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AUG context and mRNA translation in vaccinia virus

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Abstract

In this study, we examined the role of the nucleotides flanking AUG initiator codons in modulating translation of vaccinia virus genes. First, the sequence around initiation codons in the genomic sequence of vaccinia was analyzed. A preference for A in position –3 and for A or G in position +4, relative to the A of the initiator AUG codon was noted. These results are in broad agreement with known optimal consensus for the translation of eukaryotic mRNAs. Nucleotides –3 and +4, which are known to affect the efficiency of translation of cellular mRNAs over a 20-fold range, were examined by site-directed mutagenesis and functional assays in the context of vaccinia-infected cells. A collection of virus recombinants expressing β-Galactosidase (β-Gal) under the control of a strong promoter and containing all possible nucleotide substitutions in those positions was generated. Quantitation of β-Gal mRNA and β-Gal activity showed that the efficiency of translation was not affected significantly by nucleotide substitutions in positions –3 and +4. Therefore, despite the high occurrence of purine nucleotides in those positions in vaccinia mRNAs, no effect of those positions was noted in conditions where β-Gal was overexpressed.

Additional key words: β-galactosidase, poxviruses, site-directed mutagenesis, translation initiation.

Resumen

Contexto de AUG y traducción de mRNA en el virus vaccinia

En este estudio hemos examinado el papel de los nucleótidos próximos al AUG iniciador en la modulación de la traducción en genes del virus vaccinia. En primer lugar, hemos analizado la secuencia alrededor de los codones de iniciación en la secuencia genómica de vaccinia. En este análisis se detectó una preferencia por A en la posición –3, y por A ó G en la posición +4, relativa a la A en el codon de iniciación. Estos resultados coinciden en términos generales con las secuencias conocidas para la traducción óptima de mRNAs eucarióticos. Los nucleótidos –3 y +4, que afectan a la eficiencia de traducción de mRNAs celulares en un rango de hasta 20 veces, fueron examinados por mutagénesis dirigida y ensayos funcionales en el contexto de células infectadas por vaccinia. Hemos generado una colección de virus recombinantes que expresan β-galactosidasa (β-Gal) bajo el control de un promotor fuerte, y conteniendo todas las posibles sustituciones en esas posiciones. La cuantificación del mRNA de β-Gal, y de la actividad β-Gal, mostró que la eficiencia de traducción no se alteraba significativamente por las sustituciones nucleotídicas en las posiciones –3 y +4. Por tanto, a pesar de la alta frecuencia de nucleótidos de purina en esas posiciones en los mRNAs de vaccinia, no se detectó efecto de dichas posiciones en condiciones en las que la β-Gal era sobreexpresada.

Palabras clave adicionales: β-galactosidasa, iniciación de la traducción, mutagénesis dirigida, poxvirus.

Introduction[1]

Poxviruses, of which vaccinia virus is the best studied representative, are complex, DNA-containing viruses that replicate in the cytoplasm of the infected cell. During the infection cycle viral gene expression takes place in successive steps, namely early, intermediate and late. This temporal regulation is achieved by modification of the virally encoded RNA polymerase by transcription factors.

Vaccinia virus infection is known to induce a dramatic «shut-off» in the synthesis of cellular proteins. To date,

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[1] Abbreviations used: DMF (dimetil formamide), dpi (days post-infection), hpi (hour post-infection), MOI (multiplicity of infection), MOPS [3-(N-morpholino) propanesulfonic acid], ONPG (O-nitrophenyl β-D-galacto-pyranoside), ORF (open reading frame), RT (room temperature), X-Gal (5-bromo-4-chloro-3-indolyl-,-D-galactopyranoside), β-Gal (β-galactosidase).

several possibilities have been proposed to explain this process. The relative abundance of viral mRNAs may partially explain the lowered synthesis of cellular proteins, since several hours after infection the rapid mRNA turnover and inhibition of host RNA synthesis, along with the rise of viral transcripts, lead to a predominance of mRNAs of viral origin (Becker and Joklik, 1964; Sebring and Salzman, 1967; Boone and Moss, 1978; Cooper and Moss, 1979). In addition, a variety of cellular proteins and RNAs have been implicated in regulating the translation of viral *versus* cellular mRNAs. For instance, small RNAs (Bablanian *et al.*, 1978; Bablanian *et al.*, 1993; Cacoullos and Bablanian, 1993), the surface tubules of the virus particle (Mbuy *et al.,* 1982), or the proteins F17R (Person-Fernández and Beaud, 1986) and B1R (Beaud *et al.,* 1994) have been proposed as specific inhibitors of host protein synthesis.

