GLYCOPROTEIN E1 OF MHV-A59: STRUCTURE OF THE O-LINKED CARBOHYDRATES AND CONSTRUCTION OF FULL LENGTH RECOMBINANT cDNA CLONES

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INTRODUCTION

The murine coronaviruses contain two different classes of glycoproteins (for review see [1]). The envelope glycoprotein E2 resembles in its composition and biosynthetic processing the classical type of viral glycoprotein: E2 acquires N-glycosidically linked carbohydrate side chains in a cotranslational event at the rough endoplasmic reticulum (RER) [2] and in addition, it contains covalently linked fatty acids [3]. The 180K species of E2 is processed posttranslationally by proteolytic cleavage into two 90K species [2,4]. The second type of coronavirus envelope glycoprotein which was designated the matrix glycoprotein E1 is unique in many ways:

1. The transmembranal nature of E1 allows it to interact with the viral nucleocapsid like a matrix protein of other viruses [5]. The intracellular distribution of E1 in perinuclear regions of the infected cell (GERL) determines the budding site of coronaviruses [6].
2. The intramembranal hydrophobic domain of glycoprotein E1 has a capability for auto-aggregation [7].
3. In the murine and bovine coronaviruses E1 has exclusively O-linked carbohydrate side chains which are only added posttranslationally after transfer of the E1 polypeptide from the site of its biosynthesis (RER) to the trans-cisternae of the Golgi complex [2,3].

With these two classes of glycoprotein, mouse hepatitis virus A59 offers an ideal system to study the potential functions of N- versus O-glycosylation as well as the biosynthesis and intracellular

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transport of these two different molecules. Therefore, we are interested in studying the expression of the E1 gene using recombinant DNA techniques and appling site specific mutagenesis to alter the glycosylation sites of E1. We present here the structures of the carbohydrates of E1 and describe the construction of full length clones of glycoprotein E1 with the help of a synthetic oligodeoxyribonucleotide primer.

MATERIALS AND METHODS

Virus and cells. MHV-A59 was grown in 17Cl1 cells and purified as previously described [8].

Isolation of O-linked carbohydrate side chains. Glycoprotein E1 was metabolically labelled with ^{3}H-galactose, purified by preparative gel electrophoresis and subjected to ß-elimination using $NaBH_4$ as a reducing agent [3]. The released oligosaccharides were desalted by passage over a Biogel P2 column (35 x 1 cm) and separated by high performance liquid chromatography (HPLC) as indicated in the legend to Figure 1.

Permethylation analyses and combined gas chromatography-mass-spectrometry. Purified oligosaccharides were permethylated, hydrolyzed, reduced with $NaBD_4$ and analyzed as partially methylated alditol acetates by combined gas chromatography mass-spectrometry as detailed elsewhere [9].

Vibro-cholerae-neuraminidase treatment of purified oligosaccharides was carried out as described earlier [3].

Synthesis of the oligodeoxynucleotide primer. A pentadecadeoxyribonucleotide was synthesized on the polydimethylacrylamide-Kieselguhr support (C-resin, 75 µMole dC/g) following the phosphotriester method as described by Gait et al. [10]. The partially deprotected, 5'-dimethyoxytrityl-substituted oligomer was purified by reversed phase HPLC as outlined in the legend to Figure 1a. Detritylation was performed in 5 ml acetic acid:water (8:2) for 30 min at room temperature (yield after lyophilisation and ether extraction: 95 OD). Part of the material was 5'-^{32}P-labelled using T4-polynucleotide kinase (Boeheringer, Mannheim) and analyzed on a 20 % acrylamide gel (Figure 1b) using oligo(dA)$_{3-n}$ and oligo(dT)$_{3-m}$ as length markers. Radioactive length markers were prepared by elongation of oligo(dA)$_3$ or oligo(dT)$_3$ with terminal deoxynucleotidyl transferase and 5'-^{32}P-labelled [11].

