Inhibition of Translation by UAUUUAU and UAUUUUUUAU Motifs of the AU-rich RNA Instability Element in the HPV-1 Late 3' Untranslated Region*

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The human papillomavirus type 1 (HPV-1) late mRNAs contain a 57-nucleotide adenosine- and uridine-rich RNA instability element termed h1ARE in their late 3' untranslated regions. Here we show that five sequence motifs in the h1ARE (named I–V) affect the mRNA half-life in an additive manner. The minimal inhibitory sequence in motifs I and II was mapped to UAUUUAU, and the minimal inhibitory sequence in motifs III–V was mapped to UAUUUUUUAU. We also provide evidence that the same motifs in the AU-rich instability element inhibit mRNA translation, an effect that was entirely dependent on the presence of a poly(A) tail on the mRNA. Additional experiments demonstrated that the h1ARE interacted directly with the poly(A)-binding protein, suggesting that the h1ARE inhibits translation by interfering with the function of the poly(A)-binding protein.

Human papillomaviruses (HPVs)† are a group of non-enveloped, double-stranded DNA tumor viruses with tropism for epithelial cells (1, 2). Expression of the late mRNAs is restricted to the terminally differentiated cells in the upper layers of the epithelium and at least four papillomaviruses (bovine papillomavirus type 1 [BPV-1], HPV-1, -16, and -31) have been shown to contain cis-acting inhibitory RNA elements located in untranslated regions. Here we show that five sequence motifs in the AU-RNA instability element in HPV-1 late gene expression. Interestingly, the HIV Rev and RRE, and the SRV-1 CTE can overcome the inhibition (9), suggesting that the presence of high levels of HuR in the cytoplasm and the inhibitory activity of the h1ARE, whereas a primarily nuclear association of HuR is associated with inhibition of HPV-1 late gene expression. Additionally, the inhibitory effect of the h1ARE was greater at the protein level than at the mRNA level, suggesting that the h1ARE also inhibited the utilization of the mRNA. Here we present results of a mutational analysis of the h1ARE, and we provide evidence that the h1ARE inhibits mRNA translation.

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† The abbreviations used are: HPV(s), human papillomavirus(es); UTR, untranslated region; ARE, AU-rich element; nt, nucleotides; CAT, chloramphenical acetyltransferase; CMV, cytomegalovirus; hGH, human growth hormone; ELISA, enzyme-linked immunosorbent assay; GST, glutathione S-transferase; PABP, poly(A)-binding protein.

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resulting in pCC. To generate pCC(A), which contains the h1ARE in the antisense orientation, a PCR fragment amplified from pCCKH1 by using oligonucleotides H1KPNI-A (5'-GGTACCAGACTCAGTCTT-3') and H1XBA-S (5'-TCTAGATCTAGTTTCCCCAAGGCG-3') was inserted into EcoRV-digested pBlueScript (Stratagene) generating pKS-H1XK. This was followed by transfer of a KpnI-XbaI fragment from pKS-H1XK to pCC, resulting in pCC(A). Plasmids pCCXB, pCC1, and pCCC1 were generated by transfer of KpnI-XbaI fragments from the previously described plasmids pKSB2, pKSAUM/UM, pKCMV, pKCS2, and pKSC1 (10), respectively, to pCC digested with KpnI and XbaI. pCMVhGH has been described previously (17). Radiolabeled RNAs for UV cross-linking were produced from pKSB2, pKSAUM/UM, pKCS2, and pKSC1 (10).

In Vitro Transcription and Transfections

DNA transfections were performed with FuGENE (Roche Molecular Biochemicals) as described previously (10). Transfections were performed in triplicates, and mean values and standard deviations are displayed in the figures. For RNA analysis triplicate samples were pooled and analyzed by Northern blot. Each plasmid was analyzed at least three independent transfection experiments. RNA synthesis and transfections were performed as described previously (18).

