosylation). While *N*-glycosylation has been well characterized, much less is known about mucin-type *O*-glycosylation. Many questions concerning the intracellular site(s) of sugar addition, the enzymes involved, their mode of action, and their sequence specificities have not been adequately answered.

N-Glycosylation is initiated in the endoplasmic reticulum (ER)[1] by the co-translational linkage of a large oligosaccharide structure to the polypeptide. In contrast, mucin-type *O*-glycosylation starts post-translationally with the addition of a *N*-acetylgalactosamine (GalNAc) monosaccharide. The location of this event is still controversial. According to some studies GalNAc is added in the early compartments of the secretory pathway (ER and ER to Golgi intermediate compartment) (1–4). In other reports, however, this addition is localized to the Golgi apparatus (5–7). *O*-Glycosylation is subsequently completed by the stepwise addition of monosaccharides such as galactose (Gal), sialic acid (SA), *N*-acetylglucosamine, and fucose (8).

The linkage of the initial GalNAc to the polypeptide in *O*-glycosylation is carried out by UDP-*N*-acetylgalactosamine:polypeptide *N*-acetylgalactosaminyltransferases (GalNAc transferases). To date four members of the mammalian GalNAc transferase family have been cloned and expressed: GalNAc-T1 (9, 10), GalNAc-T2 (11), GalNAc-T3 (12), and GalNAc-T4 (13). Other yet uncharacterized forms have also been identified (13, 14).

Whereas for *N*-linked glycosylation a defined consensus sequence for sugar addition (Asn-Xaa-(Ser/Thr)) has been established, no such clear-cut motif has been resolved for *O*-glycosylation. Of a number of glycoproteins the *O*-glycosylated residue was identified biochemically. The presence of Pro, Ser, and Thr residues at positions adjacent to a glycosylation site were found to favor oligosaccharide addition, but amino acid sequences defining an *O*-glycosylation site were not resolved. Other studies analyzed the substrate specificities of GalNAc transferase activities in cell extracts or of purified recombinant GalNAc transferases *in vitro* (for reviews, see Refs. 14 and 15). The relative merits of these approaches have been discussed (14, 15). Wandall *et al.* (16) studied the substrate specificities of three purified recombinant GalNAc transferases (GalNAc-T1, -T2 and -T3) using short synthetic peptides. The three enzymes appeared to display distinct but partly overlapping sequence specificities.

Recently Röttger *et al.* (7) developed a glycosylation assay based on the expression of ER-retained chimeric GalNAc transferases. In this assay an ER-retained form of CD8 was shown to

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[1] The abbreviations used are: ER, endoplasmic reticulum; BHK-21 cells, baby hamster kidney cells; BFA, brefeldin A; DMJ, 1-deoxymannojirimycin; EAV M, equine arteritis virus membrane protein; endo H, endoglycosidase H; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GalNAc transferase, UDP-*N*-acetylgalactosamine:polypeptide *N*-acetylgalactosaminyltransferase; GalNAc-T1, -T2, and -T3, UDP-*N*-acetylgalactosamine:polypeptide *N*-acetylgalactosaminyltransferase 1, 2, and 3; glyco F, endoglycosidase F/N-glycosidase F; MHV M, mouse hepatitis virus membrane protein M; MVA-T7pol, recombinant modified vaccinia virus strain Ankara encoding the T7 RNA polymerase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SA, sialic acid; ST, sialyltransferase; vTF7-3, recombinant vaccinia virus encoding the bacteriophage T7 RNA polymerase; WT, wild-type.

TABLE I
Primers used in site-directed mutagenesis

No.	Sequence	Resulting mutant
1	5′-GTGTATAGATATGAAAGGTACCGTG-3′	
2	5′-GCTCTAGATTAGGCTGTCTTCTTTGCGGTGTCCG-3′	KK
3	5′-GTCTAAACATACACGGTACCTTTC-3′	
4	5′-GGGGTACCAAACATTATGGCTAGTAC-3′	S2A
5	5′-GGGGTACCAAACATTATGAGTGCTACTAC-3′	S3A
6	5′-GGGGTACCAAACATTATGAGTAGTGCTAC-3′	T4A
7	5′-GGGGTACCAAACATTATGAGTAGTACTGCTCAGG-3′	T5A
8	5′-AAGGTACCAAACATTATGGCTGCTAC-3′	A2A3
9	5′-AAGGTACCAAACATTATGAGTAGTGCTGC-3′	A4A5
10	5′-CCAAACATTATGACTGCTGCTGC-3′	3AT2
11	5′-CCAAACATTATGGCTACTGCTGC-3′	3AT3
12	5′-CAAACATTATGGCTGCTACTGCTCAG-3′	3AT4
13	5′-CAAACATTATGGCTGCTGCTACTC-3′	3AT5
14	5′-CAAACATTATGGGTGGTGGTACACAGGC-3′	3GT5
15	5′-AAGGTACCAAACATTATGAGTAGTTCTAGTCAG-3′	4S
16	5′-CCAAACATTATGAATAGTAC-3′	S2N
17	5′-CAAACATTATGACTACTCAGGC-3′	T2T3
18	5′-CAAACATTATGAGTAGTACCACTGCGGCCC-3′	Q6A
19	5′-CAAACATTATGAGTAGTACAACACAGGGCCCAG-3′	A7G
20	5′-CAAACATTATGAGCAGTACTACACAGGCCGCAGAGC-3′	P8A
21	5′-CAAACATTATGAGTAGTACTACACAAGCTCCAGAGGCCGTC-3′	P10A
22	5′-CAAACATTATGAGTAGTACAACACAAGCCGCAGAGG-3′	A8A10
23	5′-CCAAACATTATGCATCACCATCACCATCACAGTAGCACTACTCAGGCCC-3′	His
24	5′-CAGTAGGTCATTGTAGCTTG-3′	
25	5′-TCTTAAGGCCATGAGTTCTACTACAGGCGCCATAGATTCATTTTGTG-3′	EAV M + 4
26	5′-CAAGCACCAGAGCCCGGCGCCATAGATTCATTTTGTG-3′	EAV M + 9A
27	5′-GGGCTCTGGTGCTTGTGTAGTAGAACTCATGGCCTTAAGGCTC-3′	EAV M + 9A
28	5′-TCTATGGCATTTACAGCG-3′	EAV M + 9A
29	5′-CCTCGAGGGCCTACGGCAGCAAAGTCA-3′	EAV M + 9A
30	5′-GGCCATGAGTTCTTACTACASNGSNGSNGSNGGGCGCCATAGATTCATTTTG-3′[a]	EAV M + 9B–E

[a] S indicates C or G; N can be any of the bases A, C, G, or T.

be glycosylated by GalNAc-T1 and -T2 with different efficiencies whereas the protein remained unglycosylated when coexpressed with GalNAc-T3. This *in situ* glycosylation assay offers the possibility to study glycosylation of proteins in a cellular environment for each GalNAc transferase separately.