Isolation of total poly-A$^+$-RNA. Poly-A$^+$-RNA was isolated from infected 17Cl1 cells (m.o.i. = 50 PFU/cell) 16 hrs post infection, purified by oligo-dT-cellulose (P.L. Biochemicals) and assayed by *in vitro* translation [2].

Construction of full length E1-cDNA. First strand cDNA was synthesized in a mixture containing poly-A$^+$-RNA (10 µg), pentadeca oligodeoxyribonucleotide (20 ng), Tris/HCl, pH 8.3 (50 mM), KCl (30 mM), $MgCl_2$ (10 mM), sodium pyrophosphate (2 mM), RNasin (Bolton Biological), dithiothreitol (1 mM), dATP, dGTP, dTTP (1 mM

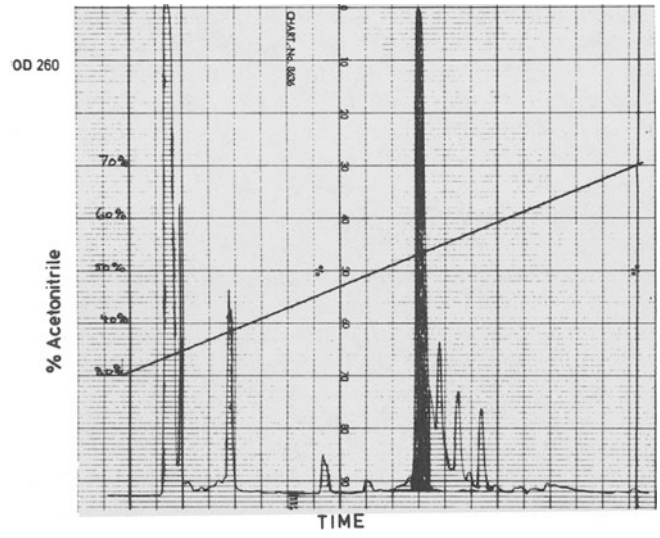

Fig. 1a:
HPLC of the partially deprotected pentadecaoligodeoxy-ribonucleotide 5'dTTCTTGCCCAGGAAC3' before detritylation. Column: 5 µ Lichrosorb RP18 (Merck); Elution: linear gradient of 0.1 M ammonium acetate (A) and 0.1 M ammonium acetate/acetonitrile (2:8 v/v) (B).

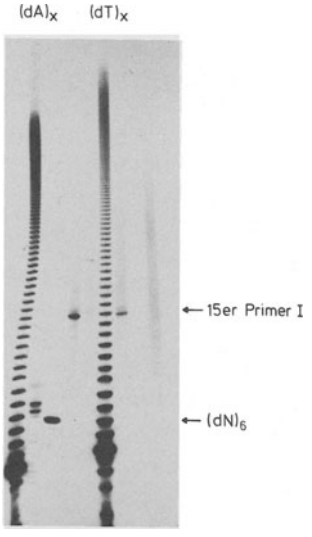

Fig. 1b:
Polyacrylamide gel electrophoresis of the 5'-^{32}P-labelled deprotected oligonucleotide. The primer was 5' labelled with polynucleotide kinase and analyzed on a 20% acrylamide gel prepared according to Frank et al.[11].

each), dCTP (0.2 mM), α-^{32}P-dCTP (50 µCi; Amersham 410 Ci/mM) and AMV reverse transcriptase (200 U, Life Sciences) in a total volume of 250 µl. Prior to the addition of dithiothreitol, dNTP's, and enzyme, the primer was annealed by incubation at 55° C for 5 min, 50° C for 10 min and 42° C for 15 minutes. The reaction was carried out at 42° C for 60 min and stopped by adjusting the mixture to 0.5 N NaCl, 20 mM EDTA pH 7.5, and 0.5 % SDS. The mixture was extracted with chloroform/isoamylalcohol (24:1), the organic phase was re-extracted with one volume of water. The dried material was taken up in 40 µl of freshly prepared 0.4 N NaOH and left for 7 hrs at room temperature to degrade the RNA. Free nucleotides were removed by passage over a Sephadex G-100 column in 10 mM NaOH (siliconized Pasteur pipette, bed volume 2 ml). The radioactive material in the void volume was collected, neutralized with HCl and precipitated with ethanol after the addition of 1/10 volume of 3.0 M potassium acetate. The addition of homopolymeric dC-tails to the 3'end of the single stranded cDNA was carried out for 10 min as described by Land et al. [12]).