To generate RNAs with a poly(A) tail of fixed length, two PCR fragments were first amplified with the following primer pairs: T7CATS (5'-GAATACGACTCACTATAGGATTGACGATGAGTGGCAGGG-3') and HPV1ANTIPA (5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
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RESULTS

Mutational Analysis of the h1ARE—To study the HPV-1 late 3′ UTR element named h1ARE (Fig. 1A) further, the 57-nt minimal element (XB) (Fig. 1B) or a functionally inactive mutant thereof (AUM/UM) (Fig. 1B) was inserted between the CAT reporter gene, driven by the human cytomegalovirus promoter, and the late HPV-1 poly(A) signals resulting in pXB and pAUM/UM (Fig. 1B). As controls, we used pΔKX (which lacks the major part of the late 3′ UTR), pCCKH1 (which contains the entire late 3′ UTR), and pCCKH1(A) (which contains the region of the late 3′ UTR containing the XB sequence in antisense orientation). HeLa cells were transfected in triplicate, the CAT production was monitored in each plate, and mean values and standard deviations are displayed in the figures. pCMVlacZ was included as an internal control in all transfections.

Analysis of CAT production in transient transfections of HeLa cells and calculation of fold difference between pCCKH1(A)/pCCKH1 and pXB/pAUM/UM revealed that the fold difference between pCCKH1(A)/pCCKH1 and pXB/pAUM/UM were similar and demonstrated that the 57-nt XB contains the major inhibitory sequence (data not shown). The mRNAs containing the 57-nt XB fragment have a short half-life (Fig. 1C). In agreement with our previous findings, the presence of the h1ARE on the mRNA also results in a higher ratio of nuclear versus cytoplasmic mRNA (data not shown). We concluded that pXB and pAUM/UM could be used for further studies of the AU-rich element.

The XB sequence contains two AUUUA motifs and three AUUUUUUA motifs that were numbered I–V (Fig. 2A). These motifs were all mutated in AUM/UM (Fig. 2A). To investigate if all motifs were required for inhibition, the motifs were mutated one by one (Fig. 2A). However, there was only a modest increase in CAT RNA and protein levels (Fig. 2B and C) for each mutant. To obtain a clearer answer on the importance of each motif, consecutive mutations were introduced in the motifs (Fig. 3A). The results revealed that higher expression levels were obtained as more motifs were mutated (Fig. 3A). In agreement with our previous findings, the presence of the h1ARE on the mRNA also results in a higher ratio of nuclear versus cytoplasmic mRNA (data not shown).
demonstrating that also the fifth motif contributed to inhibition. Interestingly, the effect was greater at the protein level than at the RNA levels, for all analyzed mutants (Fig. 3, B and C). We concluded that all five motifs contributed to inhibition in an additive manner.

**Determination of the Minimal Inhibitory Sequence of Each Sequence Motif within the h1ARE**—To determine the minimal inhibitory sequence of the AUUUA-containing motifs, point mutations were introduced in motifs I and II (Fig. 4A). Mutations in the tri-U nucleotides, the flanking As or the Us immediately flanking the As were not well tolerated (Fig. 4, B and C). However, mutations in the second nucleotide position outside the As (Fig. 4A), did not significantly affect the inhibitory activity of these motifs (Fig. 4, B and C), indicating that the smallest inhibitory motif was UAUUUAU. The two UAUUUAU were separated by a four-nucleotide spacer sequence (Fig. 4A). Substituting this sequence with four Cs did not affect the inhibitory activity of the h1ARE (Fig. 4, B and C), indicating that this spacer sequence did not contribute to the inhibitory activity.

To determine the minimal inhibitory sequence of the penta-U motifs, the nucleotides flanking the penta-Us were mutated (Fig. 5A). The results revealed that mutations in both the As flanking the penta-Us and the Us flanking the As resulted in higher CAT protein and RNA expression levels (Fig. 5, B and C). Substituting two Us in the penta-U sequence with two Cs had the strongest effect, whereas mutations in the Us flanking the As had the smallest effect on the inhibitory activity (Fig. 5, B and C). Therefore, the minimal motif was UAUUUUAU.

