mRNA Translation Regulation by the Gly-Ala Repeat of Epstein-Barr Virus Nuclear Antigen 1

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The glycine-alanine repeat (GAr) sequence of the Epstein-Barr virus-encoded EBNA-1 prevents presentation of antigenic peptides to major histocompatibility complex class I molecules. This has been attributed to its capacity to suppress mRNA translation in cis. However, the underlying mechanism of this function remains largely unknown. Here, we have further investigated the effect of the GAr as a regulator of mRNA translation. Introduction of silent mutations in each codon of a 30-amino-acid GAr sequence does not significantly affect the translation-inhibitory capacity, whereas minimal alterations in the amino acid composition have strong effects, which underscores the observation that the amino acid sequence and not the mRNA sequence mediates GAr-dependent translation suppression. The capacity of the GAr to repress translation is dose and position dependent and leads to a relative accumulation of preinitiation complexes on the mRNA. Taken together with the surprising observation that fusion of the 5′ untranslated region (UTR) of the c-myc mRNA to the 5′ UTR of GAr-carrying mRNAs specifically inactivates the effect of the GAr, these results indicate that the GAr targets components of the translation initiation process. We propose a model in which the nascent GAr peptide delays the assembly of the initiation complex on its own mRNA.

Epstein-Barr Virus (EBV) nuclear antigen 1 (EBNA-1) and latency-associated nuclear antigen 1 (LANA-1), from Kaposi’s sarcoma-associated herpesvirus (KSHV), are major latency proteins of these two gammaherpesviruses that are essential for maintaining viral episomes in infected cells (21, 22). Independent studies suggest that both proteins have evolved mechanisms to remain largely invisible to the immune system, which could otherwise eliminate latently infected cells (8, 9, 19, 25). These mechanisms act in cis and are mediated via an internal repeat region. In the case of EBNA-1 this region consists of an N-terminal glycine-alanine repeat (GAr), and for LANA-1 the region consists of a glutamine-glutamate-aspartate central repeat (QED-CR). Although the two domains do not share amino acid homology, both retard their own synthesis to reduce the production of defective ribosomal products that can be processed for the major histocompatibility complex (MHC) class I-restricted antigen presentation pathway (23, 24), highlighting the importance of translation control in regulating MHC class I-restricted antigen presentation. To compensate for their low rates of synthesis, both proteins also have slow turnover rates (4, 8).

Regulation of translation for most prokaryotic and eukaryotic mRNAs occurs at the level of initiation, but there are examples where regulation of protein synthesis depends on the elongation stage (17). The two main types of translation initiation are the classic cap-dependent and the less frequent cap-independent translation mechanisms (5, 7, 11, 14, 16). In the former, the preinitiation complex is formed around the cap structure in the 5′ untranslated region (UTR) of the message, whereas in the latter the 40S subunit is directed toward the mRNA via an internal ribosome entry site (IRES). The mechanism of GAr- and LANA-1-mediated control of translation seems different from other types of viral regulation in several aspects. The EBNA-1 GAr is 60 to 300 amino acids long, depending on virus isolate, and is positioned in the N-terminal part of the protein. The GAr message is GC rich but does not activate protein kinase R and eukaryotic initiation factor 2 phosphorylation (25). The fact that the GAr has to be encoded to suppress translation, coupled with the restricted use of GGG and GGA codons to express Gly and of GCA to express Ala in the GAr (GAT, GAG, and CAG for aspartic acid, glutamic acid, and glutamine, respectively, in the LANA sequence), could suggest that codon exhaustion might explain the effect of these repeats. However, manipulations of sequence order, orientation, and composition of the OED-CR and GAr domains and the observation that antibodies directed toward the GAr can stimulate translation in vitro instead favor a direct role for the amino acid sequence (8, 25).

Here, we have studied GAr-mediated regulation of translation in vitro and in vivo. The results presented suggest that, once synthesized, the nascent GAr peptide sequence prevents the assembly of the following upstream ribosomes. This knowledge should further understanding of how amino acid repeat sequences can affect mRNA translation in cis and should shed light on a novel type of viral control of mRNA translation and its implications in regulating MHC class I-restricted antigen presentation.
MATERIALS AND METHODS

Cell culture and transfection. Human carcinoma-derived cell lines H1299 and Saos-2 were cultivated under standard conditions in RPMI 1640 medium containing 10% fetal calf serum, 2 mM l-glutamine, 100 U/mg penicillin, and 100 µg/ml streptomycin. Cells were seeded in six-well plates at a density of 1.75 × 10^5 cells/well. The following day the cells were cotransfected with 1 µg of total expression plasmid along with 3 µl of Genejuice according to the manufacturer’s protocol (Merck Biosciences, Darmstadt, Germany).