In the present study we have used the mouse hepatitis virus (MHV) membrane (M) protein (previously called E1) as a model to study the structural requirements for *O*-glycosylation *in vivo* and *in situ*. The MHV M protein, which localizes to the Golgi complex when expressed alone (17–19), is a triple-spanning membrane protein (22–25 kDa) with a short (approximately 25 residues) amino-terminal domain exposed on the luminal side and with its carboxyl-terminal half located on the cytoplasmic side of the membrane (20). This protein was shown to be exclusively *O*-glycosylated, most likely at a cluster of 4 hydroxyl-amino acids (Ser-Ser-Thr-Thr) at the extreme amino terminus (3, 21). The number of functional acceptor sites is unknown but it was suggested that up to three side chains might be added (3, 21). The oligosaccharides are synthesized by the sequential addition of GalNAc, Gal, and SA, followed sometimes by one or two additional, unidentified sugar modifications. The maturation of the sugar side chain can be followed easily in biochemical experiments as every sugar addition gives rise to an electrophoretically detectable shift (18). Here we have identified the precise *O*-glycosylation site in the M protein and studied the structural requirements for its *O*-glycosylation *in vivo*. Structural requirements for *O*-glycosylation were subsequently investigated for three GalNAc transferases (GalNAc-T1, -T2, and -T3) using the *in situ* glycosylation assay (7). We also studied the interferential effects of an *N*-glycosylation site introduced close to the site of *O*-glycosylation. Finally we analyzed whether of the *O*-glycosylation sequence motif of MHV M was utilized when transplanted onto a reporter membrane protein.

EXPERIMENTAL PROCEDURES

Cells, Viruses, and Antibodies—Recombinant vaccinia virus encoding the bacteriophage T7 RNA polymerase (vTF7–3) was obtained from Dr. B. Moss. The recombinant modified vaccinia virus strain Ankara (MVA) encoding the T7 RNA polymerase (MVA-T7pol) (22) was a kind gift of Dr. G. Sutter. OST7-1 cells (obtained from Dr. B. Moss) and BHK-21 cells (obtained from American Type Culture Collection, Rockville, MD) were maintained as monolayer cultures in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 IU of penicillin/ml, and 100 μg of streptomycin/ml (all from Life Technologies, Ltd., Paisley, United Kingdom). The polyclonal MHV-A59 antiserum (K134) (23) and the antipeptide serum specific for the membrane protein of equine arteritis virus (EAV M) (24) have been described earlier.

Expression Vectors and Site-directed Mutagenesis—All expression vectors contained the genes under control of bacteriophage T7 transcription regulatory elements. Expression construct pTUG3M contains the MHV strain A59 M gene cloned in pTUG3 (25) as a *Bam*HI fragment (17). A mutant M gene encoding a M protein with an ER retention signal in its carboxyl terminus was made by PCR mutagenesis using a 5′ primer (primer 1; see Table I) containing the unique *Kpn*I site, present in the M gene, and a 3′ terminal primer containing the desired mutation and a *Xba*I site (primer 2). The PCR fragment was digested with *Kpn*I and *Xba*I and cloned into an intermediate cloning vector pBMΔ5 (26) that had been treated with the same enzymes. The resulting mutant M gene (M-KK) was transferred as a *Bam*HI fragment into expression vector pTUG3 and designated pTUG3M-KK. A number of mutations were introduced into the amino-terminal domain of the wild-type (WT) M and M-KK gene by PCR mutagenesis using 5′ terminal primers (primers 4 through 23) containing the desired mutations and a 3′ internal primer (primer 3) corresponding to the region of the M gene that contains the unique *Kpn*I site. PCR fragments were first cloned into the pNOTA/T7 shuttle vector (5 Prime → 3 Prime, Inc.) and subsequently excised from the plasmid with *Kpn*I and cloned into the expression vector pTUG3M and in some cases also in pTUG3M-KK, from which the corresponding M fragments had been removed using *Kpn*I. Expression cassettes coding for the hybrid proteins p33/GalNAc transferase 1/c-Myc, p33/GalNAc transferase 2/vesicular stomatitis virus-G, p33/GalNAc transferase 3/vesicular stomatitis virus-G, and p33/sialyltransferase/vesicular stomatitis virus-G (7) were cut out from

their vector pCMUIV using *Bam*HI and introduced into pTUG3 that had been digested with the same enzyme. Expression construct pAVI16 contains the EAV M gene, cloned behind the T7 promotor in pBluescript SK(−) (24). The MHV/EAV hybrid protein EAV M+4 has an insertion of the 4 hydroxylamino acids of MHV M right behind the initiating methionine of EAV M. The construct coding for this hybrid protein was generated by PCR mutagenesis using pAVI16 and primers 24 and 25. The PCR fragment was cloned into the pNOTA/T7 shuttle vector. The resulting construct was used in expression experiments. The MHV/EAV hybrid protein EAV M+9A has an insertion of 9 amino acids corresponding to the MHV M amino-terminal sequence behind the initiating methionine of EAV M. The construct encoding this hybrid protein was generated by splicing overlap extension PCR (27) using inside primers 26 and 27 and external primers 24, 28, and 29. Initially, primers 24 and 26 were used to amplify the 3′ region of EAV M; primers 27 and 28 were used to amplify the 5′ region of EAV M. The PCR fragments produced were purified, mixed, and amplified using primers 24 and 29. The resulting PCR fragment was cloned into the pNOTA/T7 shuttle vector, which was subsequently used in expression experiments. Sequencing revealed that the mutant contained an additional, unintended mutation immediately downstream of the MHV-specific sequence resulting in a glycine to alanine substitution. Mutants EAV M+9B-E also have an insertion of 9 amino acids behind the initiating methionine of EAV M. These insertions consist of the 4 hydroxylamino acids followed by 5 other amino acids. The constructs encoding these proteins were generated by PCR mutagenesis using EAV M+4 and primers 24 and 30. The latter primer is degenerated in the region encoding the 5 amino acids downstream of the hydroxylamino acid cluster. The PCR fragments were cloned into the pGEM-T Easy Vector (Promega) according to the manufacturer's instructions, excised from the plasmids with *Eco*RI, and cloned into the *Eco*RI digested expression vector pTUG3. Of four clones the mutations generated were determined by sequencing (see Fig. 7*C*).

Metabolic Labeling and Immunoprecipitation—Subconfluent monolayers of OST7-1 or BHK-21 cells in 10-cm^2 tissue culture dishes were inoculated with vTF7-3 or MVA-T7pol and subsequently transfected using Lipofectin (Life Technologies) as described (26). Where indicated brefeldin A (BFA, 6 μg/ml, from t = 3 h), 1-deoxy-mannojirimycin (DMJ, 1 mM, from t = 4.5 h), or tunicamycin (5 μg/ml, from t = 3 h) were added to the culture media. At t = 4.5 h, OST-7 cells were washed with phosphate-buffered saline and starved for 30 min in cysteine- and methionine-free modified Eagle's medium containing 10 mM HEPES, pH 7.2, and 5% dialyzed fetal calf serum. The medium was then replaced by 600 μl of similar medium containing 100 μCi of ^{35}S *in vitro* cell labeling mixture (Amersham) and cells were labeled for the indicated time periods. After pulse labeling, cells were chased with culture medium containing 2 mM methionine and 2 mM cysteine. Proteins were immunoprecipitated from lysates as described before (26). In some cases immunoprecipitates were treated with endoglycosidase F/*N*-glycosidase F (glyco F, Boehringer Mannheim) or with endoglycosidase H (endo H, Boehringer Mannheim) as described earlier (28) before analysis by SDS-polyacrylamide gel electrophoresis (PAGE) in 15 or 17.5% polyacrylamide gels.