**UAUUUAU and UAUUUUUAU Motifs Can Functionally Substitute for One Another**—The h1ARE may be divided into the B2 region with the two UAUUUAU motifs and the C1 region with the three UAUUUUUAU motifs (Fig. 6A). The B2 region, although less inhibitory than the XB sequence, as expected, inhibited CAT production and reduced mRNA levels to the same extent as the did C1 region (Fig. 6, B and C). Two B2 or two C1 regions were as inhibitory as the entire XB (Fig. 6, B and C), demonstrating that one type of motif (UAUUUAU or UAUUUUUAU) could substitute for another. Furthermore, if the two AUAUUUAU motifs were extended to two AUAUUUUAU motifs by insertion of two Us in each motif, resulting in pAUAUUUAU (Fig. 7A), or if the three penta-U motifs were all shortened to contain only three Us, as in pAUAUU (Fig. 7A), the resulting inhibitory elements were nearly as
inhibitory as the wild type h1ARE (Fig. 7, B and C). Both B2 regions and C1 regions acted by reducing mRNA steady state levels and protein production. Inhibition was also greater at the protein level than at the mRNA level. Taken together, the results demonstrated that both motifs acted in a similar manner.

Multiple Copies of the HPV-1 AU-rich Element Inhibits Protein Production >99%—For all mutants that retained inhibitory activity, we observed that the inhibitory effect was greater at the protein level than at the mRNA level (see Fig. 2, B and C, Fig. 3, B–D, Fig. 4, B and C, Fig. 5, B and C, Fig. 6, B and C, and Fig. 7, B and C). In other words, the mRNAs that contained the h1ARE, or partially active mutants thereof were utilized less efficiently by the translation machinery than mRNAs lacking the h1ARE or mRNAs containing functionally inactive sequences. To better compare the effect on protein production and the effect on the mRNA levels, multiple XB sequences were inserted into the reporter plasmid pXB, resulting in p2XB, p3XB, and p4XB (Fig. 8A), and the fold inhibition at the protein and mRNA levels was separately plotted against the number of XBxs on the mRNA. As can be seen, there is a gradual decrease in CAT protein and mRNA levels for every inserted copy of the XB fragment (Fig. 8, B and C), and the fold inhibition was greater at the protein level than at the RNA level (Fig. 8, B and C).

To study the effect of the h1ARE on translation further, we replaced the CMV immediate-early promoter with the bacteriophage T7 promoter and the cleavage and polyadenylation signal with the XhoI restriction site in the h1ARE reporter constructs pCCKH1(A) and pCCKH1 (Fig. 1B) used previously to study the h1ARE. These cloning steps resulted in pCC(A) and pCC (Fig. 9A) that were linearized with XhoI and

![Fig. 6](image)

**Fig. 6.** A, HPV-1 sequences inserted into the reporter plasmid are shown. The sequence motifs in XB are underlined and numbered. Introduced mutations are underlined. B, the indicated plasmids were transiently transfected into HeLa cells in triplicate, and protein was removed for CAT ELISA, followed by pooling of the three samples and cytoplasmic RNA extraction. An internal control plasmid was included in all transfections. B, CAT levels were monitored by CAT ELISA, and the levels are displayed as percent of CAT produced from pAUM/UM. Mean values and standard deviations are shown. C, the RNA samples were subjected to Northern blotting (lower panel) followed by phosphorimaging quantitation (upper panel). The RNA levels are displayed as percent of RNA compared with pAUM/UM. Pooled RNAs from triplicate experiments are shown.

![Fig. 7](image)

**Fig. 7.** A, HPV-1 sequences inserted into the reporter plasmid are shown. The sequence motifs in XB are underlined and numbered. Introduced mutations are underlined. Brackets mark deletions. B, the indicated plasmids were transiently transfected into HeLa cells in triplicate, and protein was removed for CAT ELISA followed by pooling of the three samples and cytoplasmic RNA extraction. An internal control plasmid was included in all transfections. B, CAT levels were monitored in CAT ELISA, and the levels are displayed as percent of CAT produced from pAUM/UM. Mean values and standard deviations are shown. C, the RNA samples were subjected to Northern blotting (lower panel) followed by phosphorimaging quantitation (upper panel). The RNA levels are displayed as percent of RNA compared with pAUM/UM. Pooled RNAs from triplicate experiments are shown.