An additional factor that might modulate translation efficiency of mRNAs is the sequence context around the initiation codon. The optimal context for translation initiation in mammalian species is GCC(A/G)CCAUGG, where AUG is the initiation codon (Kozak, 1986, 1997, 1999). Within this sequence, the –3 and +4 positions are particularly important, influencing translation efficiency up to 20 fold (Kozak, 1986). In this report we present an analysis of the viral genome showing that vaccinia virus genes follow Kozak's rules, in particular in the key positions –3 and +4. We studied the influence of these positions in the translation efficiency of vaccinia virus mRNAs, and show that of highly expressed viral late messengers is not significantly affected by mutations in those positions.

Material and Methods

Cells, viruses and plasmids

BSC-1 cells, obtained from the American Type Culture Collection (ATCC CCL 26) and CV-1 cells were grown in Eagle's minimal essential medium (EMEM) (Biowhittaker) containing 5% fetal bovine serum. The cells were maintained at 37°C in a 5% CO_2 atmosphere. The infection/transfection protocol, plaque purification and virus amplifications were carried out as previously described (Lorenzo *et al.,* 2004). Plasmid pRB20-Lz1 (Barcena and Blasco, 1998) is a derivative of pRB21 (Blasco and Moss, 1995) containing a functional F13L, flanking sequences and the LacZ gene downstream of a synthetic vaccinia early/late promoter. Oligonucleotides were synthesized by Amersham Pharmacia Biotech. Virus manipulations were carried out using standard protocols (Earl and Moss, 1991).

Isolation of recombinant viruses

To introduce mutations in the –3 and +4 positions with respect to the β-Galactosidase (β-Gal) initiator ATG, two overlapping oligonucleotides, β-Gal D and β-Gal I (see sequence below), with a mixture of four nucleotides in the respective positions, were synthesized. Two overlapping PCR products were then generated using pRB20-Lz1 as the template. Left PCR fragment was obtained with oligonucleotides HF1510 (5'-GGACAT GCTTATGTACGTAGAAGAAAA-3') and β-Gal I (5'-GAT***N***CATCT***N***GAGTTATTTATAT-3'), being ***N*** ambiguous positions, and contained vaccinia recombination flank F13L gene, synthetic early/late promoter and the sequence mutated around the initiator ATG. The right PCR fragment was obtained with oligonucleotides HF 3400 (5'-CGTTCT AAAGCTAGTGCTA TATCTCCC-3') and β-Gal D (5'-ACTC***N***AGATG***N***AT CCCGTCGTT-3') and contained the synthetic early/late promoter, LacZ gene with the mutated sequence around the initiator ATG and vaccinia recombination flank.

To generate recombinant viruses, CV-1 cells were infected with vaccinia virus F13L deletion mutant (vRB12) and subsequently transfected with the above described PCR fragments. First, 1×10^6 CV-1 cells were infected with vaccinia F13L deletion mutant vRB12 (Blasco and Moss, 1992) in fresh EMEM medium containing 2% fetal calf serum, at a multiplicity of infection (MOI) of 0.05 pfu $cell^{-1}$. One hour post-infection (hpi), cells were transfected with 1 μg of the PCR products using 25 μL of Lipotaxi transfection reagent (Roche) following the manufacturer's instructions. At 3 days post-infection (dpi), cells were scrapped, freeze-thawed three times and sonicated. Serial dilutions of progeny virus were used to infect BSC-1 monolayers in 6-well plates that were maintained at 37°C for two days under an agarose overlay. In addition to the use of F13L as a selective marker (Blasco and Moss, 1995), β-Gal expressing, plaque-forming viruses were selected by virus plaquing under an agarose overlay containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, MBI Fermentas). After a 2-day incubation 100 μL of 40 mg mL^{-1} X-Gal in dimetil formamide (DMF), in 12 mL of semisolid medium was added and large blue plaques were selected.

Single-plaque virus isolates were expanded, and their sequence around the β-Gal ATG was determined. With this aim, a fragment containing the region of the promoter-ATG was amplified from each virus isolate by PCR using primers 5'-TTCGGCAAATTTCGCCG GAACCCATTA-3' and 5'-GCGATTAAGTTGGGTA ACGCC-3' and then sequenced. Single representatives of the sixteen possibilities of nucleotides in the –3 and +4 positions were finally selected and amplified.