Indirect Immunofluorescence—Indirect immunofluorescence was performed on BHK-21 cells grown on 12-mm coverslips. The morphology of these cells makes them more convenient than OST7-1 cells for this assay. Cells were fixed at t = 6 h, permeabilized, and stained for immunofluorescence as described previously (26). The rabbit anti-MHV serum K134 was used at a 1:400 dilution.

RESULTS

Identification of the MHV M Glycosylation Site—O-Glycosylation of MHV M occurs at the exposed luminal domain of the protein, most likely at the cluster of 4 hydroxylamino acids (Ser-Ser-Thr-Thr) at the very amino terminus (Table II). Indeed, substitution of the Thr residue at position 15, the only alternative hydroxylamino acid in the ectodomain, by a valine did not influence *O*-glycosylation (not shown). In order to identify the residue(s) to which a sugar side chain is added we constructed a number of mutant M proteins, in which either 1 or 2 of the hydroxylamino acids were substituted by alanines (Table II and Fig. 1). WT and mutant M proteins were expressed in OST7-1 cells, pulse labeled for 15 min, and chased for 15, 30, or 60 min. In all cases only the unglycosylated M species (M0) was observed after the pulse. During the chases a slower migrating M species (M3) appeared for all mutants except for mutant A4A5 which remained unmodified even after 180 min (not shown); in some cases a minor additional species (M4) appeared. M3 represents a glycosylated form, which arises by the sequential addition of GalNAc, Gal, and SA; an additional, yet unknown modification gives rise to M4 (3, 18, 21). Since none of the single substitutions of the hydroxylamino acids abolished *O*-glycosylation, multiple or alternative acceptor sites apparently exist. The data indicate that both Thr residues can be used by the modifying enzymes or that substitution of the threonines disrupts the consensus sequence for glycosylation at the serine residue(s). The results are consistent with the idea that the hydroxylamino acid cluster (and not Thr^{15}) contains the acceptor site(s). It should be noted that all mutants localized to the Golgi complex similar to WT M as was verified by immunofluorescence (not shown).

TABLE II
Mutant M proteins and their O-glycosylation

M protein	Sequence[a]	Glycosylation in Vivo[b]	Glycosylation in Situ[c] T1	T2	T3
WT	M SSTTQAPEPVYQWTADEAVQFLKE	+++	+	+	+
S2A	- A----------	+++			
S3A	- -A---------	+++			
T4A	- --A--------	+++			
T5A	- ---A-------	++			
A2A3	- AA---------	+++			
A4A5	- --AA-------	−	−	−	−
3AT2	- TAAA-------	+			
3AT3	- ATAA-------	−			
3AT4	- AA-A-------	++	−	−	+
3AT5	- AAA--------	+++	+	+	+
3GT5	- GGG--------	+++			
4S	- --SS-------	++	−	−	+
T2T3	- ----------	+++			
Q6A	- ----A------	+++	+	+	+
A7G	- -----G-----	+++	+	+	+
P8A	- ------A----	++	+	−	−
P10A	- --------A--	+++	+	+	+
A8A10	- ------A-A--	++			
His[d]	-*---------	+++			
S2N	- N---------	+++			
S3N	- -N--------	+++			
ΔN[e]	- ----- ---	−			

[a] Amino acid sequence of the M protein ectodomains. Hyphens indicate residues identical to those of WT M. Gaps are introduced for maximal alignment.

[b] Comparative semiquantitative analysis of the glycosylation of the expressed M proteins: +++, ++, and + indicate efficient, less efficient, and inefficient glycosylation, respectively; − indicates no glycosylation.

[c] Glycosylation of ER-retained M proteins by ER-retained transferases. T1, T2, and T3 represent GalNAc transferase 1, 2, and 3. Whether M proteins are substrates is indicated by + and −.

[d] The asterisk indicates an insertion of 6 histidines.

[e] Mutant ΔN, which has a deletion of A7 through F22, was shown not to be glycosylated (42).

Additional Hydroxylamino Acid Substitutions—We next prepared another set of M proteins with mutations in the cluster of hydroxylamino acids. Mutants were prepared which had only 1 hydroxylamino acid, a Thr, at positions 2, 3, 4, or 5, the remaining 3 hydroxylamino acids being substituted by Ala or Gly. In addition, mutants were made with a deletion of the 2 Ser residues or with a substitution of the 2 Thr residues by Ser residues (Table II). All mutants localized to the Golgi complex as was verified by immunofluorescence. Genes were expressed as above except that the labeling was for 5 min while chase times of 60 and 180 min were used (Fig. 2). WT M was efficiently glycosylated: after 180 min of chase the unglycosylated M0 form had been fully converted into the M3 and M4 forms. Mutant 3AT3 did not become glycosylated at all, mutant 3AT2 only to a very minor extent. Mutants 3AT4 and 3AT5 were both

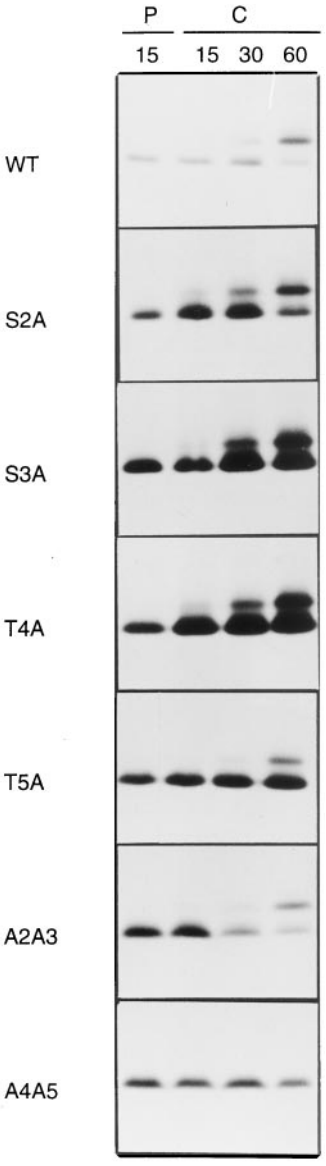

FIG. 1. **Identification of the MHV M glycosylation site.** Recombinant vaccinia virus vTF7-3 infected OST7-1 cells were transfected with a plasmid containing WT or a mutant M gene. Cells were labeled for 15 min with ^{35}S-labeled amino acids and lysed directly or chased for 15, 30, or 60 min. Cell lysates were processed for immunoprecipitation with a polyclonal anti-MHV serum (K134) followed by SDS-15% PAGE. The different M genes expressed are indicated beside each set.

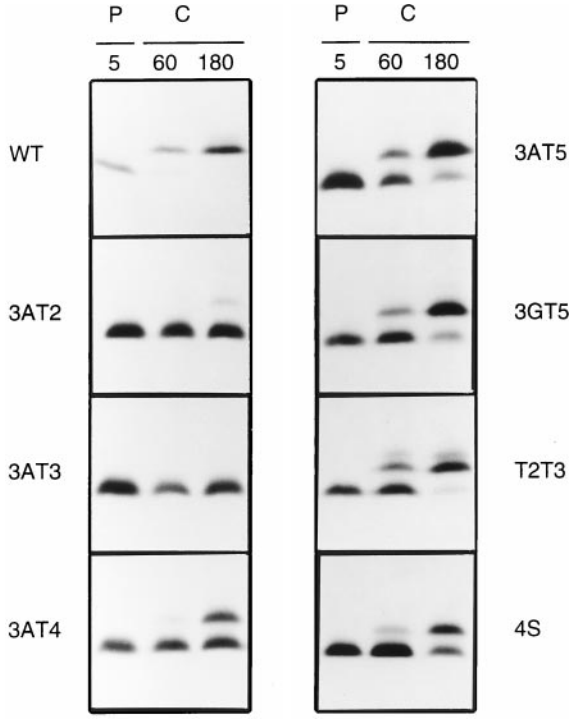

FIG. 2. **Additional hydroxylamino acid substitutions.** Genes were expressed as described in the legend to Fig. 1 except that the labeling was for 5 min while chase times of 60 and 180 min were used.

glycosylated: after 180 min of chase the majority of the unglycosylated form had been converted into the M3 form. Substitution of the hydroxylamino acids by Gly instead of Ala did not affect glycosylation. Mutants with only one acceptor site made a similar shift in apparent molecular weight (M_r) as WT M and were converted into the M3 from. Mutants 4S and T2T3 both became glycosylated.