A estimate of the inhibitory activity of one single XB sequence at protein and RNA levels (Fig. 8D) within the context of these plasmid constructs. The results clearly demonstrated that XB had a greater effect at the protein level than at the RNA level (Fig. 8D). On average from multiple experiments, we found that CAT protein levels were reduced 3.7-fold per XB and RNA levels 1.4-fold per XB in the context of the mRNAs with multiple XBxs (Fig. 8D). We concluded that in addition to the effect on mRNA half-life, mRNAs carrying the h1ARE are inefficiently utilized for translation.

Deadenylation (20, 21) could potentially cause the inhibition of translation observed here. To determine the poly(A) tail length of the mRNAs shown in Fig. 8C, they were subjected to oligonucleotide-directed RNaseH cleavage with the “RNaseH oligo” shown in Fig. 8A followed by Northern blot using a probe located downstream of the RNaseH oligo. The results revealed that all mRNAs contained poly(A) tails of similar length (Fig. 8E), demonstrating that inhibition of translation was not a result of deadenylation.

The HPV-1 AU-rich Element Inhibits Translation of the Transfected mRNAs—To study the effect of the h1ARE on translation further, we replaced the CMV immediate-early promoter with the bacteriophage T7 promoter and the cleavage and polyadenylation signal with the XhoI restriction site in the reporter constructs pCCKH1(A) and pCCKH1 (Fig. 1B) used previously to study the h1ARE. These cloning steps resulted in pCC(A) and pCC (Fig. 9A) that were linearized with XhoI and
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Fig. 8. A, the AUM/UM and XB sequences are shown. Multiple XB sequences were inserted into the reporter plasmid resulting in the indicated plasmids with one, two, three, or four XB sequences. The names of the plasmids are indicated on the left. pAL1 and pAL2, late poly(A) signals. B, the indicated plasmids were transiently transfected into HeLa cells in triplicate, and protein was removed for CAT ELISA followed by pooling of the three samples and cytoplasmic RNA extraction. An internal control plasmid was included in all transfections. CAT protein levels were monitored by CAT ELISA, and the levels are displayed as percent of CAT produced from pAUM/UM. Mean values and standard deviations are shown. C, the RNA samples were subjected to Northern blotting (upper panel) followed by phosphorimaging quantitation. The RNA levels are displayed as percent of RNA compared with pAUM/UM. Pooled RNAs from triplicate experiments are shown. D, log% CAT RNA or protein were plotted against the number of insert XB sequences. The plotted numbers represent mean values from three different experiments. The slope of each curve is shown as used as templates for in vitro synthesis of capped and polyadenylated CC(A) and CC mRNAs as described under “Experimental Procedures.” These mRNAs contained the same sequences as the mRNAs produced by the pCCKH1 and pCCCKH1(A) plasmids in the nuclei of transfected cells (Fig. 1B). Capped and polyadenylated CC and CC(A) mRNAs were transiently transfected in triplicates into HeLa cells by electroporation, and the CAT levels produced at 20 h posttransfection were quantified by using a CAT ELISA. The results revealed that CC mRNAs that contain the h1ARE in sense orientation produced ~15-fold lower CAT protein levels than the CC(A) mRNAs, which contain the h1ARE in antisense orientation (Fig. 9A). Cotransfected hGH-encoding mRNAs included in all samples as an internal control produced similar hGH protein levels (Fig. 9A).

Next, aliquots of electroporated cells were harvested at different time points, and the levels of CAT protein were monitored and plotted against time (Fig. 9B). Two interpretations of the results shown in Fig. 9B were appropriate: either the mRNAs with the HPV-1 late 3’’UTR were rapidly degraded, preventing further CAT protein synthesis, or the mRNAs were not available for further rounds of translation as a result of a direct inhibition of translation, presumably by factors binding to the h1ARE.

