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Enhancement of the vaccinia virus/phage T7 RNA polymerase expression system using encephalomyocarditis virus 5′-untranslated region sequences

(Recombinant DNA; bacteriophage T7; promoter, terminator; picornavirus; cap-independent translation; internal initiation; translation efficiency; β-galactosidase; coronavirus)

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SUMMARY

A recombinant vaccinia virus producing the bacteriophage T7 RNA polymerase was used to express foreign genes in eukaryotic cells. Translation efficiency in this expression system was enhanced significantly by employing the encephalomyocarditis virus (EMCV) 5′-untranslated region (*UTR*) which confers cap-independent translation by directing internal initiation of translation. The enhancement was accomplished by fusing open reading frames (ORFs) to the N terminus of the EMCV polyprotein coding region, thus utilizing its highly efficient translation initiation site. Expression vectors were constructed to allow cloning in all three reading frames. As reporter genes, we used the *lacZ* gene and a number of genes encoding coronavirus structural proteins: among others the genes encoding glycoproteins with N-terminal signal sequences. The signal sequences of these glycoproteins are located internally in the primary translation product. We demonstrated that this did not interfere with translocation and glycosylation and yields biologically active proteins. The usefulness of sequences that direct internal initiation was extended by using EMCV *UTR*s to express two and three ORFs from polycistronic mRNAs.

INTRODUCTION

A recombinant vaccinia virus (reVV) synthesizing the bacteriophage T7 RNA polymerase was constructed by Fuerst et al. (1986). Target genes flanked by T7 transcription regulatory sequences were expressed from a plasmid (Fuerst et al., 1986) or from a second reVV (Fuerst et al., 1987). Analysis of the T7 mRNA revealed that only a small percentage contained a 5′-terminal cap structure suggesting a low translation efficiency (Fuerst and Moss, 1989). Recently, it was shown that the polysomes contained capped mRNA (Elroy-Stein et al., 1989), indicating that the

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Abbreviations: aa, amino acid(s); βGal, β-galactosidase; bp, base pair(s); CBB, Coomassie brilliant blue; EMCV, encephalomyocarditis virus; EndoH, endo-β-*N*-acetylglucosaminidase H; ExoIII, exonuclease III of *E. coli*; FIPV, feline infectious peritonitis virus; *G*, glycoprotein-encoding gene; IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilobase(s) or 1000 bp; M, membrane; *M*, membrane protein-encoding gene; MCS, multiple cloning site; N, nucleocapsid; *N*, nucleocapsid protein-encoding gene; ORF, open reading frame; p, plasmid; *p*, promoter; *p7.5* and *p11*, promoters for the 7.5- and 11-kDa VV polypeptide-encoding genes; PAGE, polyacrylamide-gel electrophoresis; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; re, recombinant; RIPA, radioimmune precipitation assay; S, spike; *S*, spike protein-encoding gene; SDS, sodium dodecyl sulfate; TGEV, transmissible gastroenteritis virus; *TK*, gene encoding thymidine kinase; *UTR*, untranslated region; v, vaccinia; VSV, vesicular stomatitis virus; VV, vaccinia virus; wt, wild type; XGal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

majority of the RNA is not translated. We were interested in improving the translation efficiency of uncapped mRNA by using picornavirus 5′-*UTR* sequences which can confer cap-independent translation (Pelletier et al., 1988; Jang et al., 1988). This occurs by internal binding of ribosomes, as opposed to the conventional binding to the 5′ terminal cap structure. Translation efficiency in the hybrid reVV/T7 RNA polymerase expression system was enhanced significantly by EMCV *UTR* sequences (Elroy-Stein et al., 1989). The aim of this study was to enhance the translation efficiency in the T7 expression system by EMCV *UTR* sequences by fusing ORFs to the N terminus of the EMCV