Electrophoresis and Western blotting. Following separation by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, proteins were transferred to 0.45-µm-pore-size nitrocellulose membranes, and blots were blocked for 1 h at room temperature with 5% skim milk in Tris-buffered saline solution consisting of 20 mM Tris, 500 mM NaCl, and 0.1% Tween 20, pH 7.5. Blots were incubated overnight at 4°C with polyclonal antibodies against ovalbumin (Ova; Sigma-Aldrich) or polyclonal anti-GAR antibody (1:500) raised against the Gly-Ala sequence of EBNA-1 protein or the polyclonal anti-p53 CM-1. The membranes were washed before being incubated with horseradish peroxidase-conjugated rabbit anti-mouse or mouse anti-rabbit immunoglobulin antibody (1:5,000) for another 1 h and detected using enhanced chemiluminescence (Amersham Bioscience). The enhanced chemiluminescence signal was quantified using a charge-coupled-device camera and associated software (Vilber Lourimat, France). Prestained molecular markers were from Fermenta (Ontario, Canada).

Plasmid constructs. The Ova, Ova-GAR, and GAR-Ova constructs have been described elsewhere (25). All plasmids were generated using standard procedures and restriction enzymes (T, E.D.I.), and all plasmids were sequenced. Translation of the GAR sequence was verified in Saos-2 cells and c-terminal intestinal alkaline phosphatase were obtained from New England Biolabs (Ipswich, MA). Purified synthetic oligonucleotides were obtained from either MWG Biotech (Ibersberg, Germany) or Eurogentec. All Ova constructs carry a deletion corresponding to the first 50 amino acids of Ova. The c-myc IRES CDNA was provided by A. C. Prats (INSERM U589, France). The N-myc and hepatitis C virus IRESs and hairpin cDNAs were a kind gift from A. Willia (Nottingham, United Kingdom). The pcDNA3-Ova and pcDNA3-GAR constructs were obtained as described previously (25). c-myc-Ova and c-myc-Ova-GAR were generated by amplification of full-length human c-myc IRES by PCR, using the 5′ sense primer 5'-CGGATCCATGAAAGTCCGTATGGAATTCC-3′ and 3′ antisense primer 5'-TCTCGGGAGCGGGCTGGGTG-3′ and cloned into the 5′ UTR of pcDNA3-Ova and Ova-GAR BamHI sites. N-myc IRES was generated by PCR using the 5′ sense primer 5'-CGAGACTTCTGGTGTGACCCTCGGCTTGG-3′ and 3′ antisense primer 5′-CGAAAAAGTTTCTAGGACTGAGACC-3′. The fragment was inserted into the HindIII site in the 5′ UTR of pcDNA3-Ova and Ova-GAR, placing the IRES in its natural distance from the AUG initiation codon. Encephalomyocarditis virus (EMCV) IRES was generated by amplification of full-length IRES using primer pairs containing a BamHI site (5′-GGCGGATCCCTCCCTCTGGAGAAGTCT-3′ plus 3′ antisense primer 5′-GGCGGATCCGACGCCGTCTGGAT-3′). A 350-nucleotide hairpin structure was inserted in the 5′ UTR of c-myc IRES-GAR-Ova in order to create Hc-p-myc-IRES-FL-GAR-Ova (where HP is hairpin and FL is full-length). The GAR-Ova wild-type (WT) 31-amino acid (aa) construct was made by replacing the full-length GAR sequence in the FL-GAR-Ova construct with oligonucleotide sequences corresponding to 31 aa of the GAR. The same approach was used to produce Ova-32GAR with an insertion of three alanines (Ova-32GAR 3A) and Ova-31GAR with serine residues in the N and C termini (Ova-31GAR 2S), with a serine in the N terminus (Ova-31GAR 1S-N), and with a serine in the C terminus (Ova-31GAR 1S-C). For the GAR-p53 WT construct, the Ova sequence was replaced by the full-length p53. 31GAR-p53 was made by replacing the third nucleotide in each codon with thymidine. An 80-aa-long sequence was replaced by the full-length p53. 31GAR-p53 was made by PCR (qRT-PCR). Transfected cells were cultured for 36 h before treatment with 20 µM MG132 for 1 h in methionine-free medium containing 2% dialysed fetal calf serum. For quantification of the expression of the EBNA-1 gene and of the EBNA-1 gene lacking the FL.GAR sequence (EBAGAR), membranes were exposed to a storage phosphor screen (Fuji) for a few hours and analyzed on a phosphor-imager.