To synthesize the second DNA-strand, oligo(dG)$_{12-18}$ (30 µg/ml, P.L.-Biochemicals) was used as a primer. It was annealed to the template by incubating the reaction mixture in the absence of nucleotides and AMV reverse transcriptase at 65° C (5 min), 55° C (5 min), 50° C (10 min), and 42° C (15 min). The reaction conditions were the same as for the first strand synthesis omitting RNasin. After 60 min at 42° C the sample (100 µl) was cooled to 15° C and Klenow polymerase (20 U, Boehringer, Mannheim) was added. The mixture was incubated for another 8 hr at 15° C. The material was extracted with chloroform/isoamylalcohol, precipitated and separated from free nucleotides by gel filtration as above, using water as an eluent. S1 nuclease resistance of this ds-cDNA was assayed together with an aliquot of ss-cDNA and nick-translated λ -DNA in reaction mixtures containing 10.000 Cerenkov counts of the respective DNA in 50 mM sodium acetate (pH 4.5), NaCl (0.2 M), $ZnSO_4$ (1 mM), 0.5 % glycerol. For each of the three DNA samples control values were determined after 3 min boiling and subsequent quenching on ice.
A second tailing reaction was performed on ds-cDNA, adding an average of 15 dC-residues per 3'end.

Bacterial transformation with hybrid plasmid DNA. Vector pUR 250 [13]) (kindly provided by Ulrich Rüther, Köln), was restricted with AccI in the presence of alkaline phosphatase. After the addition of an average of 15 dG-residues per 3'end with terminal nucleotidyl transferase the tailed vector was purified by electrophoresis through a preparative 1 % agarose gel and recovered by electroelution. dG-Tailed pUR250 and dC-tailed cDNA were mixed in a molar ratio with a final concentration of 200 ng vector/ml in Tris-HCl, pH 7.5, 100 mM NaCl and 1 mM EDTA. The mixture was first heated to 68° C for 5 min and then kept at 43° C for 2 hrs and allowed to cool to room temperature over a 1 hr period. 10 µl of annealing solution (2.0 ng vector) was added to 100 µl of competent RRI cells. Transformation was carried out as described by Dagert and Ehrlich [14]).

Screening for plasmids containing E1 sequences. Colony hybridization of lac$^-$ colonies was performed as described by Grunstein and Hogness [15]) using an E1 specific probe obtained by reverse transcription of purified viral RNA with the synthetic primer. Plasmid DNA was prepared from chloramphenicol amplified 100 ml-cultures by lysis with alkali and purified by chromatography over a NACS 52 column (Bethesda Research Laboratories) as detailed by the manufacturer.

Subcloning and sequencing in pEMBL8 (in collaboration with J. Armstrong, EMBL.) E1-specific DNA was released from cloned p42-DNA by double digestion with HindIII and EcoRI and ligated into pEMBL8 which had been cleaved with the same restriction nu-

cleases. Dideoxy-sequencing was performed on ss-pEMBL8-DNA, obtained after superinfection of the pEMBL8-culture with wild type f1 as described by Dente et al. [16]) using a 15-base synthetic single stranded universal primer (P.L.-Biochemicals). Reaction products were analyzed on thermostated 6 % acrylamide gels [17]).