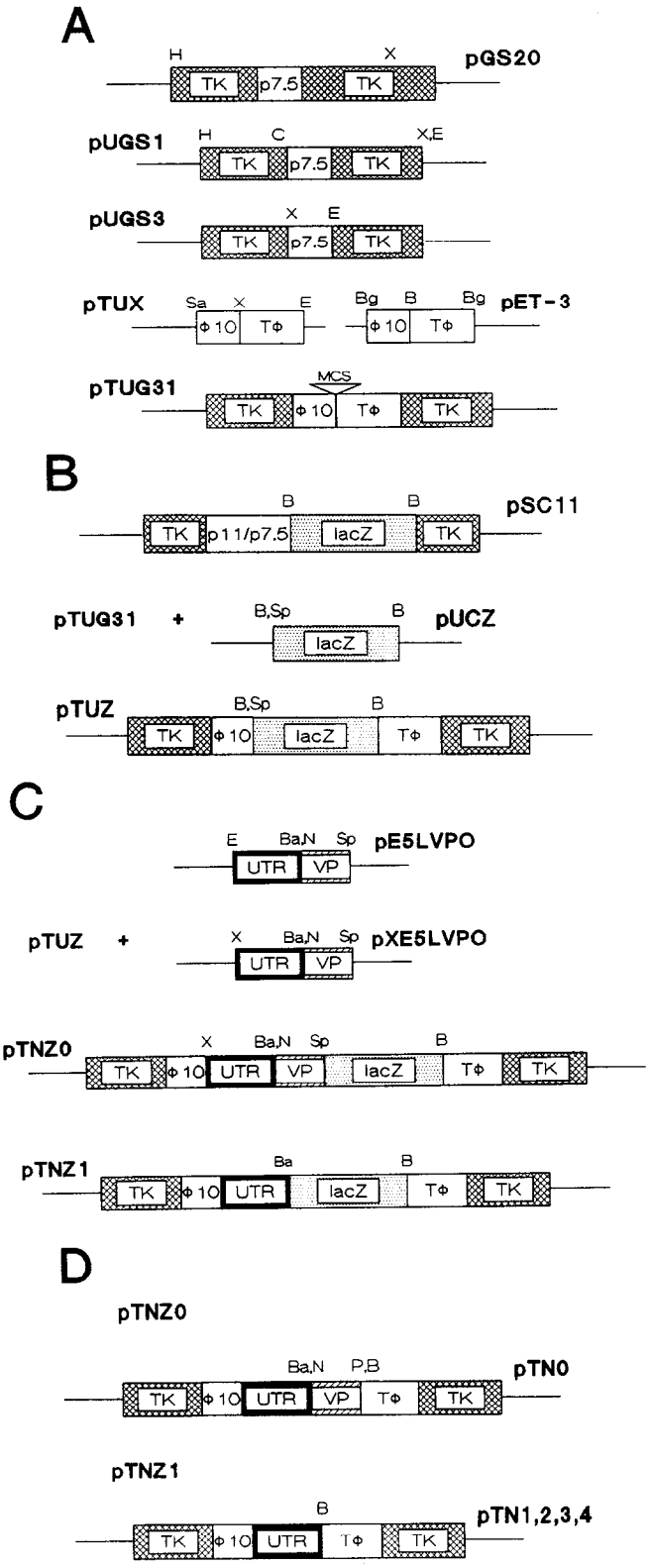

Fig. 1. Construction of expression vectors. (**A**) VV/T7 vectors. Vector pUC18 was modified to contain a unique *Xho*I site by linker insertion into a PolIk-filled-in *Eco*RI site. This vector, designated pUCX, was used to subclone a 1.7-kb *Hin*dIII-*Xho*I fragment of pGS20, to yield pUGS1. The *Xho*I site and the flanking *Eco*RI site were deleted by limited ExoIII digestion. The *Cla*I site was converted into a *Xho*I site by linker addition, yielding pUGS3. The T7 gene *10* promoter/terminator *Bgl*II cassette from pET-3 was recloned in pUC18 in the *Bam*HI site, yielding pTUC. The *Bam*HI site of pTUC, was converted into a *Xho*I site by linker addition, yielding pTUX. The *Bam*HI protruding ends were removed with mung-bean nuclease. The *Xho*I-*Eco*RI fragment of pUGS3, was replaced by the *Sal*I-*Eco*RI T7 cassette from pTUX, to yield pTUG1. The *Sma*I, *Kpn*I, *Sac*I and *Eco*RI sites of the pUC MCS were deleted by digestion with *Sma*I + *Eco*RI, and the *Xho*I-*Sal*I MCS fragment from pUCX was inserted in the *Xho*I site, yielding pTUG3. A 170-bp *Hin*dIII-*Nar*I fragment, containing the *lacZ* α-peptide-coding region was deleted from the pUC part, resulting in pTUG31. (**B**) Cloning of the *lacZ* reporter gene. The *lacZ* gene, starting from the 9th codon was isolated from pSC11 as a 3-kb *Bam*HI fragment. The fragment was PolIk-filled-in and ligated to PolIk-filled-in *Hin*dIII + *Nar*I-digested pUC18. Clones with the *lacZ* gene behind a start codon contained within the *Sph*I site (GCATGC) were identified by selection on IPTG/XGal plates and designated pUCZ. Fusion of the PolIk-filled-in *Bam*HI and *Nar*I sites regenerated a *Bam*HI site, at the 3′ end of the *lacZ* gene. The *Bam*HI site in the MCS allowed excision of the *lacZ* gene with a start codon. The *Bam*HI fragment was recloned in pTUG31, yielding pTUZ. (**C**) Construction of *UTR* vectors. EMCV *UTR* cDNA was inserted between the T7 promoter and the *lacZ* gene. The *Eco*RI site at the 5′ end of the insert of pE5LVPO was converted to an *Xho*I site by linker addition, yielding pXE5LVPO. The *Xho*I-*Sph*I fragment was recloned into pTUZ digested with *Xho*I and *Sph*I, yielding pTNZ0. The polyprotein coding region was removed by deletion of the *Nco*I-*Sph*I fragment, yielding pTNZ1. (**D**) Construction of general-purpose *UTR* vectors. The *lacZ* gene was deleted from pTNZ1 and pTNZ0 by digestion with restriction endonucleases with sites flanking it. Blunt-ended fragments containing vector sequences were isolated from agarose gel. Blunt ends were either generated by the restriction enzyme itself (*Bal*I), by removing 3′ overhanging ends with T4 DNA polymerase (*Sph*I and *Pst*I) or by filling-in 5′ overhanging ends with PolIk (*Nco*I and *Bam*HI). Digestion of pTNZ0 with *Sph*I + *Bam*HI resulted in pTN0, which was prepared to allow construction of two new vectors. Digestion of pTN0 with *Nco*I + *Pst*I resulted in pTN1. Digestion of pTNZ0 with *Nco*I + *Bam*HI resulted in pTN2. Digestion of pTNZ1 with *Bal*I + *Bam*HI resulted in pTN3. Digestion of pTN0 with *Bal*I + *Pst*I resulted in pTN4. The vectors pTN2 and pTN4 contain *Bam*HI sites in the same reading frame. The letter N in plasmid and reVV symbols refers to the presence of the 5′-nontranslated region (*UTR*), and the letter U to the absence of this region. B, *Bam*HI; Ba, *Bal*I; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; P, *Pst*I; Sa, *Sal*I; Sp, *Sph*I; X, *Xho*I; ϕ10, T7 promoter preceding gene *10* in T7 DNA; TK, VV TK sequences; Tϕ, T7 transcription terminator; *p7.5* and *p11*, promoters for the 7.5-kDa and 11-kDa VV polypeptide-encoding genes, respectively; UTR, untranslated region; VP, viral polyprotein.

polyprotein, thus utilizing the efficient translation initiation site of the polyprotein.