Sucrose gradient analysis of in vitro translation reactions. The translation reactions were incubated as described in the text and quenched with 910 µl of chilled gradient buffer (30 mM MgCl₂, 0.1 mM of cyclomexihidime, 1 mM dithiothreitol). Translation reactions from rabbit reticulocyte lysate (RRL) were analyzed on 15% to 45% sucrose gradients in a gradient buffer (15 mM Tris, pH 7.5, 0.3 M KCl, 15 mM MgCl₂, 1 mM dithiothreitol in the presence of 0.1 mg/ml of cyclomexihidime). Ultracentrifugation was performed using an SW41 rotor at 35,000 rpm for 90 min at 4°C. Gradients were analyzed using a Beckman L8-70M Ultracentrifuge equipped with a UV monitoring detector. RNA concentrations were determined by UV spectrometric measurement, and molar equivalents of each RNA were used for uncoupled in vitro translation in RRL (Promega). Reactions were stopped after 30 min by addition of an equal volume of 10 g/ml RNase A and incubation for 15 min at room temperature. Reaction products were resolved on NuPAGE XCell SureLock Gel (Invitrogen). Denaturing quantification of translation products was done using multiple exposures of each gel to ensure that the linear response range of the film was respected and that low levels of translation could be accurately quantified.

In vitro transduction/transfection. pOva, pOva-GAR, and Ova-GAR plasmids were linearized with XbaI enzyme prior to in vitro transcription reactions. To generate DNA templates, hemagglutinin (HA) tagged 43GAR-Ova (HA-43GAR- Ova) and HA-Ova PCR products were performed with T7 promoter and poly(T)50 oligonucleotides. DNA templates for in vitro transcribed T7 RNA polymerase and nMESSAGE mMESSAGE mMessage Machine high-yield coupled RNA transcription kit (Ambion). RNA concentrations were determined by UV spectrometric measurement, and molar equivalents of each RNA were used for uncoupled in vitro translation in RRL (Promega). Reactions were stopped after 30 min by addition of an equal volume of 10 g/ml RNase A and incubation for 15 min at room temperature. Product reactions were resolved on NuPAGE XCell SureLock Gel (Invitrogen). Denaturing quantification of translation products was done using multiple exposures of each gel to ensure that the linear response range of the film was respected and that low levels of translation could be accurately quantified.

RESULTS

The effect of the GAR is position and dose dependent. We performed a detailed study of GAR-dependent translocation in vitro according to mRNA concentration and GAR position within a reporter message. We used constructs where a 235-aa GAR (FL.GAR) derived from the EBNA-1 is fused to the C or the N terminus of the chicken Ova reporter sequence that...
lacks the N-terminal 50-aa signal sequence (Ova-FL.GAr and FL.GAr-Ova, respectively). These mRNA constructs were synthesized in vitro and are capped, lack poly(A) tails, and have identical flanking UTRs. In vitro translation experiments using RRL were performed for the two FL.GAr-carrying transcripts and compared with the control Ova mRNA translation profile. Three series of equimolar amounts of mRNAs were tested, and we observed that the pattern of translation is dose dependent and different for the three mRNAs (Fig. 1A). Ova mRNA translational efficiency is linearly proportional to the mRNA concentration, with the highest concentration of 300 nM providing 100% translation efficiency. FL.GAr suppression of translation is stronger when GAr is positioned in the N rather than the C terminus of Ova, which is in line with what has been shown previously (25). Interestingly, for both the Ova-FL.GAr and FL.GAr-Ova mRNAs, maximum translation efficacy is achieved between 0.4 to 3.7 nM mRNA. Higher mRNA concentrations completely block FL.GAr-Ova translation but provide around 50% of translation efficacy for Ova-FL.GAr mRNA (Fig. 1A).