β-Galactosidase assay

BSC-1 cells grown in 6-well plates were infected at a MOI of 3 pfu $cell^{-1}$. At 24 hpi cells were collected, pelleted and incubated for 10 min in 400 μL of chilled lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% NP40 and 1 mM PMSF) on ice. β-Gal activity present in cell lysates was measured using o-nitrophenyl β-D-galacto-pyranoside (ONPG) (Sigma) as substrate. The reaction was started by mixing 10 μL of cell lysate, 90 μL of H_2O and 100 μL of 0.6 mg mL^{-1} ONPG dissolved in Z buffer (60 mM Na_2HPO_4-$7H_2O$, 40 mM NaH_2PO_4-H_2O, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol). Absorbance at 414 nm was recorded at 5 min intervals until values reached saturation. β-Gal activity was quantified by comparison with known concentrations of commercial β-Gal (Sigma).

Northern blotting

BSC-1 cells grown in P100 plates were infected with virus recombinants at a MOI of 3 pfu $cell^{-1}$. At 24 hpi the cells were scraped from the plastic surface, pelleted and resuspended in 600 μL of RLT buffer (Qiagen) containing 1% β-mercapethanol. Total RNA was extracted using the «Rneasy Mini Kit» (Qiagen) according to the manufacturer's instructions. RNA was resuspended in DEPC water and the concentration was estimated by measuring the absorbance at 260 nm.

For electrophoresis of RNA, 15 μg of RNA were denatured in a buffer that contained 50% deionized formamide, 6.5% formaldehyde, 20 mM MOPS [3-(N-morpholino) propanesulfonic acid] pH 7, 8 mM sodium acetate, 1 mM EDTA for 15 min at 65°C, and cooled immediately on ice. After adding 2 μL of loading buffer (1% bromophenol blue, 10% glycerol), the samples were loaded in 1.2% agarose low EEO (Pronadisa) gel containing 6% formaldehyde in MOPS buffer (20 mM MOPS pH 7, 8 mM sodium acetate, 1 mM EDTA). Electrophoresis was performed at 3-4 V cm^{-1} until the bromophenol blue had migrated two-thirds of the length of the gel. RNA was then transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech) following the method of Sambrook *et al.* (1989).

The probes were labeled with DIG-dUTP with the PCR DIG Labelling Mix (Roche) by the incorporation of digoxigenin-11-dUTP in the polymerase chain reaction. The probe was purified with «QIAquick PCR Purification Kit» (Qiagen) and a dot blot method was used to verify DIG-dUTP incorporation.

Prehybridization of the filters was carried out in 50% deionized formamide, 6X SSC (0.15 M NaCl, 15 mM Na citrate), 5X Denhardt's solution (1% BSA, 1% ficoll 400, 1% polyvinylpyrrolidone), 0.1% SDS and 100 μg mL^{-1} denatured salmon sperm DNA. Hybridizations were performed in the same solution at 42-45°C. The labelled probes were heated for 5 min at 100°C before addition to the filters in the prehybridization solution. After hybridization, the membranes were washed at low stringency in 2X SSC, 0.1% SDS RT twice for 15 min and at high stringency in 0.5X SSC, 0.1% SDS at 68°C twice for 15 min. Hybridized probe was detected using the digoxigenin system (Roche) following the manufacturer's instructions.

Quantitative PCR

Quantitative RT-PCR was performed on RNA samples extracted by using the ABITaqMan One-Step RT-PCR (Applied Biosystems) per manufacturer's instructions. Primers 5'-CGCTCATCGATAATTTCACCG-3' and 5'-GGCTGAAGTTCAGATGTGCG -3', which amplified a 122-nt segment of β-Gal gene were used at 5 μM. Internal probe 5'-GGCAGGGTGAAACGCAGG-3' labeled with the fluorophore FAM (5'-6'FAM/ 3'-TAMRA) was synthesized by Applied Biosystems and was used at 25 μM. The reaction was carried out in an ABI Prism 7700 Sequence Detection System thermal cycler. RT-PCR conditions were: RT at 48°C-30 min, Hot Start at 95°C-10 min, followed by 50 cycles of 95°C-15 s, and 60°C-1 min.