RESULTS AND DISCUSSION

(a) Construction of T7 expression vectors

Recombinant DNA techniques were as described by Maniatis et al. (1982). Expression vectors were assembled using fragments from various sources. Vaccinia virus *TK* sequences were recloned from pGS20 (Mackett et al., 1984; obtained from Dr. B. Moss) to yield pUGS1, which was modified to pUGS3 (Fig. 1A). The bacteriophage T7 gene *10* promoter-terminator *Bgl*II cassette from pET-3 (Rosenberg et al., 1987; previously designated pAR2529, obtained from Dr. B. Moss) was modified and used to replace the *p7.5* fragment of pUGS3, to yield pTUG1. An MCS fragment was inserted downstream from the T7 promoter, yielding pTUG3, which was further modified to pTUG31 (Fig. 1A). Sequence analysis of vectors of the pTU-series revealed that a number of nt were lost downstream from the T7 promoter. The sequences which are thought to form a stable stem-loop structure at the 5′ end of T7 transcripts (Fuerst and Moss, 1989; Rosenberg et al., 1987), were still present (Fig. 2A).

(b) Cloning of the *lacZ* reporter gene

The *lacZ* gene, starting from the ninth codon was isolated from pSC11 (Chakrabarti et al., 1985; obtained from Dr. B. Moss), as a 3-kb *Bam*HI fragment, which is identical to the

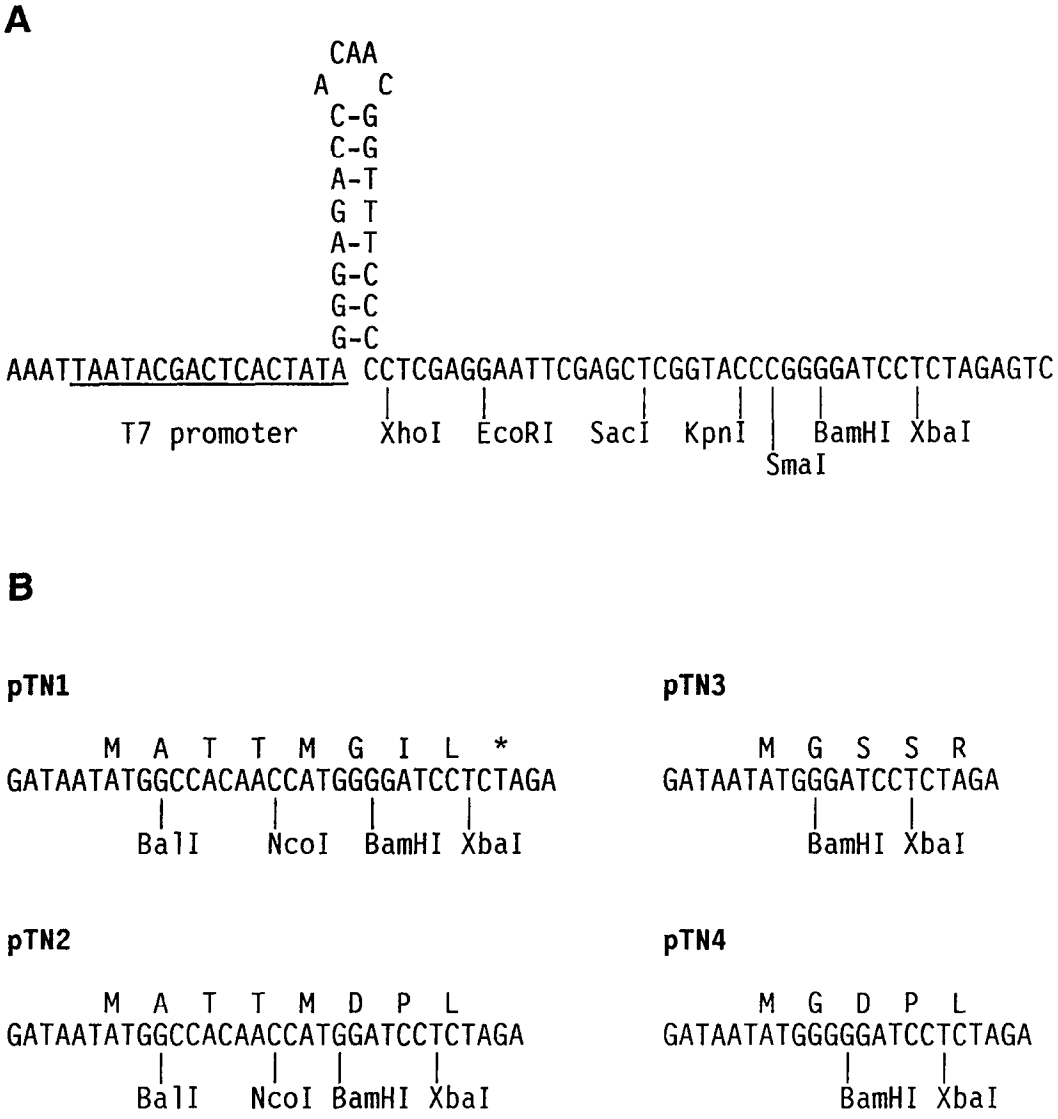

Fig. 2. Nucleotide sequences surrounding the MCS regions of VV/T7 expression vectors. All sites indicated are unique except for *Xba*I. (**A**) Vector pTUG31 (see Fig. 1A). The potential stem-loop structure in the RNA is shown (Fuerst and Moss, 1989; Rosenberg et al., 1987). (**B**) Vectors pTN1, pTN2, pTN3 and pTN4 (see Fig. 1D). The deduced aa sequences are indicated in single-letter code (aligned with the first nt of each codon); an asterisk denotes a stop codon.

fragment originally derived from pMC1871 (Casadaban et al., 1983). To obtain a *lacZ* gene with a start codon the fragment was recloned in pUC18. Clones with the *lacZ* gene fused in frame with a start codon (underlined) contained within the *Sph*I site (GCATGC) in the MCS behind the *lacZp* were identified by selection on IPTG/XGal plates and designated pUCZ (Fig. 1B). For expression in vTF7-3-infected cells the *lacZ* gene fragment from pUCZ was recloned in pTUG31, yielding pTUZ (Fig. 1B).