In order to ensure that the position-dependent effect of the GAr also occurs with other reporter genes and in vivo, the FL.GAr was fused to the N or C terminus of p53. p53-negative H1299 cells were transfected with the indicated constructs (see Fig. 2B), and qRT-PCR and Northern blot analysis were routinely performed to ensure that the GAr sequence did not affect the levels of the mRNAs to which it was fused (data not shown). A 20-min pulse-label experiment was performed to determine the rate of synthesis in the presence of proteasome inhibitor MG132 to minimize any influence caused by protein turnover. Figure 1B shows that the effect of the GAr on p53 synthesis is also greater when the sequence is localized in the N terminus than when it is in the C terminus.

Short version of GAr sequence retards translation in cis in vivo and in vitro. To test to what extent the length of the GAr affects its translation-inhibitory effect, we created shorter GAr variants. We fused a 43-aa-long GAr to the N terminus of the Ova sequence and after the human influenza HA sequence (HA-43GAr-Ova) (Fig. 2A). In this way the GAr is positioned at aa 14 to 57 in the fused protein and several amino acids in front of the AUG codon, which corresponds more to its natural position in the N-terminal part of EBNA-1 protein (from aa 90 to 238 in the human herpesvirus 4; NC_007605) than when it is positioned directly in the N terminus. The HA sequence was also directly fused to the N terminus of Ova (HA-Ova). This ensures the same nucleotide sequences within the ribosome binding site for HA-43GAr-Ova and for the HA-Ova constructs; thus, any observed differences cannot be attributed to alternative mRNA secondary structures within the region of translation initiation. The constructions built resulted in “classical,” capped and polyadenylated mRNAs with the AUG...
codons surrounded by Kozak-like sequences favorable for translation.

Plasmids containing HA-Ova control and HA-43GAr-Ova were transfected in H1299 cells. At 48 h posttransfection cells were labeled with 0.3 mCi of [35S]methionine for the indicated time (Fig. 2B) in the presence of the proteasome inhibitor MG132. We found that, indeed, HA-43GAr-Ova is translated less efficiently in vivo than the control mRNA (Fig. 2B). When three GAr constructs of different lengths were compared in the same in vivo pulse-labeling assay, it was revealed that the 43GAr construct is almost as effective in retarding its own translation as the FL.GAr under these conditions (Fig. 2C).

When we studied the translational kinetics for HA-Ova and HA-43GAr-Ova mRNAs using RRL, we observed that the first 30 min of the two translations differed (Fig. 2D). In addition to the reduced amount of protein synthesized from the HA-43GAr-Ova message, there is also a delay in translation, and there is no detectable amount of 43GAr-fusion protein before 15 min. These differences cannot be attributed to mRNA degradation, since both mRNAs have identical 5′ and 3′ UTRs, or to differences in protein degradation (25; data not shown). Thus, blocking translation takes place from the onset of synthesis, an observation that does not support the notion that the GAr would first need to reach a certain level of expression before suppressing translation. This supports the idea that the GAr acts in cis (25). If, on the other hand, GAr was synthesized well at the onset and translation was suppressed when GAr levels started to accumulate, then GAr could inhibit synthesis in trans.

The GAr changes a classical ribosomal profile by translation-dependent accumulation of a GAr-dependent peak. To better understand the mechanism of GAr-mediated retardation of translation, the assembly of FL.GAr-Ova, Ova-FL.GAr, and a control Ova mRNA into distinct translation complexes in in vitro translation assays performed in RRL was monitored by sucrose density gradients. Capped mRNAs were incubated for 15 min at 30°C, and translation reactions were quenched with chilled stop buffer containing 30 mM MgCl2 and loaded onto a 15% to 45% sucrose gradient. The gradients were fractionated after passage through a UV detector, total RNA corresponding to each gradient fraction was purified, and rRNAs were analyzed using an Agilent 2100 Bioanalyzer. The Ova mRNA showed a classical ribosomal profile: the ribosomal subunit peak (40S) was followed by joined 60S and 80S peaks and by polysomes. The UV absorbance profile for FL.GAr-Ova mRNA showed an analogous profile, but the downstream shape of the gradient differed from the control due to the presence of a strong peak early after the 80S fractions. This GAr-dependent peak (GP) also appeared from the Ova-FL.GAr message but to a lesser extent, presumably reflecting the fact that the GAr is less efficient in controlling translation in a C-terminal context (Fig. 1A). The last peaks to elute were identified as the polysomal complexes. In the control gradient without mRNA, peaks of the 40S and the 60S plus 80S subunits were observed, and the 80S ribosomes were formed by the mRNA-independent association of the 40S and 60S subunits (15) (data not shown). The position of the GP suggested that
it might be due to an accumulation of ribosomal complexes of 80S plus 40S or 80S plus 80S. When we compared the rRNA composition of the fractions corresponding to GPs of FL.GAr-Ova and Ova-FL.GAr mRNAs, we observed a proportional increase in the ratio of 18S rRNA to 28S rRNA, indicating that the GP represents a relative increase in 40S and represents a “ribosome and a half” (Table 1).