Results

Analysis of the ATG context in vaccinia virus genes

Vaccinia virus (strain WR) genomic sequence (GenBank accession number AY243312) contains 218 annotated open reading frames (ORFs). The sequences

flanking the initiator ATG codon in these sequences were analyzed. For this analysis, 99 ORFs containing the TAAAT sequence, characteristic of vaccinia late promoters, in the 30 nt upstream of the start codon were classified as late. The remaining 115 ORFs were classified as early. In many late vaccinia genes, the TAAAT element overlaps the ATG codon. Therefore, to prevent bias in nucleotide occurrence due to the presence of the TAAAT element, 58 ORFs containing the sequence TAAATG (being the underlined ATG the start codon) were excluded from the analysis.

Twelve nucleotide positions flanking the start codons at the beginning of each ORF were analyzed. The distribution of nucleotides in these positions (Fig. 1) revealed that A was present in the –3 position in 101 out of 160 (63%) ORFs analyzed. Similarly, G was the most abundant nucleotide in the +4 position (65 out of 160 ORFs, 41%). In addition, a high frequency of A was noted in position +5 (76 out of 160 ORFs, 47%). Preference of C in position –1 was also found, but exclusively in early promoters (37 out of 119 promoters, 32%). Therefore, there is an overall agreement to the Kozak consensus sequence (A/G)CC**ATG**G. In particular, key positions –3 and +4 conform to the Kozak rules both in early and late vaccinia ORFs.

Isolation of recombinant vaccinia viruses

To study the influence of positions –3 and +4 with respect to the translation start codon in the context of a vaccinia virus-infected cell, a collection of recombinant vaccinia viruses overexpressing β-Gal was isolated. Sixteen different recombinant viruses containing all the nucleotide combinations in positions –3 and +4 with respect to the initiation AUG codon of β-Gal gene were isolated. Mutagenic PCR was used to introduce the mutations, and subsequently a selection system based on plaque formation, and β-Gal staining was used to isolate the different recombinant viruses (Fig. 2). Viruses were termed VV-β-Gal NN according to the nucleotides present in the –3 and +4 positions. Thus, VV-β-Gal GC would be the version containing the sequence GAGATGC around the start codon of β-Gal gene (see Table 1).

Effect of mutations on β-Galactosidase mRNA levels

We first investigated if the mutated positions had significant effects on the accumulation of LacZ transcripts. With this aim, a northern blot analysis of total RNA isolated from BSC-1 cells infected for 24 hours with the different vaccinia virus recombinants was carried out (Fig. 3). It is known that most late vaccinia transcripts are heterogeneous in length, due to the lack of a defined 3' end (Cooper *et al.*, 1981; Mahr and Roberts, 1984). Thus, not surprisingly LacZ transcripts in late RNA gave a smear in gels, revealing heterogeneity in length. Similar amounts of LacZ mRNA were detected for the different virus mutants, indicating that the mutations introduced did not affect significantly the RNA levels. This conclusion was confirmed by measuring the

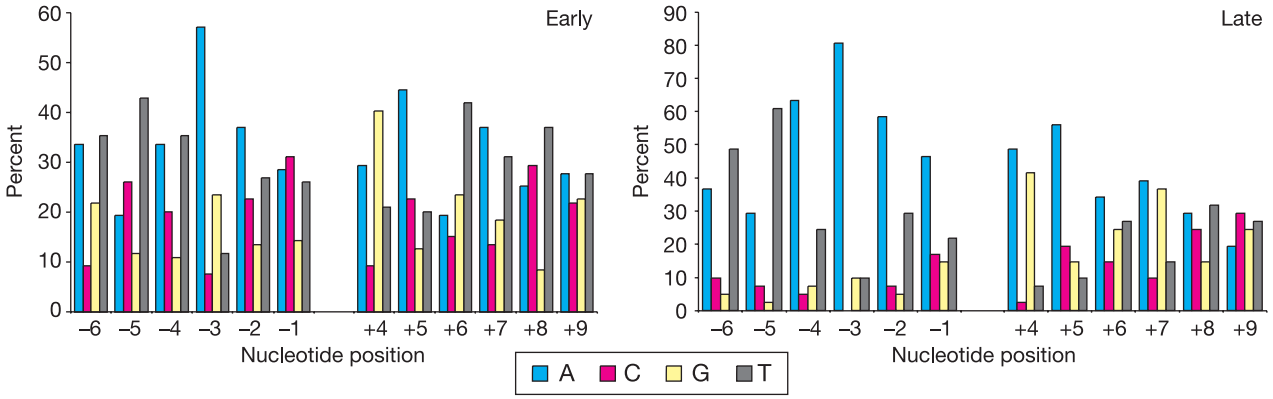

Figure 1. Analysis of AUG context in vaccinia virus ORFs (Acc. No. AY243312). Vaccinia virus (strain WR) ORFs were classified according to the absence or presence of the TAAAT element into early and late, respectively. Histograms show the distribution of nucleotides in the six positions upstream (–6 to –1) or downstream (+4 to +9) of the initiation ATG codon. Left panel shows the nucleotide distribution around the ATG of 119 ORFs classified as early. Rigth panel was derived from the sequence of late genes, excluding those containing the sequence TAAATG at the precise beginning.