To investigate if the half-lives of the transfected mRNAs were reduced by the presence of the HPV-1 late 3’’UTR, CC and CC(A) mRNA levels at 1, 3, 5, and 23 h posttransfection were determined by primer extension (Fig. 9C). CC and CC(A) mRNAs decayed at similar rates, as detected at 1, 3, and 5 h posttransfection (Fig. 9C). Longer exposures also detected similar amounts of CC and CC(A) mRNA at 23 h posttransfection (data not shown). The mRNA half-lives were calculated to be 2.6 h for CC mRNAs and 2.7 h for CC(A) mRNAs. A number of control experiments were performed using CC(A) and/or CC mRNA. These experiments verified that electroporated RNAs were not sticking to the outside of the cell and that the majority of the transfected mRNAs are normally utilized by the translation machinery (data not shown). We concluded that the inhibitory effect on the transfected mRNAs, mediated by the HPV-1 late 3’’UTR, was not a result of reduced mRNA half-life in the cytoplasm and that the HPV-1 late 3’’UTR acted by inhibiting mRNA translation. In addition, the previously observed effect on the mRNA half-life was dependent on a nuclear experience of the mRNA and was not seen when in vitro synthesized mRNAs were transfected into cells.

Inhibition of Translation by the h1ARE Requires Intact UAAUUUAU or UAAUUUUUAU Motifs in the h1ARE—To confirm that the 57-nt minimal XB sequence containing the two UAAUUUAU and the three UAAUUUUUAU motifs inhibited translation in the RNA transfection experiments, CCXB and CCAAUM/UM mRNAs (Fig. 10A) were transfected into HeLa cells in parallel. The CCAAUM/UM mRNAs produced 41-fold higher levels of CAT than the CCXB mRNAs (Fig. 10B). hGH protein levels produced from the internal control mRNAs were similar in both samples (Fig. 10B). Next, capped and polyadenylated CCXB, CCAAUM/UM, CCB2, and CCC1 mRNAs (Fig. 10A) were electroporated into HeLa cells, and the levels of CAT protein produced at 3, 6, 23, and 47 h posttransfection were

$k_{\text{CATPROT}}$ and $k_{\text{CATRNA}}$, E, the RNA samples shown in Fig. 8C were subjected to RNaseH cleavage in the presence of the RNaseH oligo indicated in Fig. 8A. The digested RNA samples were subjected to Northern blotting using a probe located downstream of the RNaseH oligo. The results demonstrate that mRNAs containing multiple XB sequences display the same length distribution of poly(A) tails as the mRNAs containing the functionally inactive AUM/UM sequence. Pooled RNAs from triplicate experiments are shown.
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The CAT protein production peaked at the 23 h time point for all four mRNAs, after which the CAT protein levels decreased. This is the expected result if the mRNAs have similar half-lives. The mRNA decay rates in the cells transfected with CCXB and CCAUM/UM mRNA were determined by primer extension on RNA extracted from the transfected cells. Fig. 10D shows that the levels of CCXB and CCAUM/UM mRNAs were similar at 1 and 2.5 h posttransfection and decayed with a rate comparable to that observed for CC and CC(A) mRNAs (compare Figs. 10D and 9C). The results confirmed that the h1ARE acts primarily by reducing mRNA translation in the RNA transfection experiments performed here and demonstrated that both the two UAUUUUAU motifs and the three UAUUUUUUAU motifs inhibit translation.

