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

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Received by W.C. Summers: 9 May 1991
Accepted: 19 July 1991
Received at publishers: 19 September 1991

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 UTRs 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...
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 XhoI site by linker insertion into a PolIk-filled-in EcoRI site. This vector, designated pUCX, was used to subclone a 1.7-kb HindIII-XhoI fragment of pGS20, to yield pUGS1. The XhoI site and the flanking EcoRI site were deleted by limited ExoIII digestion. The ClaI site was converted into a XhoI site by linker addition, yielding pUGS3. The T7 gene 10 promoter/terminator BgII cassette from PET-3 was recloned in pUC18 in the BamHI site, yielding pET-3. The BamHI site of pTUC, was converted into a XhoI site by linker addition, yielding pTUX. The BamHI protruding ends were removed with mung-bean nuclease. The XhoI-EcoRI fragment of pUGS3, was replaced by the SphI-EcoRI T7 cassette from pTUX, to yield pTUG1. The SmaI, KpnI, SacI and EcoRI sites of the pUC MCS were deleted by digestion with SmaI + EcoRI, and the XhoI-SalI MCS fragment from pUCX was inserted in the XhoI site, yielding pTUG3. A 170-bp HindIII-NarI fragment, containing the lacZ a-peptide-coding region was deleted from the part, resulting in pTUG3. (B) Cloning of the lacZ reporter gene. The lacZ gene, starting from the 9th codon was isolated from pSC11 as a 3 kb BamHI fragment. The fragment was PolIk-filled-in and digested to PolIk-filled-in HindIII-NarI-digested pUC18. Clones with the lacZ gene behind a start codon contained within the SphI site (GCATGC) were identified by selection on IPTG/XGal plates and designated pUCZ. Fusion of the PolIk-filled-in BamHI and NarI sites regenerated a BamHI site, at the 3' end of the lacZ gene. The BamHI enzyme in the MCS allowed excision of the lacZ gene with a start codon. The BamHI 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 EcoRI site at the 5' end of the insert of pESLVP0 was converted to an XhoI site by linker addition, yielding pXESLVP0. The XhoI-SphI fragment was recloned into pTUZ digested with XhoI and SphI, yielding pTNZ0. The polyprotein coding region was removed by deletion of the NcoI-SphI 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 (Bali), by removing 3' overhanging ends with T4 DNA polymerase (SphI and PstI) or by filling-in 5' overhanging ends with PolIk (NcoI and BamHI). Digestion of pTNZ0 with SphI + BamHI resulted in pTN0, which was prepared to allow construction of two new vectors. Digestion of pTN0 with NcoI + PstI resulted in pTN1. Digestion of pTN0 with PolIk + BamHI resulted in pTN2. Digestion of pTNZ1 with BalI + BamHI resulted in pTN3. Digestion of pTN0 with BalI + PstI resulted in pTN4. The vectors pTN2 and pTN4 contain BamHI 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, BamHI; Ba, BalI; Bg, BglII; C, ClaI; E, EcoRI; H, HindIII; N, NcoI; P, PstI; Sa, SalI; Sp, SphI; X, XhoI; φ10, T7 promoter preceding gene 10 in T7 DNA; TK, VV TK sequences; Tf, T7 transcription terminator; pT5 and pIl, 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 TX 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 BglII 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 BamHI 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 XbaI. (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 SphI 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
Pieonavirus 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 [35S]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-[35S]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 [35S]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 pl1. In vSC-infected cells 9–10% of the [35S]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 4.5- to twofold less than in 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 10⁴ 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.
(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 BamHI 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 protein 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).

(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 live codons of the EMCV polyprotein-encoding gene and the M gene starting from the EMCV 5'-UTR (Fig. 7A).
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.

(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

(I) 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 a Neos restriction site. In their vector, pTM1 (Moss et al., 1990), this Neos site provides the start codon. Recently, it
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.
ACKNOWLEDGEMENTS

The authors thank Dr. A.C. Palmenberg for plasmid pE5LVPO and Dr. B. Moss for providing numerous plasmids and reVV vTF7-3. H.V. and L.H. were supported by DuPhar BV, Weesp, The Netherlands.

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