The results indicate that alternative *O*-glycosylation sites exist. The M protein can be modified at both its threonines. Since Thr at position 5 was glycosylated more efficiently than Thr at position 4 (Figs. 1 and 2), Thr^5 is the most likely candidate for *O*-glycosylation in WT M. When these threonines are not available some glycosylation at the second position can occur when this residue is a threonine. A cluster of hydroxylamino acids is not required. The identity of the hydroxylamino acid at positions 4 and 5 does not appear to be essential: replacement of the threonines by serines (mutant 4S) still yielded a functional *O*-glycosylation site. Furthermore, decreasing the distance between the acceptor site and the initiating Met, by deleting the serine residues (mutant T2T3), did not affect *O*-glycosylation. Since mutants with only one acceptor site made a similar shift in M_r as WT M, it is clear that in WT M only 1 residue at a time is modified by *O*-linked sugars.

Effect of Mutations in the Flanking Regions—In search for sequence requirements around the acceptor site we investigated the effect of mutations in the regions flanking the hydroxylamino acid cluster. Replacements were made in the downstream residues at positions 6, 7, 8, and 10 and a stretch of six histidines was inserted just upstream the cluster (Table II and Fig. 3). All mutants localized in the Golgi complex as was verified by immunofluorescence.

The His mutant clearly became glycosylated. After 180 min of chase the majority of the unglycosylated M0 form had been converted into the glycosylated M3 form and an additional slower migrating form. The latter form may represent a further modification of the side chain present in M3 or, considering the relatively large mobility difference between the two forms, may carry two sugar side chains. However, this His form was not found after the cells had been treated with BFA (not shown), just as is the case for WT M4 (18). As BFA inhibits membrane transport to the trans-Golgi network (29, 30), this observation indicates that the slowest migrating His species is probably the result of an additional trans-Golgi network modification(s) just as M4. Clearly, the distance between the *O*-glycosylation site and the amino terminus of the M protein is not crucial for *O*-glycosylation.

None of the amino acid substitutions downstream of the hydroxylamino acid cluster blocked *O*-glycosylation of the M protein. Mutant proteins with mutations immediately downstream of the Thr acceptor site (Q6A and A7G) were efficiently glycosylated. Substitutions of the proline residues reduced the rate of conversion of the M protein into the M3 and M4 forms. This effect was stronger for the proline at position 8 than for the one at position 10, while replacement of both prolines together did not have a synergistic effect. Evidently, the pres-

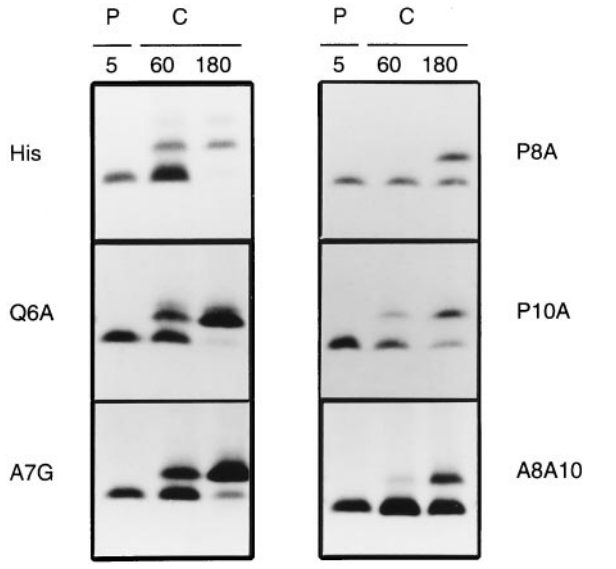

FIG. 3. **Effect of mutations in the flanking regions.** Genes were expressed as described in the legend to Fig. 2.

ence of the prolines, particularly the one located 3 residues downstream of the threonine acceptor site, is beneficial for *O*-glycosylation. The other residues downstream of the hydroxylamino acid cluster do not seem to be important for efficient glycosylation. Altogether these results point to flexible sequence requirements.

Interestingly, when we applied a recent *O*-glycosylation prediction program (Ref. 40; http://genome.cbs.dtu.dk/services/NetOglyc/) to our WT and mutant M amino-terminal sequences, the predictions were generally in good agreement with the observed results. Sequences predicted not to be glycosylated were indeed not modified in our experiments. However, not in all cases where hydroxylamino acids were predicted to be glycosylated were these residues actually used as *O*-glycosylation sites.

To further study the sequence requirements for *O*-glycosylation, we compared the amino acid sequences of the MHV-A59 M ectodomain with that of other coronaviruses known to be *O*-glycosylated. As indicated in Table III, a 1-residue insertion, even of a charged amino acid, within the hydroxylamino acid cluster is tolerated, as are several substitutions of downstream residues. Remarkably, a Pro residue at position +3 relative to the last Thr of the cluster is a conserved feature among all the M proteins that are glycosylated. A small 2-residue deletion downstream of the cluster is acceptable, but glycosylation is abolished in MHV-RI, where the deletion comprises 5 residues. Clearly, the conserved hydroxylamino acid cluster alone is not sufficient for *O*-glycosylation to occur. The lack of glycosylation in the absence of the downstream sequence may obviously be due to specific sequence requirements, but it may as well be caused by alterations in secondary structure or by the closer proximity of the glycosylation site to the membrane surface.

In Situ Glycosylation of MHV M by GalNAc-T1, -T2, and -T3—To further study the *O*-glycosylation of the MHV M protein, particularly the GalNAc transferases involved and their sequence requirements, we used a recently developed *in situ* *O*-glycosylation assay (7). This assay is based on the co-expression of ER-resident forms of the GalNAc transferases T1, T2, and T3 with substrates retained in the same compartment. Endogenous GalNAc transferase activity is not present in the ER, but the enzymes do function when retained (7). Since MHV M localizes to the Golgi complex (17–19), an ER-retained mutant (designated M-KK) was prepared carrying a cytoplasmic

TABLE III
Amino acid sequence and glycosylation of the M protein ectodomains of several coronaviruses

Coronavirus[a]	M protein ectodomain[a]	*O*-Glycosylation[a]
MHV-A59	MSS TTQAPEPVYQWTADEAVQFLKE	+
MHV-JHM	--- -----G----------	+
DVIM/MHV-S	--- --P--Q-I----------	+
MHV-RI	--- ---D ----------	−
OC-43	---K--P-- --I----------	+
BCV	---V--P-- --T----------	+

[a] Sequence and glycosylation of the M protein ectodomains of MHV-A59 (51, 52), MHV-JHM (33, 53) DVIM/MHV-S (54, 55), MHV-RI (41, 55), human coronavirus OC43 (56), and bovine coronavirus (BCV) (35, 57). Hyphens indicate residues identical to those of WT M. Gaps are introduced for maximal alignment.

