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Regulation of varicella zoster virus gene 27 translation in vitro by upstream sequences

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Summary

Northern blot analysis revealed the presence of varicella-zoster virus (VZV) gene 27 transcripts in infected cells. The SalI-G DNA fragment, located in the unique long segment of the VZV genome and containing overlapping genes 26 and 27, was analyzed in an in vitro transcription-translation system. Translation of RNA transcribed from these open reading frames showed prominent expression of gene 27. Four different subclones were constructed to contain gene 27 with and without 100 base pairs (bp) of upstream sequences. Translation of RNA from these constructs using wheat germ extract or rabbit reticulocyte lysate indicated that the sequences upstream from the predicted initiation codon (AUG) of gene 27 downregulated the expression of this gene at the level of translation and that the predicted AUG within gene 27 was preferentially used.

Varicella-zoster virus; In vitro translation; Gene regulation

Introduction

Varicella-zoster virus (VZV) is a member of the alphaherpes virus family (Mathews, 1982 and Roizman et al., 1981). The VZV genome contains a linear double-stranded DNA molecule of 124–126 kilobase pairs (kbp). Although the

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entire genome has been sequenced and 68 coding regions have been identified (Davison and Scott 1986), there is little information concerning gene regulation either at the level of transcription or translation in VZV.

Initiation of translation in eukaryotic systems has been the subject of several studies in recent years (Cavener, 1987; Jobling and Gerke, 1987; Khalili et al., 1987; Kozak, 1986 and 1984). Open reading frames present in the leader sequences have been implicated in gene regulation (Hackett et al., 1986; Kaufman et al., 1987; Khalili et al., 1987). When two overlapping reading frames exist, in some systems, the downstream AUG codon appears to be the preferred site for initiation of translation (Budzilowicz and Weiss, 1987; Van Duin et al., 1986, Yokota et al., 1986). In these instances, the upstream AUG codon is in a less favorable context (Kozak, 1986) for initiation of translation. Based on Kozak's "scanning" model, a 40S ribosomal subunit binds initially at the 5'-end of a mRNA and migrates linearly until it reaches the first AUG codon. If the first AUG codon lies in an optimal context (CACCAUGG in higher eukaryotes), the 40 S subunit does not migrate further, but instead couples with a 60 S subunit and the initiation of protein synthesis takes place uniquely at that site. If the context is less favorable around the first AUG (especially A in the -3 position and G in the +4 position are important), some of the 40S subunits bypass that site and initiation occurs at the next site (Kozak 1986).

We have studied the expression of VZV gene 27 using an in vitro transcription-translation system. The results indicate that when two overlapping reading frames (genes 26 and 27) are present, the one downstream (gene 27) is preferentially translated and that sequences upstream from the predicted initiation codon in gene 27 downregulate its translation.

Materials and Methods

Cells and virus

VZV was propagated in African green monkey kidney cells (BSC-1) by co-cultivation of semiconfluent cultures with trypsinized infected cells as described (Gilden et al., 1978, 1982).

Cloning the VZV SalI-G fragment

The VZV SalI-G DNA fragment (5.6 kb) was cloned into the transcription vector pGem2 (Promega Biotec) at the SalI site as described (Vafai et al., 1986).

Construction of subclones carrying gene 27

Clone 1. The recombinant plasmid containing the SalI-G fragment was cleaved with EcoRI (located close to the SP6 promoter) and SstII. DNA fragments were separated by agarose gel electrophoresis and the largest DNA fragment containing gene 27 and the plasmid vector was electroeluted and blunt-ended using the large
fragment of DNA polymerase I. After addition of EcoRI linkers, DNA was purified by gel electrophoresis, religated and used to transform _E. coli_ strain HB101 (Maniatis et al., 1982). Clone 2. The recombinant plasmid from clone 1 was linearized with _NsiI_ (Fig. 2). The initiation codon (ATG) was removed by blunting the ends. The DNA was then religated and used to transform _E. coli_. Clone 3. The recombinant plasmid from clone 1 was cleaved with _HindIII_ (located close to the T7 promoter) and _HpaI_. The larger DNA fragment containing gene 27 and the plasmid vector was purified by gel electrophoresis and blunt-ended. After the addition of _HindIII_ linkers, DNA was repurified, religated and used to transform _E. coli_. Clone 4. The recombinant plasmid from clone 1 was cleaved with _NsiI_ and EcoRI. The largest DNA fragment containing gene 27 and the vector was purified by gel electrophoresis and blunt-ended. After the addition of _NcoI_ linkers (5'TCCATGGG3') to prevent any alterations in the gene 27 reading frame, the DNA was repurified, religated and used to transform _E. coli_. VZV sequences downstream from the _HpaI_ site were deleted as described in clone 3.