In gradients without mRNA, the 40S and 60S/80S subunit peaks were observed, and the last peak was formed by the mRNA-independent association of the 40S and 60S subunits (15) (data not shown).

**TABLE 1.** rRNA analysis of fractions derived from Ova, FL.GAr-Ova, and Ova-FL.GAr constructs

<table>
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<tr>
<th>mRNA fraction</th>
<th>rRNA value of the indicated construct*</th>
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<td></td>
<td>Ova</td>
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<td>18S</td>
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<td>3</td>
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<td>4</td>
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<tr>
<td>9</td>
<td>2.5</td>
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<td>10</td>
<td>3.2</td>
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* Relative rRNA values were determined from 15-in in vitro translation using chicken Ova, FL.GAr-Ova, and Ova-FL.GAr constructs, followed by sucrose gradient analysis. Values are derived from curves obtained using a standard protocol for Agilent 2100 Bioanalyzer results quantification. Fractions 6 and 7 possess the GP (Fig. 3A). The relative increase in 18S rRNA in comparison with 28S rRNA in the FL.GAr-Ova and Ova-FL.GAr fractions 6 and 7 indicates that there is relatively more 40S in these fractions. There are fewer polysomes in the heavy fractions of Ova than in the GAr-Ova or Ova-GAr constructs, indicating that translation of this message has reached near saturation at 15 min, whereas translation of the GAr-carrying mRNAs is still ongoing.
We also tested a shorter GAr sequence to see if the length of the GAr would affect the appearance of the GP and to ensure that flanking sequences do not affect its formation. After 15 min of translation, the gradient profiles for HA-43GAr-Ova and HA-Ova mRNAs slightly differed due to the presence of the GP early after the 80S fraction. The intensity of this peak was enhanced after 30 min of translation for HA-43GAr-Ova and not seen at all for the control mRNA at the same time point (Fig. 3B), indicating that the 40S plus 80S ribosomal complexes accumulate between 15 and 30 min only on GAr-carrying mRNAs.

We also tested the effect of the GAr on the distribution of the mRNA in the polysomal fractions. Labeled mRNAs from EBNA-1 and from an EBNA-1 lacking the FL.GAr sequence (EBΔGAr), as well as from EBΔGAr in which a hairpin sequence has been inserted in the 5' UTR, were translated in vitro and subjected to polysomal fractionation. As expected, the presence of the hairpin in front of the EBΔGAr open reading frame results in a decrease in the amount of mRNA associated with polysomal fractions (Fig. 3C). This distribution of mRNA resembles that observed from the WT EBNA-1 mRNA, further indicating that the GAr is controlling the assembly of ribosomes on its own message.

Changes in amino acid sequence, but not RNA sequence, affect GAr's capacity to suppress translation. Antibodies directed toward the GAr have previously been shown to repress GAr-dependent inhibition of mRNA translation in vitro (25), suggesting that the amino acid sequence, at least to some extent, is responsible for its effect as an mRNA translation regulator. However, the GAr sequence is GC rich and depleted of uracils, and it has been proposed that this is, in fact, the more important aspect of the GAr (20). To further investigate the relative importance of the RNA sequence versus the amino acid sequence in controlling mRNA translation, we examined whether silent mutations in the third position of each codon could restore translation. We changed the nucleotide composition by introducing uracil in the alanine and glycine codons [GCA and GG(G/C) to GCU and GGU, respectively] in a 31-aa-long GAr fused to p53 (31GArT-p53) and compared the rate of synthesis of this construct with one carrying the corresponding WT GAr (31GAr-p53). Metabolic [35S]methionine labeling for 20 min of H1299 cells expressing 31GArT-p53, 31GAr-p53, p53, or empty vector for 24 h shows that translation of 31GArT-p53 is notably less efficient than the control p53 mRNA and similar to that observed with the corresponding 31-aa WT GAr sequence (Fig. 4A). Similar results but less striking ones were obtained in vitro when the same constructs were translated over different RNA concentrations using RRL (Fig. 4B).