(c) Construction of *UTR* vectors

Picornavirus *UTR* sequences were inserted between the T7 promoter and the *lacZ* gene to assess their influence on the translation efficiency. The EMCV *UTR* cDNA was derived from clone pE5LVPO (Parks et al., 1986; obtained from Dr. A.C. Palmenberg), and recloned in pTUZ (Fig. 1C). The polyprotein coding region was removed, yielding pTNZ1 (Fig. 1C). This plasmid encoded a fusion protein which consists of the N-terminal 5 aa of the EMCV polyprotein and βGal, starting from the ninth aa residue.

(d) Enhancement of βGal synthesis

Synthesis of βGal was monitored in vTF7-3-infected HeLa cells using the following infection-transfection assays: in situ staining with XGal; labeling and SDS–PAGE analysis, and SDS–PAGE analysis and CBB staining. A comparison between constructs pTNZ1 and pTUZ, with and without EMCV *UTR*, respectively, demonstrated that the translation efficiency was significantly enhanced by the *UTR*. To make accurate comparisons the same constructs, designated vTNZ and vTUZ, respectively, were used to prepare reVV by procedures described in Mackett et al. (1984). Recombinants were twice plaque-purified and identified by coinfection of HeLa cells with

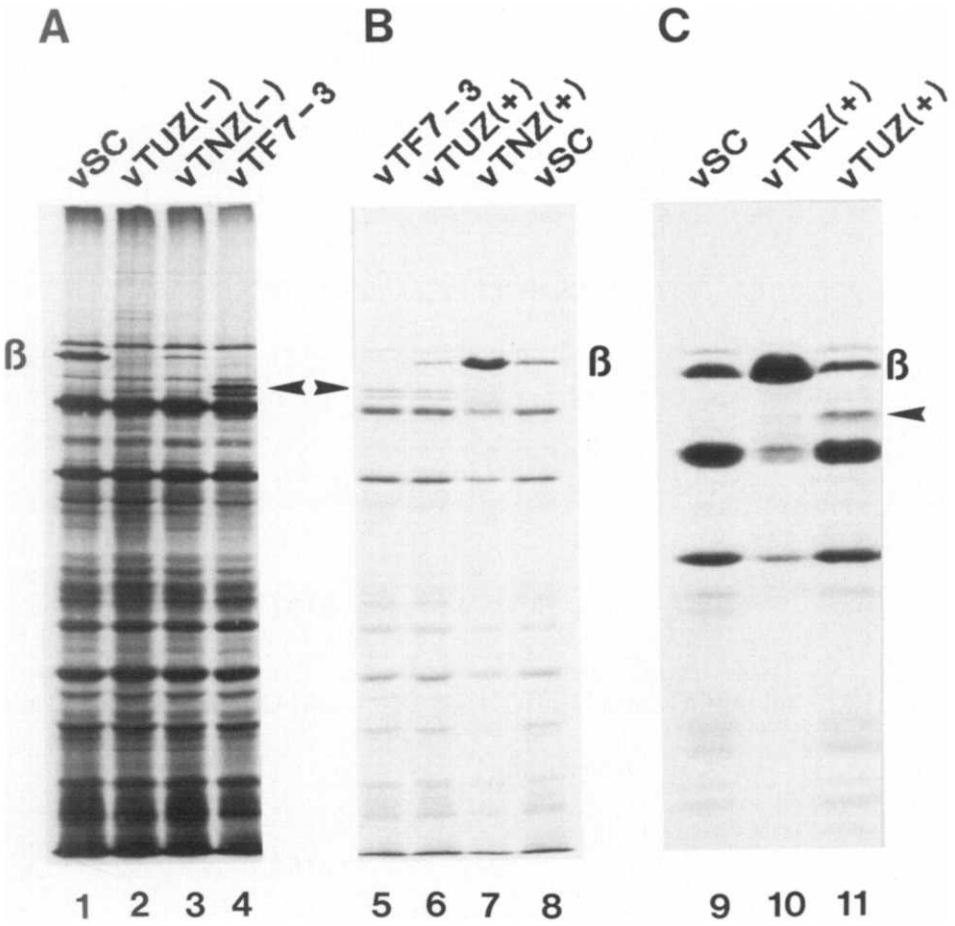

Fig. 3. Enhancement of βGal synthesis. HeLa cells were infected with the reVV indicated above the lanes. Single infections, indicated with (−), were carried out with vTUZ and vTNZ. Double infections with the same recombinants and vTF7-3 are indicated with (+). The βGal band is indicated with β. The T7 RNA polymerase band is indicated with arrowheads. Infected cells were labeled for 1 h with [^{35}S]methionine in methionine-free (panels **A** and **B**) or in normal (panel **C**) medium and harvested. Samples were boiled in Laemmli sample buffer and analyzed by 0.1% SDS–10% PAGE. The autoradiograms are shown. βGal bands, identified by CBB staining, were cut from the gels and the radioactivity was counted as well as for the rest of the lanes. Samples were analyzed for cells infected with vSC (lanes **1**, **8** and **9**), constructed with pSC11 (Fig. 1B); vTUZ (lane **2**), constructed with pTUZ (Fig. 1B); vTNZ (lane **3**), constructed with pTNZ1 (Fig. 1C); vTF7-3 (lanes **4** and **5**), vTUZ + vTF7-3 (lanes **6** and **11**), or vTNZ + vTF7-3 (lanes **7** and **10**).