**Preparation of VZV RNA probes**

For Northern analysis, the DNA insert from the recombinant plasmid of clone 4 was removed using _HindIII_ and EcoRI. This DNA fragment was cloned into pGem3, linearized with EcoRI. RNA, complementary to the coding region for gene 27, was transcribed using SP6 RNA polymerase in the presence of [³²P]UTP as described (Vafai et al., 1986).

**RNA isolation and poly(A)⁺ selection**

RNA from uninfected and VZV-infected cells (4 × 10⁹) were extracted using the procedure described (Cathala et al., 1983) except that proteinase K (200 μg/ml) was included in the solubilization buffer. Poly (A)⁺ RNA was purified on oligo-d(T) cellulose (Bethesda Research Labs.) column according to the manufacturer's instructions.

**Gel electrophoresis and Northern hybridization**

Aliquots of RNA solution (10 μg) were denatured with dimethyl sulfoxide and glyoxal and electrophoresed through 1.2% agarose gel as described (McMaster and Carmichael, 1977; Maniatis et al., 1982). The gel was stained with 0.5 μg/ml of ethidium bromide for 30 min, destained in water for 30 min and irradiated on an ultraviolet transilluminator for 20 min. RNA was transferred onto 0.1 μm nitrocellulose filters as described (Thomas 1980). Filters were prehybridized for 4 h in a solution containing 50% formamide, 5 × Denhardt's solution, 5 × SSPE (1 × = 0.15 M NaCl, 0.01 M PO₄, pH 7.4, and 1 mM EDTA), 200 μg/ml denatured salmon sperm DNA, 100 μg/ml yeast tRNA, and 0.1% SDS. ³²P-labeled RNA probes (3 × 10⁹ cpm) were hybridized to RNA bound to nitrocellulose at 42°C for 20 h in
the prehybridization buffer. The filters were then washed twice with $2 \times SSC$ ($1 \times SSC = 0.15 \text{ M }\text{NaCl}, 0.015 \text{ M sodium citrate}$), 0.1% SDS at room temperature for 10 min each, twice with $0.1 \times SSC$, 0.1% SDS at room temperature for 10 min each and then once with $0.1 \times SSC$, 0.1% SDS at $55^\circ\text{C}$ for 30 min. Filters were dried, covered with plastic wrap and exposed to Kodak XRP film with intensifying screen.

In vitro transcription and translation

The recombinant plasmids were cleaved with HindIII downstream from the gene 27 and used as templates for RNA transcription. The transcription reactions were performed as described (Vafai et al., 1986) using SP6 RNA polymerase (Promega Biotec). After RNA transcription, the reaction mixture was digested with DNAse (Promega Biotec), extracted with chloroform, and the RNA was precipitated with ethanol. RNA was redissolved in sterile water ($3 \mu\text{g}/10 \mu\text{l}$) and used for in vitro translation reaction.

RNA ($3 \mu\text{g}$) was translated in vitro using either rabbit reticulocyte lysate (Promega Biotec) or wheat germ extract (New England Nuclear) according to the manufacturer's instructions in the presence of 50–60 $\mu\text{Ci}$ of $[^{35}\text{S}]$methionine (New England Nuclear) per reaction. The translation reaction was incubated for 30 min at $30^\circ\text{C}$. After translation, 1 $\mu\text{l}$ of the reaction mixture was spotted on a glass microfibre filter (Whatman 934-AH), boiled in 10% trichloroacetic acid (TCA) for 4 min, washed twice with water, once with 95% ethanol, once with acetone, dried and the radioactivity was counted. Aliquots of the translation products containing 50000 to 100000 cpm were resuspended in an equal volume of TNE buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 5 mM EDTA) and 1/3 volume of $3 \times$ sample buffer (150 mM Tris–HCl, pH 7.0, 6% SDS, 15% 2-mercaptoethanol, 0.03% bromophenol blue), heated in boiling water for 4 min, cooled on ice and analyzed by 8–12% SDS-PAGE as described (Laemmli, 1970). Gels were run at 50 V for 16 h, stained in 50% methanol–7% acetic acid–0.2% Coomassie brilliant blue and de-stained in 50% methanol–7% acetic acid. Gels were processed for fluorography (Enlightning; New England Nuclear), dried, and exposed to Kodak X-Omat AR film at $-70^\circ\text{C}$.