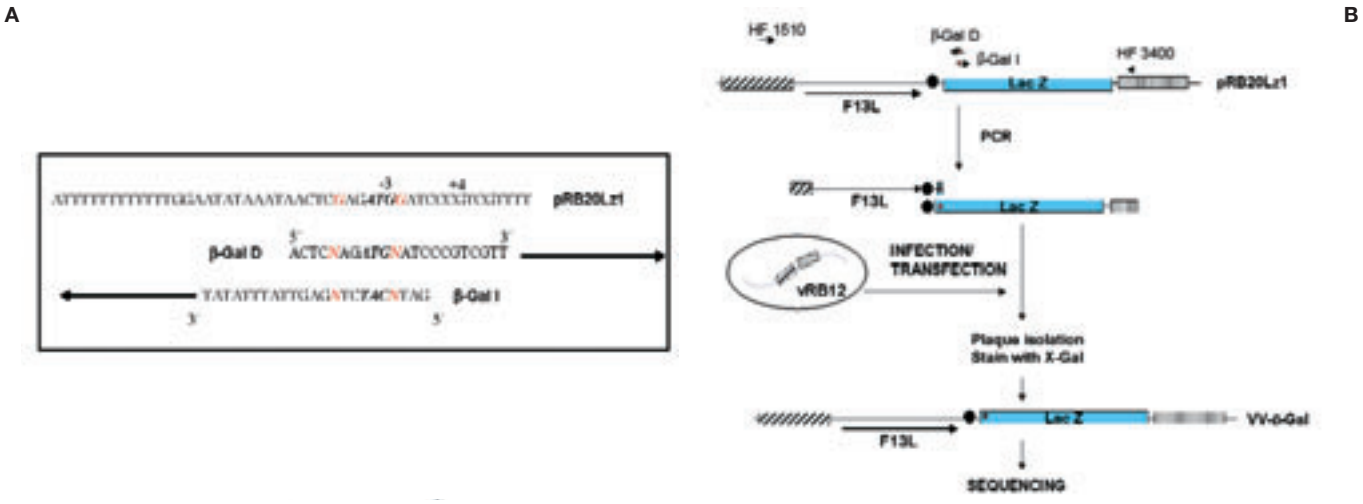

Figure 2. Isolation of recombinant viruses with mutations in the ATG context. A) Sequence of oligonucleotides used for mutagenesis around the initiator ATG. To introduce all the possible mutations in the positions –3 and +4 around the β-Gal ATG, two overlapping oligonucleotides were designed, that included a mixture of the four nucleotides in the positions subject to study. These oligonucleotides were used to generate two overlapping PCR products that would carry the sixteen possibilities of nucleotides in these positions. B) Isolation of recombinant virus with point mutations around the ATG initiator. The products carrying the mutations were amplified by PCR and transfected in BSC-1 cells infected with vaccinia virus vRB12, a virus mutant deficient in F13L. Virus plaques were selected using the F13L selection system and β-Gal staining. Finally, plaque-purified virus clones were expanded, and the different versions were selected by sequencing. Black circle indicates the position of the promoter and sequence preceding β-Gal initiator ATG. Hatched boxes represent the left and right flanks for recombination into the viral genome.

amount of LacZ mRNA by quantitative RT-PCR (Fig. 3B). Slight differences (±25%) were noted when measured levels for the mutants were compared to that of the control VV-Lz2 virus. Therefore, the mutations –3 and +4 with respect to the AUG did not seem to affect significantly mRNA levels.

Expression of β-Gal by recombinant viruses

We next determined β-Gal levels in extracts prepared from cells infected with the different viruses at 24 hpi. Figure 4 shows the β-Gal activity relative to that of control VV-Lz2 virus from four independent experiments.