vTF7-3 followed by in situ staining with XGal. A comparison of βGal synthesis levels between these recombinants was made in co-infections with vTF7-3. Lysates of infected HeLa cells were prepared after metabolic labeling with L-[^{35}S]methionine (Amersham Corp.) as described (Vennema et al., 1990). Analysis by SDS–PAGE was carried out as described (Laemmli, 1970). Typical results of these experiments are shown in Fig. 3. The βGal protein was identified by co-electrophoresis of purified βGal (Boehringer, Mannheim) and CBB staining (Fig. 4). Its identity was confirmed by RIPA using monoclonal antiserum against βGal (Promega, Madison, WI), followed by autoradiography (data not shown). Translation efficiency was determined by incorporation of [^{35}S]methionine. Liquid-scintillation counting of bands cut from stained gels of several experiments showed that 25–30% of the total radioactivity was incorporated into βGal using a construct containing *UTR* sequences. Using the standard hybrid/T7 system without *UTR* sequence incorporation into βGal was 3% of the total. The improvement of expression due to *UTR* sequences was therefore eight- to tenfold. The level of expression was also compared to recombinant vSC (Vennema et al., 1991) which was constructed with the cloning vector pSC11 (Chakrabarti et al., 1985), with the *lacZ* gene driven by the strong late VV *p11*. In vSC-infected cells 6–7% of the [^{35}S]methionine was incorporated into βGal. Production of βGal in the T7/*UTR* system was therefore approx. four times more efficient than that with vSC. Remarkably, incorporation into other proteins, presumably of VV, in vTNZ + vTF7-3-infected cells was 1.5- to twofold less than in the vTUZ + vTF7-3 and vTF7-3-infected cells. This reduction was not due to methionine limitation since it was also observed when normal medium was used for metabolic labeling, instead of methionine-free medium (Fig. 3C). The same observation was made with another reporter gene (see section **f**). Among the affected proteins was the T7 RNA polymerase (indicated with an arrowhead). When single infections were carried out with vTNZ and vTUZ, synthesis of βGal still occurred with recombinant vTNZ but not with vTUZ (Fig. 3A). This can be explained by translation through internal initiation of transcripts starting at late VV promoters, located upstream from the *lacZ* gene in the recombinant genome. Late in infection transcription termination does not occur and therefore long transcripts are synthesized (Mahr and Roberts, 1984), which may span the reporter gene. The same phenomenon was observed with another reporter gene (see section **f**). To estimate total production levels the amount of accumulated βGal was compared to standard amounts of purified βGal in CBB-stained gels (Fig. 4). The βGal band was most prominent in lysates of vTNZ + vTF7-3-infected cells; in vSC-infected cells it had about the same intensity as the major VV bands. Comparison to purified βGal showed that 10^4 cells infected with vTNZ + vTF7-3 produced approx. 1 μg βGal. The amount of βGal produced in pTNZ transfected and vTF7-3-infected cells was approximately the same as in vTNZ + vTF7-3-infected cells. In our hands the standard hybrid/T7 system gave approximately half the expression level of vSC. Using similar constructs the same ratio was reported (Falkner and Moss, 1990), indicating that the expression level with our construct in the standard hybrid/T7 system was no underestimate.

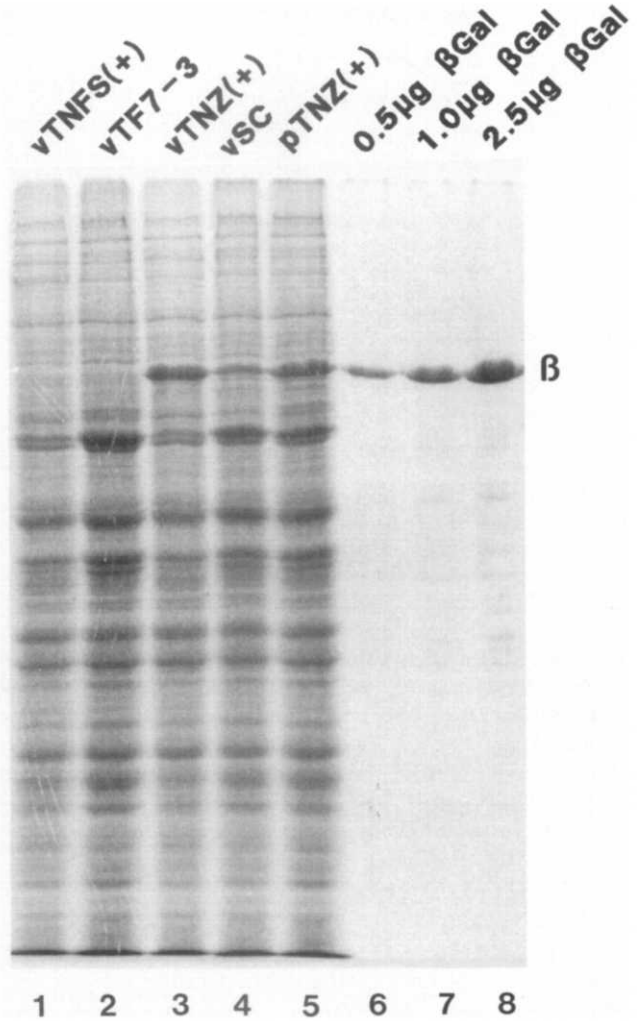

Fig. 4. Total βGal production levels. HeLa cells were infected with the reVV indicated above the lanes. Double infections with vTF7-3 are indicated with (+). Samples from 10^4 infected cells were prepared at 24 h after infection, boiled in Laemmli sample buffer and analyzed on 0.1% SDS–10% PAGE. After CBB-staining the βGal bands were compared to standard amounts of purified enzyme (Boehringer, Mannheim). Samples were analyzed for cells infected with vTNFS, constructed with pTNFS (Fig. 7A), and vTF7-3 (lane **1**), vTF7-3 (lane **2**), vTNZ + vTF7-3 (lane **3**), vSC (lane **4**), or vTF7-3 transfected with pTNZ (lane **5**). Recombinants vTNZ and vSC are described in the legend to Fig. 3. Samples of 0.5 μg (lane **6**), 1.0 μg (lane **7**) and 2.5 μg (lane **8**) βGal were also analyzed. The CBB-stained gel is shown.