Results

Expression of VZV gene 27 in the VZV-infected monkey kidney cells

Northern-blot hybridization of poly (A)$^+$ RNA from VZV-infected cells with a $^{32}\text{P}$-labeled RNA probe complementary to the coding region of gene 27 revealed a 1.2–1.3 kb band corresponding to a mRNA for gene 27 indicating its transcription (Fig. 1).
In vitro expression of genes 26 and 27 located within the VZV SalI-G fragment

The VZV SalI-G fragment (5.6 kb), located in the unique long segment of the VZV genome, was cloned at the unique SalI site of an in vitro transcription vector.

Fig. 1. Northern blot hybridization analysis of transcripts from VZV-infected cells. Ten μg of glyoxalated poly (A)^+ RNA from uninfected and from VZV-infected cells were size-fractionated on 1.2% agarose gel at 100 V for 2 h. RNA was then transferred onto nitrocellulose and hybridized to a ^32^P-labeled RNA probe complementary to VZV gene 27. Lane 1, RNA from VZV-infected cells; lane 2, RNA from uninfected cells. λ DNA digested with HindIII and EcoRI and bacteriophage φ X174 DNA digested with TaqI were end-labeled using ^32^PATP and used as internal markers.
Fig. 2. Construction of the recombinant DNA carrying VZV SalI-G fragment and expression of this fragment in vitro. (A) The physical map of the VZV DNA which consists of two covalently linked segments, a unique long (U₁) and a unique short (Uₛ) segment bounded by inverted repeat sequences (IRₛ/TRₛ). (B) The SalI-G fragment (5.6 kb), located in U₁ segment of the VZV genome and carries genes 26 and 27. SalI-G contains three SsrII (designated as S) and two NsiI (designated as N) restriction sites. One of the SsrII sites is located 100 base pairs upstream from the initiation site (AUG) of gene 27 and one of the NsiI sites is located at the AUG of gene 27. An HpaI (designated as H) site is located 100 base pairs upstream from the termination codon for gene 27. (C) In vitro translation of transcripts from the SalI-G fragment. The recombinant plasmid carrying the SalI-G fragment in pGem2 was linearized using HindIII and transcribed in vitro using SP6 RNA polymerase. The transcripts were translated using rabbit reticulocyte system in the presence of [³⁵S]methionine as described in Materials and Methods. The translation products were analysed by a SDS–10% PAGE and autoradiographed (lane 2). As a control (lane 1), no RNA was included in the translation reaction. Lysozyme (14300), β-lactoglobulin (18400), α-chymotrypsinogen (25700), ovalbumin (43000), bovine serum albumin (68000), phosphorylase B (97400), and myosin (200000) were used as internal standards. (pGem2). When RNA was transcribed from one strand containing the overlapping genes 26 and 27 and translated using the rabbit reticulocyte system, a major protein band with an apparent molecular weight of 36000 was detected by SDS–PAGE (Fig. 2). In addition, longer exposure of the gel resulted in the detection of a protein band of 65000 (data not shown). These bands correspond to the size of polypeptides...
predicted by the DNA sequence of genes 27 and 26, respectively (Davison and Scott, 1986). The greater intensity of the 36000 band than of the 65000 suggests more efficient translation of gene 27 than gene 26 and a preference for initiation codon (AUG) of the open reading frame for gene 27. In addition to these 36000 and 65000 protein bands, other minor protein bands were also detected by SDS–PAGE (Fig. 2). These proteins could arise due to the translation of premature RNA molecules or due to random initiation at different initiation (AUG) codons. Similar protein patterns were observed when both capped or uncapped RNAs were translated in vitro (data not shown).