To further test the effect of the mRNA versus the peptide sequence, we constructed a series of short GAr mutants fused to Ova (Fig. 4C). The GAr sequence consists of single alanines separated by one, two, or three glycines. When we introduced an alanine residue next to another alanine on three locations in a 30-aa-long GAr (GAr 3A), the mutation slightly disrupted the capacity of the GAr to suppress translation in vivo. When we instead introduced two serines in the N- and C-terminal parts of the 30GAr (GAr 2S), we observed a strong interference with GAr function. Interestingly, when a serine residue was inserted in the C-terminal part of the 30GAr sequence (GAr 1S-C), the efficacy of protein synthesis remained low. However, a single serine in the N-terminal part (GAr 1S-N) had a strong disruptive effect on GAr function, indicating that an intact nascent N terminus of the GAr is more important for the capacity to suppress translation than the C terminus. The introduction of serines in the N or C terminus of the GAr involved the identical nucleotide substitutions. The GAr-like sequence derived from the Papio virus carried single serine residues inserted at every seventh amino acid and had little effect on the rate of protein synthesis.

We also fused a polyQ sequence to the p53 in order to compare the effect of another GC-rich repeat sequence. Increasing the length of polyQ above 35 aa is associated with several types of human disease including Huntington's, and like the GAr, polyQ also causes disruption in protein processing. The polyQ sequence consists of CAG repeats, and thus, like the GAr mRNA, the repeat sequence lacks uracils. However, an 80-aa-long repeat fused to the N terminus of p53 had little or no effect on the rate of protein synthesis (Fig. 4D).

These results demonstrate the specificity by which the GAr affects mRNA translation and support the idea that the retardation of translation by GAr is strongly amino acid sequence-dependent and that the RNA sequence plays little role in suppressing mRNA translation.

Overriding the effect of the GAr by altering the 5' UTR. The observation that changes in the codon sequence have little effect on GAr-mediated translation suppression compared to major disruptions caused by single substitutions in the amino acid sequence would indicate that the ribosome has no difficulties, per se, to translate the GAr, which argues against the idea that the GAr targets the elongation process. This instead suggests that the GAr might affect mechanisms linked to initiation. To further test this idea, we investigated if changes in the mechanisms of translation initiation can affect GAr's capacity to suppress protein synthesis, and we fused several eukaryotic and viral IRESs to the 5' UTRs of the FL.GAr-Ova and the EBNA-1 messages (Fig. 5 and data not shown). Surprisingly, the 5' UTR from the c-myc mRNA, which has been shown to contain IRES activity (13, 18) but not the EMCV-derived IRES, can override the effect of the GAr in both constructs in vivo (Fig. 5A and C). This effect is not observed when the 5' UTR of the c-myc message is located in the 3' UTR of the FL.GAr-Ova construct (data not shown) and is not due to an increase in the levels of c-myc-FL.GAr-Ova mRNA in the cells (Fig. 5B). The fact that the EMCV IRES cannot override the GAr indicates that the effect of the c-myc 5' UTR to override the GAr is not simply to bypass cap-dependent translation but is mediated via a more specific, yet unknown, effect of the c-myc 5' UTR. In support of this, we find that the introduction of a hairpin structure upstream of the c-myc IRES (HPc-myc-IRES-FL.GAr-Ova), which prevents the scanning of the preinitiation complex, results in the abrogation of protein synthesis (Fig. 5A). However, even though this hairpin structure has previously been shown not to suppress cap-independent translation in a bicistronic setting, it is still possible that this hairpin structure may disrupt the c-myc IRES structure when inserted in the 5' UTR. Nevertheless, these results reveal unsuspected, but interesting and specific, aspects of the c-myc 5' UTR that will be investigated separately. Importantly, these results show that changes in the 5' noncoding region of
GAr-carrying mRNAs are sufficient to override its translation-inhibitory effect; hence, nonspecific translation-inhibitory effects due to codon exhaustion, structures in the coding region of the mRNA, or any effects on the translation elongation by the GAr are unlikely.