Table 1. Collection of recombinant virus. The ATG context of LacZ gene is shown. The point mutations around the ATG initiator (underlined) are marked in red

	–3	+4	
ATTTTTTTTTTTTGGAATATAAATAACTCGAGATGGATCCCGTCGTT			VV–Lz2
ATTTTTTTTTTTTGGAATATAAATAACTCAAGATGAATCCCGTCGTT			VV AA
ATTTTTTTTTTTTGGAATATAAATAACTCAAGATGCATCCCGTCGTT			VV AC
ATTTTTTTTTTTTGGAATATAAATAACTCAAGATGGATCCCGTCGTT			VV AG
ATTTTTTTTTTTTGGAATATAAATAACTCAAGATGTATCCCGTCGTT			VV AT
ATTTTTTTTTTTTGGAATATAAATAACTCCAGATGAATCCCGTCGTT			VV CA
ATTTTTTTTTTTTGGAATATAAATAACTCCAGATGCATCCCGTCGTT			VV CC
ATTTTTTTTTTTTGGAATATAAATAACTCCAGATGGATCCCGTCGTT			VV CG
ATTTTTTTTTTTTGGAATATAAATAACTCCAGATGTATCCCGTCGTT			VV CT
ATTTTTTTTTTTTGGAATATAAATAACTCGAGATGAATCCCGTCGTT			VV GA
ATTTTTTTTTTTTGGAATATAAATAACTCGAGATGCATCCCGTCGTT			VV GC
ATTTTTTTTTTTTGGAATATAAATAACTCGAGATGGATCCCGTCGTT			VV GG
ATTTTTTTTTTTTGGAATATAAATAACTCGAGATGTATCCCGTCGTT			VV GT
ATTTTTTTTTTTTGGAATATAAATAACTCTAGATGAATCCCGTCGTT			VV TA
ATTTTTTTTTTTTGGAATATAAATAACTCTAGATGCATCCCGTCGTT			VV TC
ATTTTTTTTTTTTGGAATATAAATAACTCTAGATGGATCCCGTCGTT			VV TG
ATTTTTTTTTTTTGGAATATAAATAACTCTAGATGTATCCCGTCGTT			VV TT
NEGATIVE CONTROL – WR			