(e) Construction of general-purpose *UTR* vectors

To obtain general-purpose cloning vectors the *lacZ* gene was deleted from pTNZ1 and pTNZ0. This was achieved by digestion with restriction endonucleases with sites flanking the *UTR* and the *lacZ* gene and by religation. Different combinations resulted in the construction of vectors with unique *Bam*HI sites in all three reading frames. Details are given in the legends to Fig. 1D and 2B.

(f) Expression of glycoproteins with N-terminal signal sequences

Apart from internal initiation directed by the *UTR*, the efficient start site of the EMCV polyprotein probably contributed to the translation efficiency. Deletion of the start codon to allow expression of foreign genes with a downstream start codon was expected to reduce translation enhancement. Therefore, we assessed whether synthesis of fusion proteins was generally applicable. In particular, we wanted to see if the N-terminal signal sequences of glycoproteins function normally when these are synthesized as fusion proteins with N-terminal extensions. We cloned several glycoprotein genes in frame with the EMCV start codon. Restriction fragments containing the FIPV and mouse hepatitis virus *S* genes and the VSV *G* gene were obtained as described (Vennema et al., 1990) and recloned into the appropriate expression vector. In infection-transfection assays each of these proteins was produced in a biologically active form (data not shown). This was demonstrated by the induction of cell fusion in the proper target cells as we have shown with reVV (Vennema et al., 1990). It was described before that an internalized signal sequence of the VSV glycoprotein functions normally in transfected cells (Rottier et al., 1987). Comparison of the *S* gene constructs in transfection experiments showed increased synthesis of *UTR*-containing constructs (data not shown). Recombinant VV were prepared of the FIPV *S* gene preceded by a T7 promoter with or without EMCV 5′-*UTR* sequences, and designated vTNFS and vTFS, respectively. Metabolic labeling experiments and SDS–polyacrylamide gel analysis showed that insertion of the *UTR* resulted in an approx. fivefold enhancement of the translation efficiency (Fig. 5). As with *lacZ*, a reduced incorporation into VV proteins was observed in cells infected with vTNFS + vTF7-3. The combined effect of enhanced translation of FS mRNA and reduced translation of other mRNAs resulted in S protein band intensity comparable to the most abundant VV proteins synthesized late in infection. When cells were infected with vTNFS alone, synthesis of the S protein was still observed (Fig. 5) as it was with *lacZ* as reporter. The level of expression by vTNFS alone was comparable with that of vFS (Vennema et al., 1990), which contains the *S* gene driven by *p7.5* (data not shown).

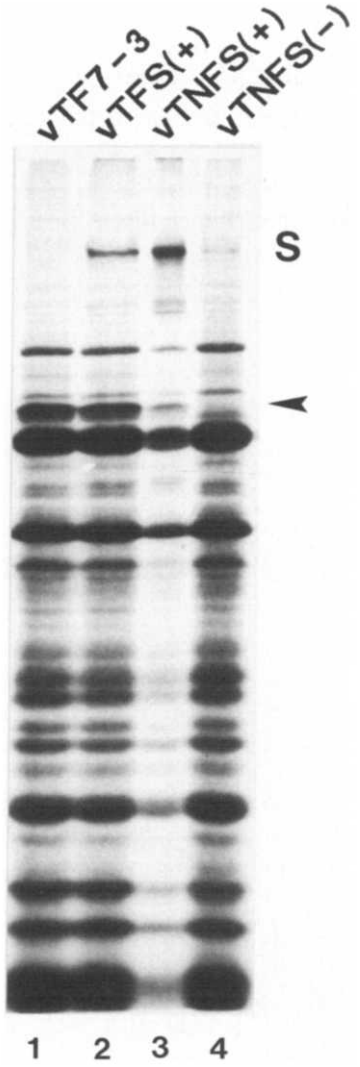

Fig. 5. Enhancement of glycoprotein synthesis. HeLa cells were infected with the reVV indicated above the lanes and labeled for 1 h with [^{35}S]methionine at 16 h after infection in methionine-free medium and harvested. Samples were boiled in Laemmli sample buffer and analyzed by 0.1% SDS–10% PAGE. The autoradiogram is shown. Samples were of cells infected with vTF7-3 (lane **1**), vTFS (section **f**) and vTF7-3 (lane **2**), vTNFS, constructed with pTNFS (Fig. 7A), and vTF7-3 (lane **3**), or vTNFS alone (lane **4**). Double infections with vTF7-3 are marked (+). Single infections are marked (−). The FIPV S protein and the T7 RNA polymerase bands are indicated with **S** and an arrowhead, respectively.

(g) Expression of ORFs without start codon

The coronavirus M protein contains three transmembrane domains which provide signals for membrane integration and anchoring. In addition, the M proteins of FIPV and TGEV have cleavable N-terminal signal sequences. To study the membrane integration properties of the FIPV M protein without this signal sequence, part of the *M* gene was cloned in pTN1, omitting the part that encoded the signal sequence. The construct was designated pTFM*. The fusion product was encoded by the first five codons of the EMCV polyprotein-encoding gene and the *M* gene starting

from the 12th codon. The complete *M* gene cloned in pTUG31 was designated pTFM. The fusion protein migrated slightly slower than the wt M protein (Figs. 6A and 7B, lanes 9 and 10), indicating that the signal sequence of the wt expression product was cleaved. Digestion with EndoH (Boehringer, Mannheim) showed that the expression product was completely glycosylated (Fig. 6A). Consequently, the signal sequence of the FIPV M protein is not required for translocation and glycosylation, as had been demonstrated before for the TGEV M protein (Kapke et al., 1988). In this case, however, the experiment had been performed by in vitro translation in the presence of microsomes, and glycosylation occurred at a very low level.