RESULTS AND DISCUSSIONS

Structure of the O-linked carbohydrate side chains of glycoprotein E1. Glycoprotein E1 of MHV-A59 contains exclusively O-linked carbohydrate side chains. They could be released from the protein backbone in an intact form by ß-elimination in the presence of $NaBH_4$ to avoid peeling of the carbohydrate chain [3]). Two peak fractions designated A and B were separated by HPLC (Fig.2, upper panel). By removal of sialic acid residues fraction A and B were converted into an identical product which eluted in the position of an aminosugar-containing disaccharide alditol (Fig. 2, lower panel).

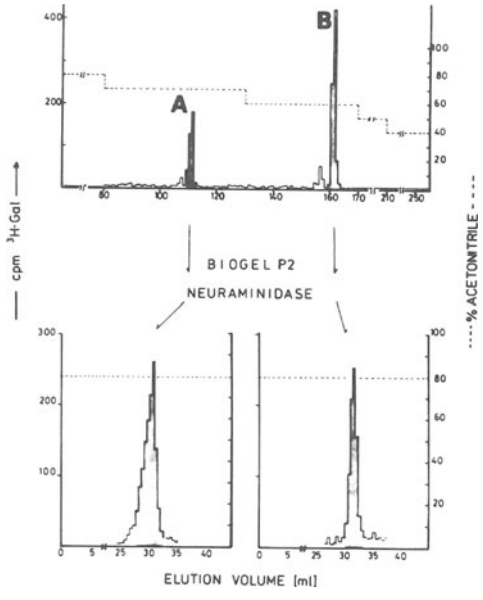

Fig. 2: HPLC of reduced carbohydrate side chains obtained from E1 by ß-elimination
column: 5 µ Lichrosorb-NH_2 (Merck, Darmstadt)
flow rate: 0.5 ml/min
elution: Acetonitrile/15 mM KH_2PO_4, pH 5.5, in a stepwise gradient.
(peak fraction A: 70 % acetonitrile/30 % KH_2PO_4
peak fraction B: 60 % acetonitrile/40 % KH_2PO_4)

Part of fractions A and B (about 5 µg each) were permethylated, hydrolyzed, reduced and analyzed as partially methylated alditol acetates by gas-chromatography-mass-spectrometry. Figure 3 shows the gaschromatogram obtained from peak fraction B. The two major peaks were in an equimolar ratio and the underlying compounds were identified by mass-spectrometry to be derived from a 3-sub-

stituted galactose (2,4,6,-Gal) and a 3,6-di-substituted N-acetyl-galactosaminitol (1,2,4,5-GalNAc). None of the other peaks in Figure 3 showed a sugar-specific fragmentation pattern in mass-spectrometric analysis.

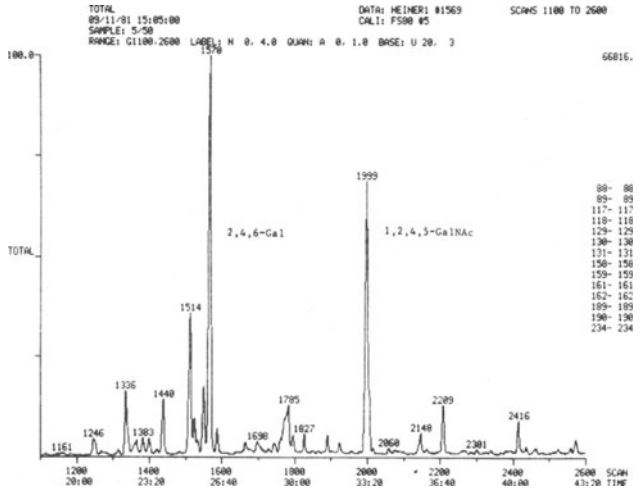

Fig. 3: Gas-chromatogram of partially methylated alditol acetates derived from oligosaccharide B. column: 25 m fused silica capillary column; OV101 stationary phase temperature: 100 - 240° C, 3°/min