KK*XX* ER retrieval signal, KKTA as in another type III membrane protein 3-hydroxy-3-methylglutaryl-coenzyme A reductase (31). ER localization of this mutant was verified by immunofluorescence (Fig. 4). Using the anti-MHV serum, WT M expressed in BHK-21 cells showed a typical Golgi staining pattern. In contrast, the mutant M-KK appeared in a reticulate staining pattern typical for proteins localized in the ER. In pulse-chase experiments no trace of glycosylation of the mutant protein could be detected even after 2 h of chase (not shown), demonstrating that retention was very effective and that no endogenous GalNAc transferase activity, able to glycosylate MHV M, was present in the ER.

The mutant M-KK protein was subsequently tested in the *in situ* glycosylation assay by coexpression with each of the GalNAc transferases in OST7-1 cells. Cells were pulse-labeled for 30 min followed by a 60-min chase. Immunoprecipitation experiments revealed that GalNAc-T1, -T2, and -T3, as well as an ER-retained form of sialyltransferase (ST) used as a negative control, were each well expressed; immunofluorescence confirmed their retention (not shown). As Fig. 5*A* shows, when the M-KK mutant was expressed alone or in combination with ER-retained ST, only unglycosylated M was seen. However, when coexpressed with ER-retained GalNAc-T1 and -T3, the protein was fully converted into the slightly slower migrating GalNAc-modified form M1 (3, 18). When coexpressed with ER-retained GalNAc-T2 conversion was not complete: a fraction of M-KK had remained unglycosylated. These results indicate that MHV M can serve as a substrate for all three GalNAc transferases, but with different efficiencies.

To further study the substrate sequence preferences of the 3 GalNAc transferases we tested a selection of our M mutants in the *in situ* *O*-glycosylation assay. These mutants were each provided with the same ER retrieval signal as was used in mutant M-KK. As is clear from Fig. 5*A*, mutants 3AT5-KK, Q6A-KK, A7G-KK, and P10A-KK behaved quite similar to M-KK. They all served as a substrate for each of the GalNAc transferases, but were less efficiently glycosylated by GalNAc-T2. Interestingly, an additional glycosylated species was observed when mutant P10A-KK was coexpressed with GalNAc-T3. The mobility of this form is indicative of the addition of a second GalNAc unit. Consistently, further conversion occurred when the chase period was extended to 3 h (Fig. 5*B*). Although we cannot rule out the addition of other residues such as Gal to GalNAc, this seems very unlikely as it did not occur with other mutants. Mutant A4A5-KK was not used as a substrate by any of the GalNAc transferases (Fig. 5*A*), as was expected from the *in vivo* experiments (Fig. 1). Distinct substrate preferences were observed with mutants P8A-KK, 3AT4-KK, and 4S-KK; the former protein exclusively served as a substrate for GalNAc-T1, while the latter two proteins were only modified by GalNAc-T3 under the conditions used. The modification of 4S was not complete: the majority remained unglycosylated. When

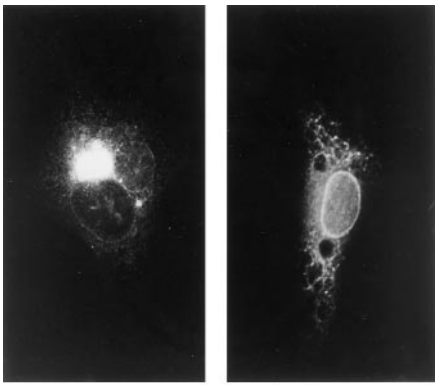

FIG. 4. **Localization of WT M and a M mutant with an ER retrieval signal (M-KK).** Genes encoding WT M and M-KK were expressed in BHK-21 cells using the MVA-T7pol expression system. Cells were fixed at 6 h post-infection and processed for indirect immunofluorescence using the anti-MHV serum K134.

the chase time was extended to 3 h conversion was almost complete (Fig. 5*B*).

An interesting finding was the small but significant difference in the mobility shift caused by GalNAc addition to mutant 3AT4-KK protein as compared with the same modification of other M proteins such as M-KK and 3AT5-KK. This difference was subtle in our standard 15% polyacrylamide gels but became evident by extended electrophoresis in longer gels (Fig. 5*B*). Consistently, modification of Thr^4 (mutant 3AT4-KK) by GalNAc-T3 caused a smaller shift in electrophoretic mobility than modification of Thr^5 (mutant 3AT5-KK). Importantly, the shift in mutant 3AT5-KK protein compares with that in M-KK. From this we infer that WT-M protein is normally glycosylated at Thr^5.

The results indicate that GalNAc-T1, -T2, and -T3 have overlapping specificities *in situ* with the provided substrates. All three GalNAc transferases modified Thr at position 5 in its natural environment. GalNAc-T2 and -T3 but not GalNAc-T1 needed the Pro residue at position 8. Only GalNAc-T3 was able to modify Thr at position 4, when this was the only potential glycosylation site present. GalNAc-T3 was also the only enzyme able to modify both Thr and Ser residues, although modification of Ser seemed to be less efficient.

Interferential Effects of an N-Glycosylation Site—Next we investigated the interferential effect of an *N*-glycosylation site close to the site of *O*-glycosylation. Therefore we introduced an *N*-glycosylation consensus sequence (Asn-Xaa-(Ser/Thr)) in the MHV M protein by substituting Ser at position 2 by Asn. Cells expressing this mutant S2N protein were pulse-labeled for 30 min and chased for 30 and 90 min (Fig. 6*A*) or they were labeled continuously for 3 h (Fig. 6*B*). Prior to gel electrophoresis some immunoprecipitates were treated with glyco F or with endo H. Glyco F removes all *N*-linked sugars from proteins, while endo H only removes immature *N*-glycans, *i.e.* oligosaccharide side chains not yet modified by Golgi enzymes. Fig. 6*A* demonstrates that the newly introduced *N*-glycosylation consensus sequence was functional. Most of the pulse-labeled mutant S2N protein had become *N*-glycosylated as evidenced by the sensitivity of the approximately 28-kDa protein to the endoglycosidases. Removal of the *N*-linked sugars with glyco F yielded a protein that comigrated with the small fraction of unglycosylated M protein (about 23 kDa). Deglycosylation using endo H, however, leaves one *N*-acetylglucosamine residue attached yielding a protein that migrated slightly slower. Analysis of the chase samples demonstrated that the *N*-glycosylated protein also became modified by *O*-linked sugars. During chase the amount of endo H-sensitive 28-kDa M protein decreased while that of glyco F-sensitive protein remained constant, indicating the formation of endo H-resistant S2N. This material was differentially and heterogeneously glycosylated and could therefore not be distinguished from the background. The endo H-sensitive material remained unmodified by *O*-linked sugars.