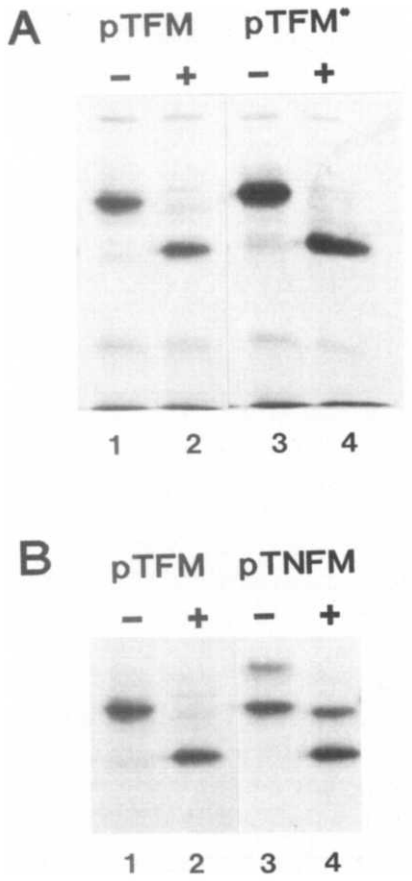

Fig. 6. synthesis of the FIPV M protein. The *M* gene was expressed with constructs described (section **g**). The structure of the plasmids is given schematically in Fig. 7A. The *M** ORF of pTFM* contains the coding sequence of the M protein except for the N-terminal signal sequence. Plasmids pTNFM and pTFM contain the complete *M* gene, with and without *UTR*, respectively. Expression was monitored by transfection of vTF7-3-infected HeLa cells. Plasmids used for transfection are indicated above the lanes. Cell lysates, prepared after metabolic labeling with [^{35}S]methionine, were processed for RIPA and analyzed by 0.1% SDS–12.5% PAGE after mock treatment or treatment with EndoH, carried out as described (Vennema et al., 1990), indicated by – and +, respectively, above the lanes. The autoradiograms are shown. (Panel **A**) Comparison of pTFM (lanes **1** and **2**) and pTFM* (lanes **3** and **4**), after pulse-labeling with [^{35}S]methionine for 1 h. (Panel **B**) Comparison of pTFM (lanes **1** and **2**) and pTNFM (lanes **3** and **4**), after pulse-labeling with [^{35}S]methionine for 1 h and chase for 3 h in normal medium.

(h) Translation of two and three ORFs from one mRNA

The EMCV *UTR* enabled internal initiation of translation as demonstrated by the expression of a reporter gene with an *UTR* from di- and tricistronic mRNAs, regardless of its position in the mRNA (Jang et al., 1988). To establish if several *UTR* sequences could be used to express more than one ORF, constructs were prepared encoding two or three different proteins (Fig. 7A). The reporter genes, cloned into the appropriate vectors, encoded FIPV structural proteins, which were analyzed by RIPA using a polyvalent anti-FIPV serum. The results of infection-transfection experiments are shown in Fig. 7B. Lane 5 shows that the *M* and *N* genes were both expressed from pTFMN, at levels slightly below those of pTNFM and pTNFN (lanes 2 and 3, respectively). Cleavage of the signal sequence of the M protein was apparently affected by the presence of the N-terminal extension, which was long and highly charged in this case. During the pulse-labeling experiments an extended precursor was found (Fig. 7B), indicating that the majority of translation products was initiated at the EMCV polyprotein start site. After chase incubations the precursor was partly converted to the cleaved product (Fig. 6B). Digestion with EndoH showed that both forms were glycosylated, indicating that the N-terminal extension did not affect translocation (Fig. 6B). In view of the results with pTFM* (section **g**), we should consider the possibility that a transmembrane segment provided the translocation signal instead of the internalized N-terminal signal sequence. The signal sequence was cleaved correctly, albeit more slowly than for the wt protein. Construct pTFMNS, having *UTR* sequences upstream of the *N* and *S* genes, induced synthesis of the N and S proteins. Apparently, downstream *UTR* sequences had a deleterious effect on translation of the part of the mRNA for the M protein, which had no cap and no *UTR*, since the M protein was readily produced from a monocistronic T7 mRNA without *UTR* (Fig. 7B, lane 9). Finally, pTFM*NS was prepared which efficiently expressed three genes. This plasmid consisted of the *M* gene of pTFM* combined with the *N* and *S* genes of pTFMNS, and contained *UTR* sequences upstream from three ORFs. The synthesis of the N and S proteins and the M* protein was driven by this construct (Fig. 7B, lane 8).

(i) Conclusions

(*1*) The reVV-bacteriophage T7 expression system was considerably improved. A similar approach was chosen as recently described by Elroy-Stein et al. (1989); these investigators changed the EMCV translation start site to contain an *Nco*I restriction site. In their vector, pTM1 (Moss et al., 1990), this *Nco*I site provides the start codon. Recently, it

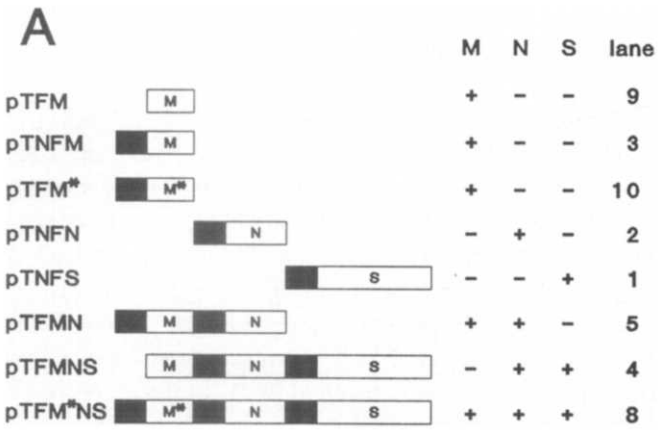

Fig. 7A. Vennema et al.