**DISCUSSION**

The GAr sequence of the EBV-encoded EBNA-1 and the QED-CR of the LANA-1 of KSHV both play key roles in respective virus strategies to evade MHC class I-restricted antigen presentation and the immune system. Previous reports have shown that the capacity of these repeats to prevent MHC class I antigen presentation is dependent on their suppression of mRNA translation in cis. In this report we have further investigated the effect of the GAr sequence on mRNA translation initiation. Previous work has shown that the GAr needs to be within the coding sequence to be effective and that antibodies against the GAr sequence can override some of its translation-inhibitory effect in vitro, and this has led to the suggestion that the GAr peptide sequence and not the RNA sequence is responsible for its effect. This notion is now further strengthened by the results presented here. We find that a GAr sequence in which we have introduced thymidine in the third position of each codon to create silent mutations retains the capacity to suppress translation in vitro as well as in vivo. In contrast, small changes to the N-terminal part of the GAr peptide sequence have strong disruptive effects. Furthermore, introduction of long non-GAr-encoded GC-rich RNA sequences that are also free of uracils, such as an 80-aa polyQ, has no effect on protein synthesis.

The observation that introduction of a serine in the N-terminal part of the GAr peptide has a more pronounced effect on GAr function than inserting serines in the C terminus sug- gestion that the nascent GAr peptide is more important than the trailing end. This, together with the observation that GAr is more potent when located in the N terminus of a fusion protein.
and at high concentrations, could be effects that reflect different sides of the same coin and be explained by a common model (Fig. 6). Hence, when the GAr is located in the 5′ encoded region of the mRNA, the nascent GAr appears first and in close proximity to the initiation site, and the local concentration of the GAr associated with the polysome is higher than when it is fused to the C terminus. The idea that more GAr at the polysome is more effective in disrupting translation is also supported by the observation that longer GAr sequences when fused to the same substrate are more potent inhibitors of translation. Similarly, when the mRNA concentration is high in the in vitro assays, there is more GAr in the lysate relative to its target factor, making the GAr more potent than it is at low mRNA concentrations. The fact that the GAr is translated at low concentrations indicates that the GAr does not act as a negative factor on translation per se but, instead, prevents the action of a positive-acting translation factor. For example, if the mRNA sequence encoding the GAr was difficult to read through, it would not be expected to be capable of independent translation. The model in which the GAr competes with a translation initiation factor can also encompass other observations. According to rRNA analysis, the GAr causes an increase in the ratio of 18S to 28S on the mRNA, indicating that there is a relative accumulation of preinitiation complexes on the mRNA, which could be explained by the nascent GAr’s preventing the formation of 80S complexes. This is supported by the observation that preventing the scanning of the preinitiation complex of a non-GAr mRNA results in a similar distribution of mRNA throughout the gradient as the EBNA-1 mRNA. The relative increase in 18S supports the notion that the GAr first needs to be synthesized in order to prevent the assembly of the following upstream ribosomes. In this context it is worth noting that when the GAr is fused to the C terminus, we observe the peak appearing at the same position, even though it is less pronounced, showing that the GAr affects the polysomal profile the same way independently of its position on the mRNA. If the GAr caused a direct block on elongation, it is likely that the peak would instead be formed where the GAr is being synthesized, and in the case of the Ova-GAr this would be further down the polysome and, thus, in a heavier fraction.

The idea that the effect of the GAr is predominantly mediated via translation initiation is further underlined by the observation that changing the 5′ UTR of GAr-carrying mRNAs is sufficient to overcome its translation-inhibitory effect and that small changes in the nascent peptide sequence have a more pronounced effect than changes to the trailing end. Neither observation is likely if the GAr acts via mechanisms related to elongation, including difficult ribosome readthrough in GC-rich regions, codon exhaustion, or more specific mechanisms related to elongation. Furthermore, fusion of the c-myc 5′ UTR to the 3′ UTR has no effect on GAr-dependent translation control, making it unlikely that it could act by stabilizing the ribosome on the GAr. These results, together with the lack of effect of the 80-aa polyQ, show that codon exhaustion is an unlikely mechanism for causing disruption of synthesis of repeat sequences in vivo. The effect of the GAr on controlling its own synthesis in cis seems unique, and future work will determine if the LANA-1 acts via a similar mechanism. There are, to our knowledge, no other similar examples described (6) where the encoded peptide affects its own synthesis, and peptide-mediated control of synthesis in cis is normally linked to short reading frames upstream of the main reading frame (10, 12).

The fact that disruption of every seven residues inactivates the GAr can perhaps help explain the discrepancies between the results presented here and those of a recent report suggesting that the RNA sequence of the GAr would prevent the elongation of the ribosome along the GAr (20). However, in this report the investigators compare translation of GAr-carrying mRNAs in which isoleucine residues were inserted to disrupt the GAr every 30 aa. Most of the results presented here and in previous studies (25) go against the idea that the GAr mRNA prevents translation elongation.