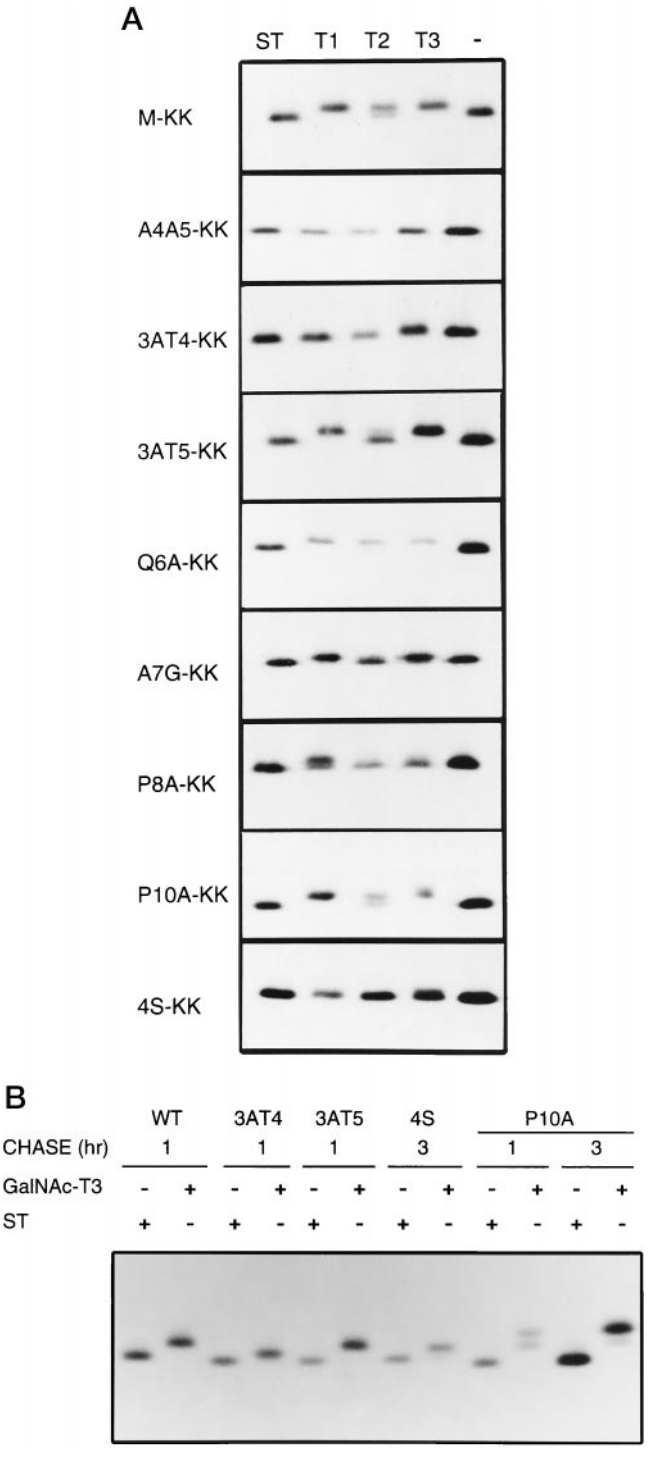

FIG. 5. **Glycosylation of mutant M proteins by GalNAc-T1, -T2, and -T3 *in situ*.** ER-retained M mutants were expressed in OST7-1 cells using the MVA-T7pol expression system, alone or in combination with ER-retained GalNAc-T1, -T2 and -T3, as well as an ER-retained form of ST. Cells were pulse-labeled for 30 min followed by a 60- or 180-min chase. Cell lysates were processed for immunoprecipitation using the anti-MHV serum K134. Immunoprecipitates were subjected to electrophoresis for 800 V-h using a standard 15% polyacrylamide gel (*Panel A*) or for 4000 V-h using a long 15% polyacrylamide gel (*Panel B*).

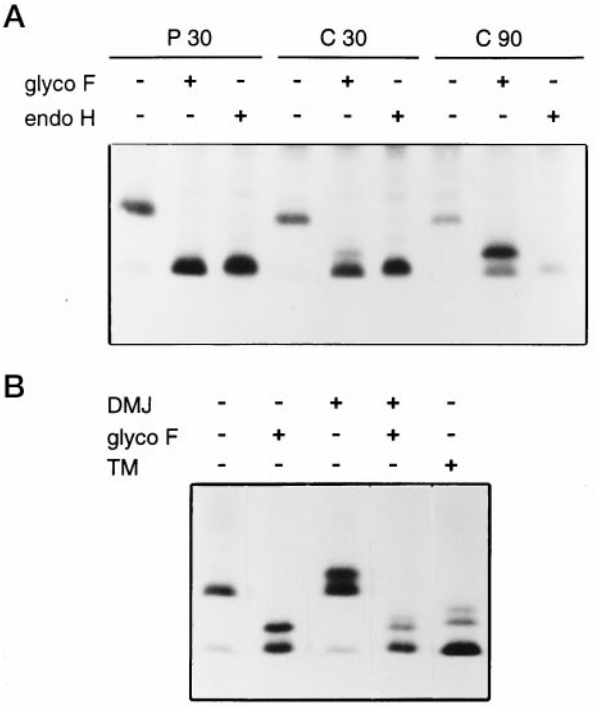

FIG. 6. **Interferential effects of an *N*-glycosylation site.** Mutant S2N (containing a *N*-glycosylation consensus sequence) was expressed as described in the legend to Fig. 1, except that labeling was for 30 min while chase times of 30 and 90 min were used (*Panel A*) or labeling was continuously for 3 h (*Panel B*). Prior to gel electrophoresis some immunoprecipitates were treated with glyco F or endo H. When indicated DMJ or tunicamycin were added to the culture media.

Removal of all *N*-linked sugars by treatment with glyco F resulted in the appearance of the typical pattern of differently *O*-glycosylated M species. After 90 min of chase the majority of S2N was present in the M3 form. These data demonstrated that S2N was both *N*- and *O*-glycosylated and suggest that *O*-glycosylation was initiated in the Golgi complex.

Because the heterogeneous maturation of the *N*-linked sugars obscured the analysis, we treated the S2N-expressing cells with DMJ, which interferes with the action of α-mannosidase I, thereby keeping the sugars in a simple, endo H-sensitive form (32). *N*-Glycosylated M proteins synthesized in the presence of DMJ appeared as 28- and 30-kDa species (Fig. 6*B*). When these proteins were deglycosylated with glyco F, the removal of the *N*-linked sugars resulted again in the typical pattern of differently O-glycosylated M species, indicating that the 30-kDa form represents S2N protein that is both *N*- and *O*-glycosylated. Finally, treatment of the cells expressing the S2N protein with tunicamycin, which blocks *N*-glycosylation, resulted again in the appearance of the typical pattern of differently *O*-glycosylated M species.

We also tested a mutant carrying a similar substitution of the Ser residue at position 3 instead of at position 2 (mutant S3N). This mutation also generates a *N*-glycosylation consensus sequence. This mutant S3N protein became both *N*- and *O*-glycosylated just as mutant S2N (data not shown). These results confirm that mutations of the Ser residues at positions 2 and 3 do not disturb the *O*-glycosylation recognition sequence. Furthermore, the results show that co-translational *N*-glycosylation of an Asn very close to a site of *O*-glycosylation did not interfere with the post-translational addition of *O*-linked sugars. Strikingly, a hydroxylamino acid that is part of an *N*-glycosylation consensus sequence (Asn-Xaa-(Ser/Thr)) can apparently be modified by *O*-linked sugars.