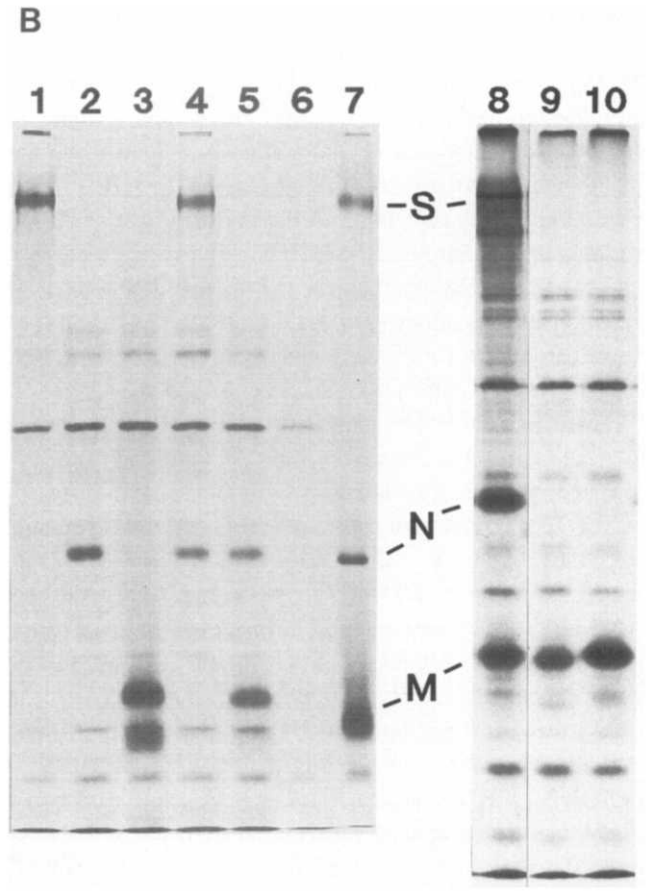

Fig. 7. Synthesis of FIPV structural proteins. (**A**) Expression constructs for mono-, di- and tricistronic mRNAs are represented schematically. Open boxes represent the ORFs of the *M*, *N* and *S* genes of FIPV and blackened boxes represent the *UTR* sequences. The table to the right summarizes the results of infection/transfection experiments, shown in panel **B**. Lane numbers for the gels shown in panel **B** are given in the column at the far right. (Panel **B**) RIPA and SDS–PAGE analysis of [^{35}S]methionine-labeled, vTF7-3-infected and transfected HeLa cells. Plasmids used for transfection are listed in scheme **A**. Lanes **6** and **7** contain samples of mock and FIPV-infected feline kidney cells, respectively, prepared as described (Vennema et al., 1990). FIPV structural proteins are indicated. Lanes **1–7** and **8–10** are from 10 and 12.5% PAGE (0.1% SDS), respectively, which explains why the bands did not comigrate. The autoradiograms are shown.

was reported that elimination of this start codon caused a decrease in expression (Moss et al., 1990). In our system the translation start site was not modified by mutagenesis but instead the highly efficient EMCV polyprotein start site was used. Internal binding apparently occurs directly at the translation start site of EMCV (Kaminski et al., 1990). Therefore, changing the position of the start codon may diminish the enhancement achieved with the *UTR*.

(*2*) Extensive comparison made between expression with and without *UTR* sequences showed that translation efficiency could be improved eight- to tenfold. Taking into account that 5–10% of the T7 transcripts are capped and translated in the standard hybrid system, the fraction of transcripts involved in translation approaches 100% using EMCV *UTR* sequences. We were not able to further enhance the expression level by treatment with hypertonic medium (unpublished data) in contrast to previous observations (Elroy-Stein et al., 1989).

(*3*) Our strategy not only allowed efficient expression of *lacZ* but also of a number of genes of glycoproteins with N-terminal signal sequences. Due to the cloning strategy these signal sequences were located internally in the translation product. Nevertheless, they functioned normally, as demonstrated by biological activity and glycosylation of the polypeptides.

(*4*) The observation that *UTR* containing reVV express reporter genes even without coinfection with vTF7-3 adds a novel option to the use of our expression vectors, namely to use the reVV derived from them for immunization. The use of picornavirus *UTR* sequences obviates the need for transcription regulatory elements, since internal initiation of translation occurs on late read-through transcripts of VV.

(*5*) The preparation of constructs that synthesize fusion proteins allows expression of ORFs without a start codon. This was done for the FIPV *M* gene lacking the N-terminal signal sequence. The fusion product was completely glycosylated, proving that the signal sequence of the FIPV M protein is not required for translocation and glycosylation. Our T7/*UTR* vectors with a unique cloning site in three reading frames allow expression of virtually any ORF.

(*6*) *UTR* sequences were employed to express several genes from one transcription unit. In this way the complete set of structural proteins of FIPV was synthesized.

(*7*) Recently, *UTR* sequences were shown to be required for a cytoplasmic expression system based on constitutive synthesis of T7 RNA polymerase in mammalian cells (Elroy-Stein and Moss, 1990). The vectors developed in the present study are directly applicable for this type of expression system.

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