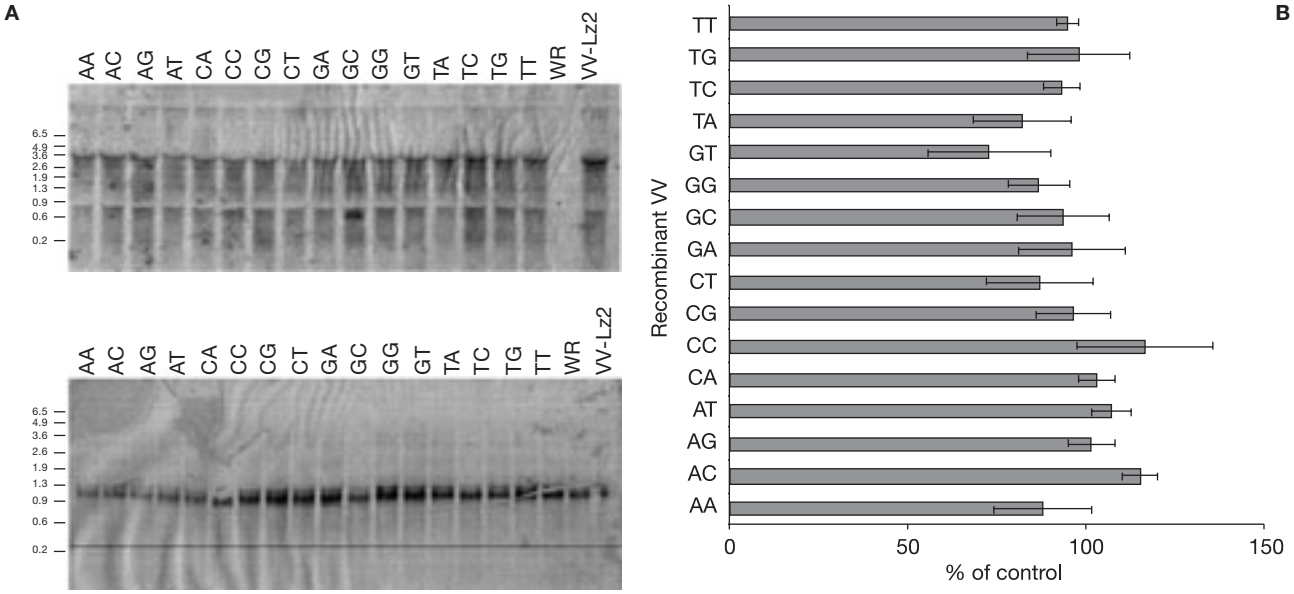

Figure 3. Quantitation of LacZ mRNA. A) Northern blot. BHK-21 cells grown in T-75 flask were infected with the virus collection at a MOI of 3 pfu cell^{-1}. At 24 hpi, cells were scraped from the plastic, pelleted and the total RNA was extracted. RNA was separated by electrophoresis and transferred to a nylon membrane. The hybridization was performed with LacZ (top) or F17L (bottom) specific probes labeled with digoxigenin. B) Quantitative RT-PCR. BSC-1 cells were infected at a MOI of 5 pfu cell^{-1} with the virus collection, WR or VV-Lz2. At 24 hpi the cells were collected and the RNA was extracted with the Rneasy Mini Kit (Quiagen). A quantitative RT-PCR reaction was performed with the TaqMan One-Step RT-PCR Kit. Data are presented as the percentage of LacZ expression with respect to the positive control VV-Lz2, and are the mean results from three independent experiments.

Small differences were noted between the different clones, but these were within 30% of the average values. Significantly, the values obtained with clone VV-β-Gal GG were 40% lower than those of VV-Lz2 virus, that contains the same sequence around the initiator AUG in β-Gal gene. Therefore, we consider that most, if not all, the differences detected in β-Gal activity between individual clones is within the experimental error.

Since the β-Gal protein product is identical in all of our constructs (except for the first amino acid, which is not present in the wild-type enzyme), we assume that the specific activity and stability of β-Gal is identical in all cases. With this assumption, the amount of β-Gal relative to the amount of LacZ mRNA levels is a direct indication of the translation efficiency of the respective mRNAs.

Discussion

Translation initiation of most eukaryotic mRNAs takes place after a preinitiation complex binds to the 5' cap, scans the mRNA in the 5' to 3' direction, and finds a particular AUG codon as the initiation site. Selection of the correct initiation codon is influenced, at least partially, by its flanking residues; in mammalian cells, the core of the «Kozak» consensus is (G/A)CCAUGG, where the A residue of the AUG is designated position +1. The purine residue in –3 is considered the most important flanking residue, followed by G in +4 (Kozak, 1987, 2002). In this study we have examined the effects of mutations in those two key positions flanking a vaccinia virus translation initiation ATG codon. For this purpose, recombinant vaccinia viruses containing all possible nucleotide substitutions in positions –3 and +4 were constructed. β-Gal gene was chosen as a reporter gene to facilitate quantitation of gene expression levels. Because of the experimental design used, the results refer exclusively to vaccinia late mRNAs, since although a combined early/late promoter was used to direct β-Gal expression, only a late time point (i.e., 24 hpi) was used in the experiments.

We have found that, in our experimental system, neither position significantly affected the levels of β-Gal enzymatic activity present in infected cells. To rule out possible transcriptional effects of the mutations, mRNA levels were quantitated and shown to be similar for all the virus recombinants used in the study. Therefore, we have to conclude that in our recombinant viruses overexpressing β-Gal, there is no major effect of the nucleotides in positions –3 and +4 in the translation efficiency of vaccinia mRNAs. However, analysis of