Transfer of the MHV M O-Glycosylation Site—To investigate

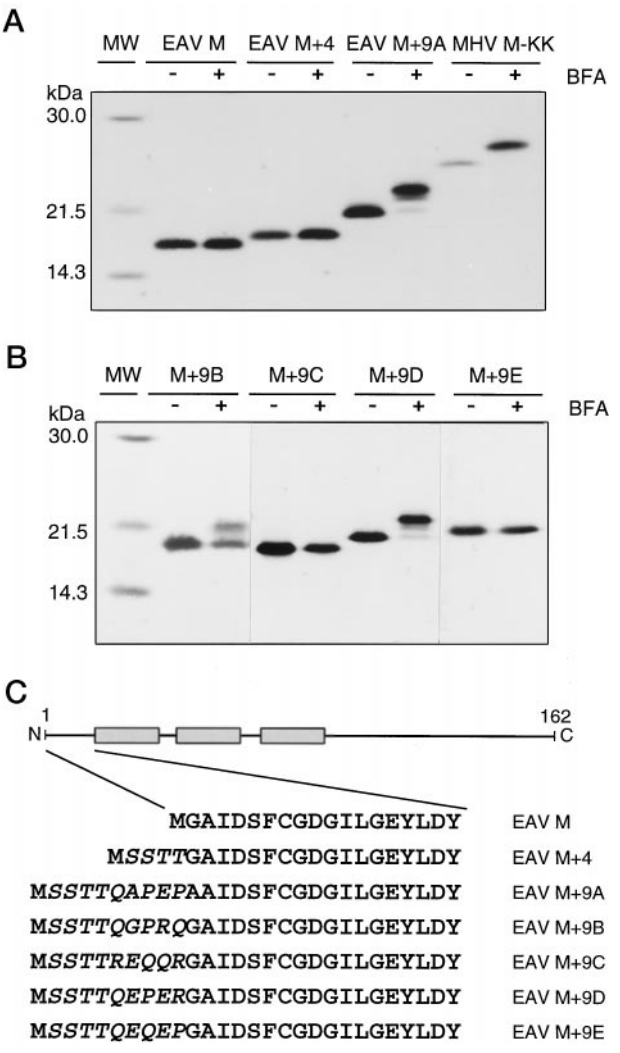

FIG. 7. **Transplanting the MHV M *O*-glycosylation site.** Genes encoding EAV M, the hybrid proteins EAV M+4 and EAV M+9A, and the ER-retained MHV M mutant (MHV M-KK) were expressed as described in the legend to Fig. 1, except that cells were pulse-labeled for 30 min followed by 60 min chase (*Panel A*). *Panel B* shows a similar experiment in which genes encoding proteins EAV M+9B through E were expressed. When indicated, BFA was added to the culture medium. Cell lysates were processed for immunoprecipitation using the anti-MHV serum K134 (MHV M-KK) or an anti-EAV M serum (EAV M, EAV M+4, and EAV M+9A through E). Immunoprecipitates were subjected to SDS-17.5% PAGE. In *Panel C* a schematic representation of the structure of the EAV M protein is shown. The three transmembrane domains are indicated by *boxes*. The amino acid sequence of the amino-terminal domain of EAV M and of the 6 hybrid proteins are shown. Inserted amino acids are printed in *italics*. Mutant EAV M+9A contained an additional unintended mutation, immediately downstream of the MHV-specific sequence resulting in a glycine to alanine substitution.

whether the amino-terminal sequence of the MHV M protein can be transplanted onto a foreign protein to introduce a functional *O*-glycosylation site, we constructed two hybrid proteins. These proteins consisted of the EAV M protein extended at its very NH_2-terminal end with either 4 or 9 MHV M-derived amino acids inserted right behind the initiating methionine. As a result, a protein designated EAV M+4 was obtained that only acquired the MHV M hydroxylamino acid tetrapeptide, and a protein designated EAV M+9A, which in addition carried the downstream sequence containing the two prolines (see Fig. 7*C*). The EAV M protein is a type III membrane protein with a similar topology as the MHV M protein (24). Its short amino-terminal domain (approximately 19 residues) is not modified by

N- or *O*-glycans. Cells expressing the M proteins were pulse-labeled for 30 min and chased for 60 min in the presence or absence of BFA. BFA causes a rapid redistribution of Golgi enzymes to the ER (30) and was used to allow *O*-glycosylation of the ER-retained EAV M proteins (28). WT EAV M appeared as an unglycosylated 18-kDa protein, which did not become modified when BFA was present (Fig. 7*A*). The ER-retained form of MHV M (M-KK), which was used as a positive control, was not glycosylated in the absence of BFA but became modified in its presence, presumably to the M3 form (18). The hybrid proteins EAV M+4 and M+9A migrated slower than EAV M due to the insertions made. The presence of BFA did not affect the electrophoretic mobility of the EAV M+4 protein, indicating that it did not become *O*-glycosylated. In contrast, addition of BFA caused a shift in mobility of EAV M+9A similar to that of MHV M-KK; in addition, a slightly slower migrating species was also detected. Thus, transferring only the hydroxylamino acid cluster to a reporter protein was not sufficient to create a functional *O*-glycosylation site. However, when the 5 downstream amino acids were included, a functional glycosylation site was apparently created.

While the difference in glycosylation of EAV M+4 and EAV M+9A may result from specific sequence requirements, an alternative explanation might be that the 5-residue stretch in EAV M+9A just acts as a spacer, displacing the glycosylation site away from the membrane and thereby allowing access of the GalNAc transferases. To investigate this issue, we made several additional constructs with different 5-residue sequences downstream of the hydroxylamino acid cluster designated EAV M+9B through E (see Fig. 7*C*). Expression of these constructs in the absence of BFA revealed that the unglycosylated proteins had slightly different electrophoretic mobilities despite their identical protein length (Fig. 7*B*). These effects apparently result from the primary sequence differences which are known to sometimes affect the binding of SDS and thereby migration in gel (34). The glycosylation of the mutant proteins was again evaluated by looking at the effect of BFA. Clearly, the electrophoretic mobility of EAV M+9C and M+9E was not changed, indicating that these proteins did not become modified. However, under the same conditions a significant part of M+9B and the majority of EAV M+9D showed the characteristic shift in mobility indicative of *O*-glycosylation. Interestingly, both these proteins have a proline residue at position +3 relative to the last Thr of the hydroxylamino acid cluster. The results indicate that the residues downstream of the hydroxylamino acid cluster are not solely acting as tethers distancing the glycosylation site away from the membrane. Obviously, sequence requirements play an important role.

DISCUSSION

Our results indicate that in MHV M only 1 residue of the hydroxylamino acid cluster is glycosylated. The shift in electrophoretic mobility of WT M upon glycosylation is similar to that of mutant M proteins that have only one possible acceptor site. The number of oligosaccharide side chains added to MHV M was hitherto unknown. Based on the increase in M_r after GalNAc addition and on the complexity of the pattern of glycosylated species observed it was speculated earlier that MHV M might contain up to three functional *O*-glycosylation sites (3, 21). The cluster of 4 hydroxylamino acids (SSTT) in MHV M is identical to the sequence present at the extreme amino terminus of the human erythrocyte membrane protein glycophorin A. Both proteins were shown by chemical analysis to contain identical types of oligosaccharide structures (21). However, in glycophorin A all but one (the first Ser) of the 4 hydroxylamino acids in the cluster were identified as glycosylation sites by Edman degradation (36). Differences in downstream sequences and in secondary structures between MHV M and glycophorin A as well as cell-type specific variations in *O*-glycosylation might explain this discrepancy.