Why we observe that only the 5′ UTR of the c-myc message that includes an IRES can override the GAr and no other tested eukaryotic IRESs is puzzling (Fig. 5 and data not shown). However, this special effect of the c-myc 5′ UTR in the context of the GAr appears to be cap dependent since fusion of a hairpin structure upstream of the c-myc 5′ UTR blocks translation. Hence, this indicates that this effect is not related directly to cap-independent translation but, rather, to a special feature of the c-myc 5′ UTR, and it is possible that the IRES activity of this RNA sequence and its capacity to override GAr-dependent translation suppression represent two inde-

FIG. 5. The c-myc IRES overrides the translation-inhibitory effect of the GAr. (A) Autoradiograph of an in vivo pulse-labeling experiment shows that fusion of the c-myc IRES in the 5′ UTR of the FL.GAr-Ova message restores mRNA translation. This is not observed using the EMCV IRES, indicating that this effect is not mediated by cap-independent translation per se. Introduction of a hairpin structure 5′ of the c-myc IRES (HPc-myc-IRES-FL.GAr-Ova) blocks its capacity to stimulate synthesis of the FL.GAr-Ova mRNA. The c-myc 5′ UTR has no effect when fused to the Ova by itself (c-myc-IRES-Ova). (B) Northern blot analysis shows the integrity of the c-myc-IRES-FL.GAr-Ova mRNA and indicates that fusion of the GAr to a message does not increase its levels in the cells. (C) Fusion of the c-myc IRES in the 5′ UTR of the EBNA-1 message leads to an approximately twofold increase in EBNA-1 steady-state levels similar to those of the EBΔGAr. The data are representative of more than three separate experiments. WB, Western blotting.
ependent functions. However, unlike many IRESs that constitute complex structures, the c-myc IRES has been reported to require only a short 50-nucleotide sequence to support cap-independent translation, highlighting its potentially unique characteristics (2). A recent report identified four common factors binding and activating the c-, L-, and N-myc IRESs (3). None of these is linked to cap-dependent translation, and they are unlikely to be related to the capacity to override the GAr since, for example, the N-myc IRES has only a weak influence over GAr-dependent translation control (data not shown). Further work elucidating the exact target for the GAr should also clarify how the c-myc IRES functions.

It is tempting to try to draw a connection between the effect of the c-myc IRES, the GAr of EBNA-1, and Burkitt’s lymphoma since in its characteristic form, this tumor type is characterized by the expression of EBNA-1 and the translocation of the c-myc oncogene (26). However, we have not observed any effect of the GAr on stimulating the c-myc IRES or any evidence that the GAr affects c-myc expression in Burkitt’s lymphoma cells, indicating that the GAr–c-myc IRES relationship is likely to work only one way and probably only by chance. Immunological experiments have concluded that the GAr and the QED-CR allow EBV and KSHV to express EBNA-1 and LANA in infected cells without causing the immune system to detect and kill the host cells. This is mediated by preventing antigenic peptides from reaching the MHC class I pathway. Hence, the function of these repeats in controlling the translation of each mRNA is vital for the survival of the viruses. In this respect, it is interesting that the GAr acts immediately on the rate of translation. This implies that this mechanism of evading the MHC class I pathway acts from the onset of viral protein synthesis. In contrast, trans-acting viral factors that are known to disrupt the function of the MHC class I pathway need to be built up to certain levels before becoming functional, leaving the host cell exposed during the early stages of infection. Even though the sequences are different, the QED-CR and the GAr both cause a similar suppression of mRNA translation in cis, suggesting that different types of repeats can have the same effect; even though these sequences are unique, the concept might be widely used among other viruses. The GAr and the QED-CR of the LANA-1 sequences also have the capacity to affect protein degradation. In the case of the GAr this effect is likely due to interference of substrate unfolding at the 26S proteasome (4).
Understanding how viral mechanisms control mRNA translation might have direct practical implications. For example, EBV-infected individuals carry CD8\(^+\) T cells toward EBNA-1 epitopes, but these T cells fail to recognize and kill EBNA-1-expressing cells due to the effect of the GAr on mRNA translation (1, 19). So, disrupting the mechanisms of action of the GAr via therapeutic intervention has the potential to increase the presentation of viral antigens on MHC class I molecules and thereby to trigger an immune reaction toward virus-carrying tumor cells.

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