Expression of VZV gene 27 in vitro

Four different subclones of the SalI-G DNA fragment containing gene 27 were constructed as detailed in Materials and Methods. RNA transcribed from each subclone was translated in both the wheat germ and rabbit reticulocyte systems in the presence of [35S]methionine. Aliquots (1 µl) of each translation reaction were precipitated with trichloroacetic acid (TCA), counted and analyzed by SDS–PAGE (Fig. 3).

Our results indicated that (1) using clone 1 which contained the predicted translational initiation codon for gene 27 and clone 2, in which this codon had been deleted (see Fig. 3 legend) a reduced efficiency of translation was observed (46000 cpm to 17000 cpm in wheat germ and 73000 cpm to 23000 cpm in rabbit reticulocyte lysate) as shown in Fig. 3; (2) in experiments using clone 3 in which all the upstream sequences up to the initiation codon for gene 27 were retained and clone 4, in which these sequences were deleted, the translational efficiency was increased (46000 cpm to 58000 cpm in wheat germ and 80000 cpm to 300000 cpm in rabbit reticulocyte). These results suggested that sequences immediately upstream from gene 27 may be downregulating its expression.

Analysis by SDS–PAGE and autoradiography after translation (Fig. 3) showed that in the wheat germ translation system, the protein encoded by gene 27 was detected in clone 1, which contains the AUG initiation codon, but not in clone 2 which lacks the codon. In addition, deletion of upstream sequences resulted in enhanced expression of gene 27 (clones 3, 4). Furthermore, in translation reactions using the rabbit reticulocyte system, a protein with reduced molecular weight (34000) was detected in the absence of the predicted initiation codon (Fig. 3; clone 2) suggesting that the AUG codon downstream from the predicted initiation codon might have been used (see discussion). Again, deletion of sequences upstream from gene 27 resulted in enhanced expression of gene 27. Fig. 3 illustrates the autoradiograph carrying the 36000 protein band encoded by gene 27. The protein encoded by this gene was the major translation product detected by SDS–PAGE analysis.

To determine whether the enhanced expression of gene 27 was due to any specific sequences in the vector, this gene was transcribed under the control of another promoter (e.g., T7 promoter). The insert from the clone 4 recombinant, which lacks the sequences upstream from gene 27, was removed using HindIII and EcoRI restriction endonucleases. This DNA was then cloned into pGem3, linearized with
Fig. 3. Construction of subclones carrying gene 27 and their expression in vitro. Constructs 1 and 2 contain 100 bp of upstream sequences and 750 bp of the downstream sequences. In construct 2, the predicted translational initiation codon for gene 27 was deleted. Construct 3 contains 100 bp of upstream and 100 bp of downstream sequences. In construct 4, the sequences up to the initiation codon for gene 27 were deleted. Recombinant plasmids carrying these clones were linearized using HindIII downstream from gene 27 and transcribed using SP6 RNA polymerase. The transcripts were translated in vitro using either the rabbit reticulocyte or wheat germ system in the presence of $[^{35}S]$methionine. Aliquots (1 µl) of translational reaction mixtures were precipitated with trichloroacetic acid and counted. Incorporated cpm for each of the clones are given on either side. The translation products were separated on a SDS–8% PAGE and autoradiographed. As a control (−RNA), no RNA was used in the translation reaction. The numbers above the bands indicate the clones used for translation.

HindIII (downstream from gene 27), transcribed using T7 RNA polymerase and translated in vitro using the rabbit reticulocyte system. The radioactivity incorporated in the translation products was counted and the products were analyzed by SDS–PAGE (data not shown). These studies indicated that similar to the results using SP6 polymerase, the efficiency of translation was enhanced.
Discussion

In this study we analyzed the regulation of VZV gene expression in vitro at the level of translation. Using the VZV SalI-G DNA fragment cloned into an in vitro transcription vector, we have shown that the initiation codon AUG for the downstream gene 27 is used preferentially over that of gene 26, despite the fact that the putative initiation codon for gene 26 is in a relatively better context (GAGAUGG) than that of gene 27 (CGTAUGGC) for eventual protein synthesis.

Based on the Kozak's relaxed scanning mechanism (Kozak, 1981) the 40 S ribosomal subunit could slide by the 5'-proximal AUG (if it is in an unfavorable context) and start protein synthesis at a following initiation codon. VZV seems to elude this mechanism since the 5'-proximal AUG is in a better context. Also, our result is at variance with several reports indicating that when two overlapping open reading frames exist, the AUG which is in the better context is used (Kozak, 1986; Budzilowicz and Weiss, 1987; Van Duin et al., 1986; Yokota et al., 1986). One possible explanation for our observation could be the increased downregulation of translation of gene 26 by its upstream sequences relative to that of gene 27.