The h1ARE Inhibits Translation of mRNAs Carrying a Cap and a poly(A) Tail—The poly(A) tail is required for efficient mRNA translation (23). We therefore wished to investigate if the HPV-1 AU-rich element interfered with the function of the poly(A) tail. We transfected polyadenylated and unpolyadenylated capped CC and CC(A) mRNAs and monitored the levels of CAT protein in the cells at 24 h posttransfection. Fig. 11A shows that the polyadenylated CC mRNA, which contains the HPV-1 AU-rich element in sense orientation, produced 56-fold lower CAT protein levels than polyadenylated CC(A) mRNA, which contains the HPV-1 AU-rich element in antisense orientation. In contrast, the presence of the h1ARE on the unpolyadenylated CC mRNA did not inhibit CAT production significantly (Fig. 11A). The stimulatory effect of the 3′-poly(A) tail, when added to the capped CC mRNA, which contains the h1ARE, was only 1.5-fold, compared with 56-fold when added to the CC(A) mRNA. Translation of co-transfected hGH mRNA was similar in all samples (Fig. 11A). Similar results were obtained in a time course experiment using the XB sequence and the inactive AUM/UM mutant (Fig. 11B), demonstrating a connection between the 57-nt XB sequence and the poly(A) tail.

Taken together, the results demonstrated that inhibition of CAT production by the h1ARE was dependent on a 3′-poly(A) tail. Therefore, the results showed that the stimulatory effect on translation mediated by the 3′-poly(A) tail on cap-dependent translation was perturbed by the h1ARE.

Also here could the inhibitory effect of the AU-rich RNA element on translation be indirect through deadenylation. To investigate if the h1ARE promoted deadenylation of the in vitro synthesized mRNAs that were transfected into the HeLa cells, mRNAs with a poly(A) tail of fixed length were transfected into the cells and the length of the poly(A) tail was determined at 1 or 4 h posttransfection by Northern blotting. Capped mRNAs with and without the h1ARE or poly(A) tail were analyzed. The results revealed that the mRNAs were not deadenylated at 1 h posttransfection (Fig. 11C). In addition, analysis of the polyadenylated mRNAs containing the h1ARE at 4 h posttransfection showed that the poly(A) tails remained intact (Fig. 11C). Therefore, inhibition of translation was not caused by deadenylation.

The h1ARE Interacts with the Poly(A)-binding Protein—Having established that the h1ARE inhibited the function of the poly(A) tail, it was reasonable to speculate that the h1ARE, or h1ARE binding factors, interact directly with the poly(A)-binding protein (PABP). We therefore tested if PABP bind directly to the XB RNA. GST-PABP was UV cross-linked to XB RNA or AUM/UM RNA. UV cross-linking of GST-PABP revealed that GST-PABP bound strongly to the XB RNA but only weakly to
the AUM/UM RNA (Fig. 12A); GST-PABP did not bind to an unrelated RNA derived from the L1 coding region in HPV-16 (Fig. 12A). The GST-HuR protein was used as positive control and interacted only with XB, as expected (13), and GST-PCBP did not bind to any of the RNA sequences (Fig. 12A). To confirm that the binding of GST-PABP to the XB sequence was sequence specific, a competition experiment was performed. The XB RNA competed efficiently with the RNA probe for binding to GST-PABP, whereas the HPV-16 L1-derived RNA did not (Fig. 12B), demonstrating that the interaction with XB was sequence-specific. Analysis of the deletion mutants B2 and C1 that were shown to inhibit translation to a similar extent also interacted with the PABP (Fig. 12A). Therefore, binding of PABP to the different mutants (B2, C1, and AUM/UM)) correlated with their inhibitory effect on translation.