Our observations strongly suggest that in MHV M the sugar side chain is added to Thr at position 5. Glycosylation at Thr^4 also occurs, but only when Thr^5 is not available due to mutation. Interestingly, GalNAc addition to either of these residues resulted in proteins which, despite their identical M_r, had subtly different electrophoretic mobilities. Although both threonines can function as acceptor sites, double glycosylation was never observed for WT M. This may be due to steric hindrance precluding the modification of adjacent residues. Double glycosylation, however, seemed to occur with one mutant (P10A) but here the acceptor sites were not identified. This additional glycosylation was only observed when the protein was retained in the ER. Many mucin-type glycoproteins are heavily *O*-glycosylated (*e.g.* glycophorin A; Ref. 36). It is not yet clear to what extent prior *O*-glycosylation at one site alters the addition of *O*-linked sugars at vicinal sites. That such interference can occur was recently demonstrated *in vitro* using peptide substrates (37). Many glycoproteins carry both *O*- and *N*-linked sugars. In some of these, *O*-glycosylation was shown to occur at the hydroxylamino acid of a predicted *N*-glycosylation tripeptide sequence (Asn-Xaa-(Ser/Thr)) (38). We show here that *O*-glycosylation is indeed not affected by prior *N*-glycosylation. Introduction of a functional *N*-glycosylation site adjacent to the site of *O*-glycosylation in MHV M did not significantly affect the addition of *O*-linked sugars. Evidently, the transferases are not sterically hindered by the prior addition of *N*-linked sugars.

No unique motifs for *O*-glycosylation of MHV M could be identified. Most single, double, and triple substitutions in the amino terminus of MHV M did not greatly affect *O*-glycosylation. Also the identity of the hydroxylamino acid was not essential since both Ser and Thr residues could serve as acceptor sites. Furthermore, M proteins with a deletion of the serine residues or with insertions upstream of (6 histidines) or within the hydroxylamino acid cluster (Val and Lys; coronaviruses OC43 and BCV, respectively) became *O*-glycosylated. Only mutant M proteins with hydroxylamino acids exclusively at positions 2 or 3 and alanine residues at positions 4 and 5 did not become glycosylated at all (A4A5 and 3AT3) or only to a very minor extent (3AT2). Presumably the sequence contexts of these mutants impede efficient *O*-glycosylation. *O*-Glycosylation sites in clusters of hydroxylamino acids are common in many other glycoproteins (38–40). However, transfer of just the cluster of 4 hydroxylamino acids onto a reporter protein was not sufficient to create a functional *O*-glycosylation site; inclusion of 5 additional MHV M amino acids downstream of the glycosylation site made the transfer successful. Consistently, deletions as small as 5 (coronavirus MHV-RI; 41) or as large as 16 amino acids (mutant ΔN; 42) in this region abolished *O*-glycosylation. Apparently, the sequence downstream of the glycosylation site is in some way crucial for sugar addition. In the absence of downstream sequences, the acceptor sites may become too close to the membrane, thus becoming inaccessible to the GalNAc transferases. However, the downstream sequences are not solely acting as spacers, since several reporter proteins (EAV M+9C and M+9E) containing alternative sequences downstream of the hydroxylamino acid cluster did not become *O*-glycosylated. The data indicate that the glycosylation apparatus in OST7-1 cells displays very flexible primary sequence requirements for *O*-glycosylation. Previously, the GalNAc transferases in other cell types were also shown to tolerate a broad range of changes in the sequence flanking the *O*-glycosylation site (43).

The lack of sequence requirements for *O*-glycosylation is conceivably due to the occurrence in cells of multiple GalNAc transferases (9–14). Therefore we decided to study *O*-glycosylation using the *in situ* glycosylation assay which allowed us to analyze the primary sequence requirements of MHV M for three GalNAc transferases separately in a cellular environment. In contrast to *in vitro* assays which use short acceptor peptides, in the *in situ* approach the influence of substrate conformation on *O*-glycosylation is also accounted for. GalNAc-T1, -T2, and -T3 showed largely overlapping, but distinct substrate specificities. MHV M served as a substrate for all three GalNAc transferases. GalNAc-T2 and -T3, but not GalNAc-T1, needed the Pro residue at position 8. Examination of the sequences surrounding glycosylation sites shows a high frequency of Pro residues especially at position −1 and +3 relative to the acceptor site (38, 39, 44, 45). Consistently, several studies using GalNAc transferase activities from bovine and human colostrum also indicate an important role for Pro residues at position +3 (46–48). Other studies using purified recombinant GalNAc-T1, -T2, and -T3 found that a short acceptor peptide not containing proline was glycosylated exclusively by GalNAc-T3 (12, 16). This difference in proline requirement might be explained by the use of different substrates and assays. GalNac-T3 was the only enzyme able to modify both Thr and Ser residues in the MHV M protein context, although modification of Ser was less efficient. This result is in agreement with several studies, which showed that one GalNAc transferase may utilize both Ser and Thr residues, serine residues generally being used less efficiently (10, 11, 16, 49). Our results indicate that, although many mutations were allowed, the primary sequence remains an important factor in determining the site of *O*-glycosylation. The sequence requirements for *O*-glycosylation by the individual GalNAc transferases were more strict than for glycosylation of substrates by the total pool of cellular GalNAc transferases. Moreover, M mutants that were efficiently glycosylated under these latter conditions served as substrates for all three GalNAc transferases tested. In contrast, M mutants that were less efficiently glycosylated by the cellular enzyme pool served as substrates for only one GalNAc transferase (Table II). These data support the hypothesis that *O*-glycosylation in cells reflects the combined activities of all GalNAc transferases present and may explain why an *in vivo* *O*-glycosylation consensus sequence has not been identified.

The intracellular site of *O*-glycosylation initiation remains a matter of debate. GalNAc addition has been localized to several compartments of the secretory pathway (ER, ER to Golgi intermediate compartment, and Golgi apparatus) (1–7). Earlier reports suggested *O*-glycosylation of MHV M to start in the ER to Golgi intermediate compartment (3, 4). However, *O*-glycosylation of mutant S2N protein could not be detected for molecules containing endo H-sensitive *N*-linked sugars. Only M proteins containing *N*-sugars modified by Golgi enzymes were *O*-glycosylated, indicating that *O*-glycosylation of MHV M was initiated in the Golgi complex. This is in agreement with other studies that localized initiation of *O*-glycosylation to the Golgi complex (5–7).

All coronavirus M proteins are either *N*- or *O*-glycosylated. The function of M protein glycosylation is not known, but presumably it is in some way beneficial for the virus. While the M protein is required for budding, its glycosylation is not. Recently we showed that mutant M protein A4A5, which does not become glycosylated, was efficiently assembled into virus-like particles (26). These observations are consistent with earlier studies that used tunicamycin (50) and monensin (51) to inhibit glycosylation in infected cells. More likely, glycosylation plays a role in virus-host interactions. The Ser-Ser-(Xaa)-Thr-Thr motif and the Pro residue at position +3 relative to the last Thr of the hydroxylamino acid cluster are very well conserved. Since glycosylation may vary from cell to cell depending on the expressed repertoire of GalNAc transferases (14), these conserved features may serve to increase the opportunities for the M proteins to become glycosylated in many different cell types.

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