2,4,6-Gal = 2,4,6-tri-O-methyl-1,3,5-tri-O-acetyl-galactitol

Permethylation analysis of material from peak A (Fig. 2) indicated that this oligosaccharide also contained galactose and N-acetyl-galactosamine in a 1:1 ratio. By mass-spectrometry it was shown that the N-acetyl-galactosamine derivative present in A was only mono-substituted in position 3 (data not shown). In both structures, however, the galactosamine residue had to be the innermost sugar linked to the protein backbone. The combined data lead to the following structures for oligosaccharides A and B:

A

NeuNAc 2 $\xrightarrow{\alpha}$ 3 Gal 1 $\xrightarrow{\beta}$ 3 GalNAcOH

B

NeuNAc
2
6 $\downarrow\alpha$
NeuNAc 2 $\xrightarrow{\alpha}$ 3 Gal 1 $\xrightarrow{\beta}$ 3 GalNAcOH

Similar carbohydrate structures have been reported for mucin type glycoproteins (for review see [18]), but this is the first time that any O-linked carbohydrate structure has been elucidated from

viral glycoproteins. The comparison of the molecular weight of the non-glycosylated precursor of E1 and the fully glycosylated E1 species suggests that about 3 carbohydrate side chains are attached per molecule of E1.

When this work was begun, very little was known about the structure and the function of the glycosylated portion of E1 extending on the outside of the virus particle [1]. Studies with monensin have shown that glycosylation of E1 is inhibited in the presence of this ionophore while virus particle formation continues into the lumen of the RER [2]. The amino-terminal part of E1 may also contain specific signals for the intracellular transport, defining the characteristic budding sites of coronaviruses in the RER and the Golgi [6]. The O-linked carbohydrates of E1 could contribute to the rigidity of the viral membrane as was proposed for the anti-freeze glycoprotein from polar fish [19].

Construction of full length E1 clones. To study possible functional interactions at a molecular level, full-length recombinant DNA of E1 was constructed in order to eventually express this gene in eucaryotic cells using an appropriate vector. Cloning of the E1 gene was facilitated after the structure of mRNA 7 (encoding the nucleocapsid protein) had been elucidated [20]. A pentadeca-oligodeoxyribonucleotide d(TTCTTGCCCAGGAAC) complementary to nucleotides 10 to 24 of the 5'coding region of mRNA 7 was synthesized following the phosphotriester method and applied as a primer in first strand cDNA synthesis as shown in Figure 4. The primer sequence contains restriction sites for EcoRII, TagX1, BstN1. The computer search for further base sequence complementary between the primer and the mRNA 7 sequence showed that the primer should bind specifically only in the desired position. The method outlined in Fig. 4 was originally described by H. Land et al. [12]. The C-tailing of the single-stranded cDNA and the application of the oligo(dG)$_{12-18}$ as a primer for second strand synthesis protects the extreme 3'terminal region of the ss-cDNA (corresponding to the 5'terminus of the mRNA and thus to the N-terminus of the protein). Part of these sequences would have been lost, if self-primed second strand synthesis and S1 nuclease digestion of the hair pin loop had been employed. The products of the first strand synthesis were analyzed together with those of the first C-tailing reaction on a 2 % alkaline agarose gel (Fig. 5, lanes 1, 2). The presence of discrete bands in the expected molecular weight range showed that primer synthesis had been successfull. When oligo(dT)$_{12-18}$, was used as a primer, the bands appeared to be less discrete and they corresponded as to be expected to products of higher molecular weight, indicating that the synthetic primer had primed cDNA-synthesis internally (Fig. 5, lane 3).
Synthesis of the second strand was followed by determination of the amount of radioactive cDNA that was rendered S1 nuclease resistant. 85 % of the cDNA was resistant indicating that the C-