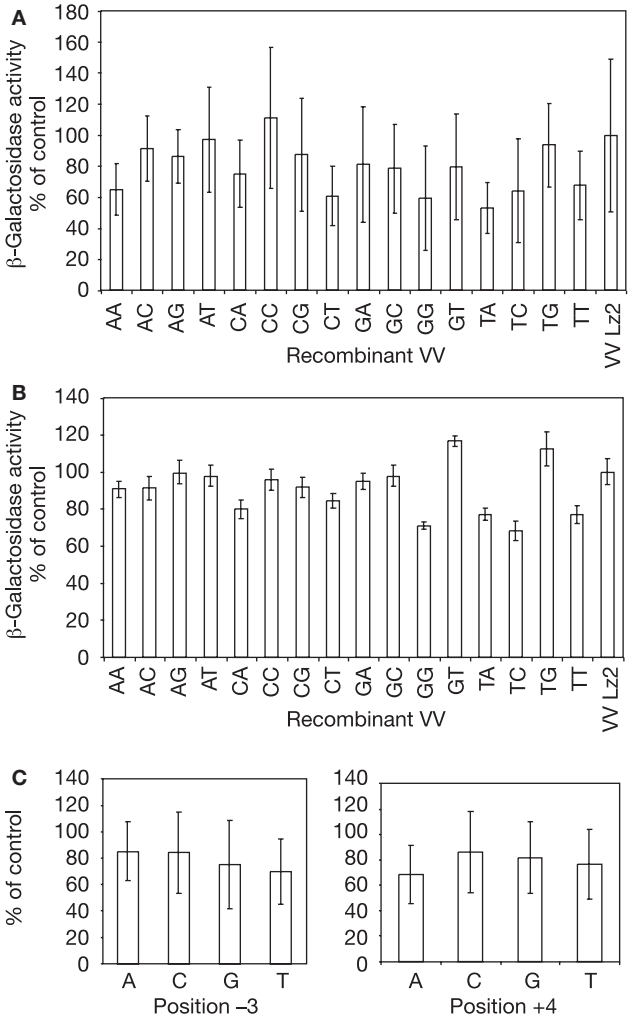

Figure 4. β-Galactosidase activity. A) BSC-1 cells were infected with the virus collection or positive control VVLz2 at a MOI of 5 pfu cell^{-1}. Cells were lysed at 24 hpi and the β-Gal activity was determined using ONPG as substrate. β-Gal levels relative to those of the VVLz2 are represented as the average ± SD from four independent experiments. B) Translation efficiency of β-Gal mRNA. β-Gal activity levels were corrected with respect to the levels of β-Gal mRNA of each one of the recombinant clones. The values are represented as the percent of those obtained with VV-Lz2. C) Average levels obtained from all the recombinants regarding nucleotide positions –3 (left) or +4 (right). The values were obtained by averaging the values from all the recombinants harboring the nucleotide specified.

the ORFs in the genomic sequence of vaccinia showed a preference for A in position –3 and G or A in position +4, suggesting that those positions conform to the Kozak consensus. One possible explanation for our inability to show differences in expression due to mutations in these positions is that the promoter used to express the marker gene β-Gal is extremely strong (Blasco and Moss, 1995; Chakrabarti *et al.*, 1997). This promoter was derived from a mutagenesis study of strong natural promoters to maximize promoter strenght (Davison and Moss, 1989a, b). As a consequence, saturation of the translation machinery with a specific mRNA might have precluded the detection of differences in mRNA translation.

An alternative explanation is that, in contrast to uninfected cells, the AUG context in vaccinia virus late genes does not affect translation efficiency. To date, there is little information about how vaccinia infection modifies the cellular translation machinery. It has been long known that vaccinia virus infection produces a dramatic inhibition on the synthesis of cellular proteins. This «shut-off» of cellular protein synthesis may in part result from the rapid turnover of mRNAs during infection, leading to enrichment of viral mRNAs. In fact, several hours after infection, viral transcripts account for most of the mRNA in the cytoplasm of the infected cell (Boone and Moss, 1978; Cooper and Moss, 1979). In addition, direct effects on translation have been assigned to virion proteins (Mbuy *et al.*, 1982; Person-Fernández and Beaud, 1986; Beaud *et al.*, 1994), early proteins (Beaud and Dru, 1980), or RNAs (Rosemond-Hornbeak and Moss, 1975; Gershowitz and Moss, 1979). Vaccinia late mRNAs are similar to cellular mRNAs in several ways; they are capped and polyadenilated. However, they contain unusual features like the presence of a poly-A sequence in the 5'end of the mRNA (Patel and Pickup, 1987; Ahn and Moss, 1989; Ink and Pickup, 1990). How the unique 5' structure feature of vaccinia late transcripts relate to the lack of relevance of the –3 and +4 positions is not known.

Vaccinia late promoters contain the motif TAAAT (Hanggi *et al.*, 1986; Davison and Moss, 1989b) which in most cases is closely placed upstream of the initiation codon. Many highly expressed late genes contain the sequence TAAATG, where the TAAAT motif overlaps the translation initiation codon. Of note, in this sequence the –3 position (T) does not conform to the canonical Kozak sequence, which reinforces the idea that vaccinia highly expressed genes may not, in some instances, follow the «–3» Kozak rule.

Our results indicate an unambiguous compliance of key nucleotide positions in vaccinia genes with the Kozak rules. However, these positions not always lead to significant differences in gene expression. Further studies will be necessary to test the hypothesis that viral proteins and/or RNAs functionally modify the cellular translation machinery.

Acknowledgments

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