At present, the start site for the 1.2 kb transcript (Fig. 1) coding for VZV gene 27 is not known. Since all eukaryotic mRNA carry 5' untranslated regions, it can be assumed that a part or all of 100 bp sequences upstream from the translational start site (AUG) would be present in the mRNA. Our in vitro translation data obtained using a fragment of VZV gene 27 with 100 bp of the upstream sequences deleted (Fig. 3, clone 4) indicate that these upstream sequences may act to downregulate gene 27 expression. Downregulation of gene expression by upstream sequences has also been demonstrated in other eukaryotic systems (Geballe et al., 1986, Kaneda et al., 1987; Pelletier and Sonnenburg, 1985) suggesting that the downregulation may be a more widespread phenomenon than heretofore recognized.

Posttranscriptional regulation of gene expression has been studied in cytomegalovirus (Geballe et al., 1986). In these studies, by the addition of sequences within the 5' leader region of early (β) cytomegalovirus transcripts to a region upstream from an immediate early (α) gene, the expression of this (α) gene was converted to the early (β) class. These regulating sequences contained dyad symmetry that could promote the formation of hairpin structures. The authors speculated the presence of such hairpin structures to be a contributing factor in the regulation. In addition, Kaneda et al. (1987) showed that the human thymidylate synthase (TS) gene contains a triple tandem repeat of 30 nucleotides each immediately upstream from the TS AUG codon. Deletion of the first two tandem repeats and a portion of the third resulted in an increased translational efficiency of the TS gene, whereas deletion of only the first two tandem repeats did not affect translational efficiency. Therefore, these authors speculated that the formation of stem-loop structures within the upstream sequences could account for the translational efficiency of the TS gene. In fact, Pelletier and Sonnenburg (1985) demonstrated that the efficiency of translation decreased with the introduction of hairpin structures upstream from the initiator codon of the herpes simplex virus type 1 thymidine kinase gene. However, our analysis of sequences (100 bp) upstream from the initiator codon of
VZV gene 27 using the RNAFOLD program (M. Zuker, personal communication) did not reveal any unique structural features, suggesting that regulation of VZV gene 27 may involve different mechanism(s).

The preferential translation of gene 27 over gene 26 and downregulation by upstream sequences in gene 27, observed in our studies, were similar in both rabbit reticulocyte and wheat germ extract systems. It has been shown that in wheat germ extracts, the translation is initiated only at the authentic AUG codon (Kozak and Shatkin, 1977). However, in the rabbit reticulocyte system, ribosomes appear to initiate inappropriately at each successive AUG codon, thus generating an array of translation products (Parker et al., 1986). Our results indicated that in the absence of the predicted initiation codon of gene 27 (Davison and Scott, 1986), the 36K protein was not synthesized in wheat germ extract. However, a smaller size protein of 34K was detected in rabbit reticulocyte lysate, suggesting the usage of a downstream AUG codon in the latter translation system. The second AUG codon in gene 27 is 45 nucleotides downstream from the first one. That would predict the size of the truncated in vitro translation product to be approximately 34000. This was in agreement with our observation in Fig. 3, construct 2 using rabbit reticulocyte lysate.

Northern blot hybridization of poly(A)⁺ RNA from VZV-infected cells indicated the presence of a 1.2–1.3 kb mRNA for gene 27. Maguire and Hyman (1986) have also identified a 1.1 kb RNA species from this region of the VZV genome.

The highly cell-associated nature and low virus yields in tissue culture of VZV have hampered progress in studies involving VZV gene regulation. Our in vitro studies provide an opportunity to understand the regulation of VZV genes. This is the first time a VZV gene has been studied using a novel approach.

In summary, the results of our study show that in case of two overlapping reading frames in the VZV genome, the downstream initiation codon is preferentially used. Furthermore, sequences upstream from the AUG initiation codon in VZV gene 27 downregulate its expression at the level of translation. Experiments are underway to test the ability of these sequences to downregulate other genes. In addition, the existence of such downregulating sequences upstream from the AUG initiation codon of different VZV genes awaits further investigation.

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