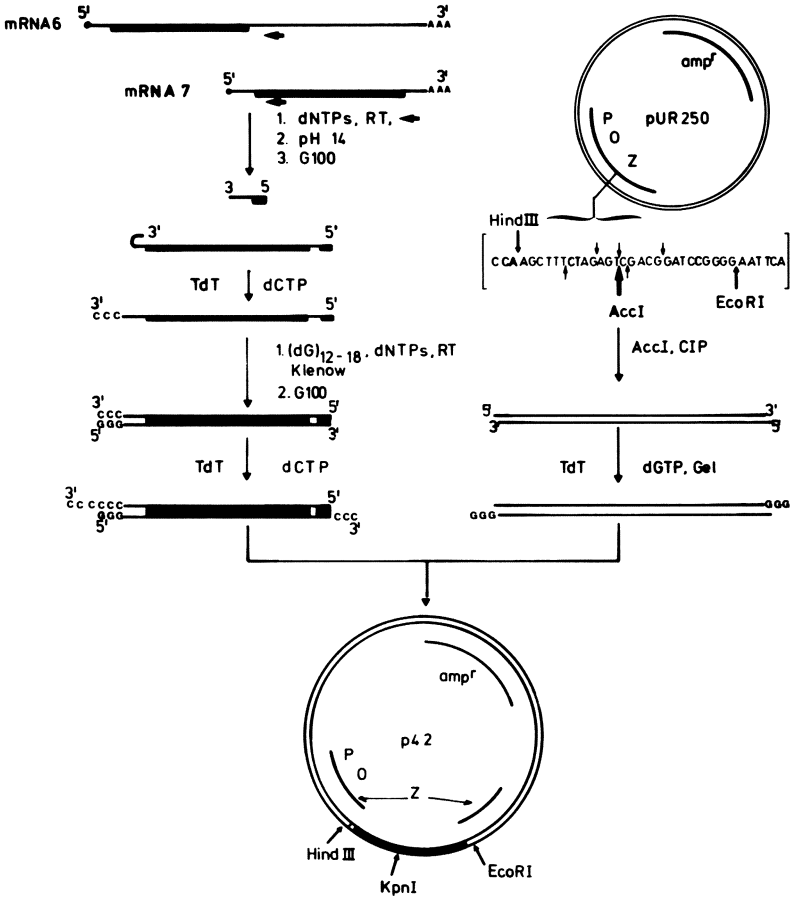

Fig. 4: Construction of full length E1 clones.

In A59-infected cells a nested set of 7 mRNA molecules is produced, which extend from a common 3'-end towards the 5'-end of the genome [21]. Each mRNA functions monocistronically.
Only mRNA 7 (encoding the nucleocapsid protein) and mRNA 6 (encoding E1) are shown. Thick strands illustrate the coding regions. The arrow indicates the synthetic primer. For further details see Materials and Methods.

tailing and oligo$(dG)_{12-18}$-priming had been efficient. In comparison second strand synthesis with non-tailed cDNA as template yielded only 45 % nuclease resistance (data not shown).

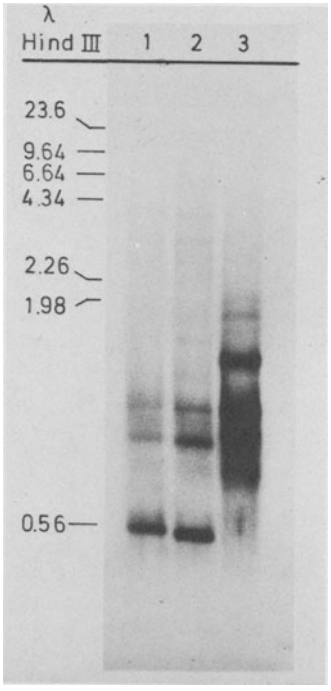

Fig. 5: Analysis of single-stranded cDNA on a 2 % horizontal alkaline agarose gel.

lane 1: dC-tailed cDNA obtained with the synthetic primer.

lane 2: cDNA obtained with the synthetic primer.

lane 3: cDNA obtained with oligo $(dT)_{12-18}$ as a primer.

λ-HindIII: molecular weight markers in Kb.

The pUR250 vector [13]) was chosen as a cloning vehicle (Fig. 4), because any GC-tailed material cloned into six of the eight unique restriction sites of the 36 base polylinker region could easily be recovered by cleavage at adjacent restriction sites. In addition, the presence of similar polylinker regions in the pUC-M13-,or pEMBL-families allowed an easy conversion from ds-DNA to ss-DNA in any desired orientation. The AccI site (Fig.5) was chosen as the cloning site, because GC-tailed inserts could eventually be sequenced according to Maxam and Gilbert [22]) without time consuming strand separation [13]).

Characterization of recombinant clones. A total of 70 recombinant clones were obtained with material corresponding to 1 µg of starting poly-A^+-RNA. These clones were assayed by colony hybridization and in part characterized by restriction mapping. Figure 6 shows the results obtained with clone p42. As expected, it could be linearized with either HindIII or EcoRI yielding identical cleavage products of 3.5 Kb (as compared to 2.7 Kb for pUR250). The 800 bp insert contained restriction sites for KpnI (Figure 6) and BgII (data not shown).

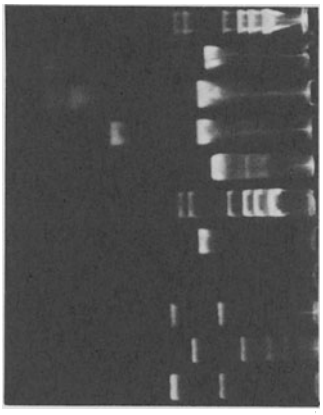

Fig. 6: Restriction digestion of p42. Plasmid DNA was isolated from clone 42, purified by chromatography over a NACS52 column and restricted as indicated. λ-HindIII-fragments were used as molecular weight markers on the 1.6% agarose gel. Staining: ethidium bromide.

The position of these sites was in agreement with the sequence of E1 as determined by J. Armstrong by shotgun cloning. The EcorRI-HindIII fragment, containing the recombinant DNA, was ligated into pEMBL8, and ss-DNA was sequenced to characterize the termini of the insert. The insert is flanked on either end by homopolymeric GC-tails (21, 25 bp in length) which are followed at the one end by the synthetic primer sequence and the E1 carboxy terminus. At the end corresponding to the 5'end of mRNA 6, a 76 base pair-noncoding leader sequence is present (Fig. 7).

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-76    -70         -60         -50         -40        -30
CCTATA AGAGTGATTG GCGTCCGTAC GTACCCTCTC AACTCTAAA CTCTTGTAGT

-20         -10      -1  +1                                    30
TTAAATCTAA TCCAAACATT   ATG AGT AGT ACT ACT CAG GCC CCA GAG CCC GTC
                        Met Ser*Ser*Thr*Thr*Gln Ala Pro Glu Pro Val
                                          60
TAT CAA TGG ACG GCC GAC GAG GCA GTT CAA TTC CTT AAG GAA TGG
Tyr Gln Trp Thr*Ala Asp Glu Ala Val Gln Phe Leu Lys Glu Trp
               90
AAC TTC TCG TTG GGC ATT ATA CTA CTC TTT...
Asn Phe Ser Leu Gly Ile Ile Leu Leu Phe...
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Fig. 7: Sequence of the 5'region of mRNA 6. A76 nucleotide noncoding leader sequence precedes the coding region. Note the cluster of four hydroxy amino acids in series at the NH_2-terminus of E1 as potential attachment sites for O-linked carbohydrate side chains.

While the sequence of this leader may be interesting with respect to the splicing mechanism which leads to the formation of the nested set, the amino acid sequence of the E1 NH_2-terminus is quite unique for several reasons: i) There is no hydrophobic signal sequence as commonly present in viral glycoproteins. ii) The cluster of four hydroxy-amino acids adjacent to the initial methionine are presumably the attachment site of the O-linked carbohydrate side chains (marked with an asterix in Figure 7). iii) The potential N-glycosylation site involving Asn(27) (underlined in Figure 7) is just at the beginning of one of the three hydrophobic domains which presumably span the membrane. Our present studies focus at the expression of the E1 gene and the construction of primers in order to alter or delete the O-glycosylation sites.

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