**DISCUSSION**

The mutational analysis presented here revealed that the HPV-1 AU-rich element consists of the two UAUUUAU heptamers and the three UAUUUUUAU nonamers. Previous studies on the c-fos ARE led to the conclusion that the UAUUUUAU motif was sufficient for mRNA destabilization but not optimal, and that multiple copies were needed for significant destabilization (24). This is in agreement with the results presented here. Based on a mutational analysis and sequence alignments, these authors concluded that the minimal motif may be UUAUUUA(U/A)(U/A). In another article on the c-fos ARE, a deletion analysis of the c-fos ARE led to the conclusions that the UUAUUUAUU nonamer, and not the UAUUUAU heptamer, was the shortest destabilizing motif (25). These authors also found that mRNAs containing multiple copies of the nonamer are degraded more rapidly than mRNAs with only one copy. Using the HPV-1 ARE, we also found that multiple copies of the motif were more inhibitory than one copy. However in our system, mutations outside of the heptamer UAAUUUA did not affect its ability to reduce mRNA levels. In the context of the HPV-1 ARE, the UAAUUUA was the shortest motif with inhibitory activity, indicating that the sequence context may affect the potency of an AU-rich element. In contrast to the HPV-1 AU-rich element that contains two UAAUUUA and three UAUUUUUAAU motifs, the AU rich element on the IL-3 mRNA contains six AUUUA motifs. Mutations in three of these motifs had the same effect as deleting the entire element (26). The HPV-1 AU-rich element contains five motifs, and we show that all five contribute to inhibition in an additive manner. Similarly, mutations in all three AUUUA motifs in the c-fos ARE resulted in mRNA stabilization (27). It appears that multiple copies of the "AUUUA"-related motifs are required for full transcription is indicated. The sequences of the minimal h1ARE (XB), the mutant AUM/UM sequence, and the two deletion mutants of the h1ARE (B2 and C1) are shown. Mutations in AUM/UM are underlined. Plasmid names are indicated on the left. The functionally important sequence motifs are numbered and underlined. Numbers refer to nucleotide positions in the HPV-1a genomic clone (37). B, the histogram shows mean values and standard deviation of the quantified CAT levels produced at 20 h posttransfection in HeLa cells transfected with capped and polyadenylated CCXB and CCAUM/UM mRNAs. A representative experiment is shown. Mean values and standard deviation of the quantified hGH protein levels produced from the hGH-encoding mRNA included as internal control are shown below the histogram. C, the graph shows quantified CAT levels produced from capped and polyadenylated CCXB, CCAUM/UM, CCB2, and CCB1 mRNAs at various time points posttransfection into HeLa cells. The inset shows the hGH protein levels at the same time points produced from the hGH-encoding mRNAs included as internal control. D, cytoplasmic CCXB and CCAUM/UM mRNA levels detected by primer extension in transfected HeLa cells at different time points posttransfection as indicated. The arrow indicates the specific extension products of the transfected mRNAs.
function of the various AU-rich RNA elements.

We have previously shown that the mRNA half-life is reduced by the presence of the h1ARE when using DNA transfections in which the mRNAs are synthesized in the cell nuclei (9, 10). In contrast, when the same mRNAs were introduced directly into the cytoplasm as described here, bypassing the nucleus, there was no effect on the mRNA half-life by the h1ARE. These results indicate that a nuclear experience of the h1ARE-containing mRNAs is necessary for rapid mRNA degradation, suggesting that the mRNAs are either modified in the nucleus or interact with nuclear factors that induce premature mRNA degradation. Recent results on the ARE-containing c-fos mRNA showed that HuR mediates nuclear export of c-fos mRNAs (28). HuR also increases the c-fos mRNA half-life, suggesting that nuclear export and mRNA stability are connected and that inefficient mRNA export leads to premature degradation. In a previous article, we reported that the HIV-1 mRNA export factor Rev in combination with RRE or the SRV-1 CTE could overcome the inhibitory effect of the h1ARE (9), demonstrating that export of the h1ARE-containing mRNAs through an alternative, productive pathway overcomes inhibition and results in high expression. These results also demonstrated that the h1ARE has an inhibitory function in the nucleus, in addition to its inhibitory effect of translation in the cytoplasm described here.

The h1ARE binds PABP and may inhibit the interaction between PABP and eIF4G, thereby preventing circularization of the mRNA and the subsequent loading of ribosomes on the mRNA. This is not without precedent since it was recently shown that the rotavirus mRNA 3'-end-binding protein NSP3 interacts with eIF4G and that NSP3 competes with the PABP for the eIF4G (29). Alterations of either the poly(A)-PABP or cap-eIF4E complexes lead to access to the poly(A) tail and cap by the poly(A) ribonuclease (PARN/DAN), resulting in deadenylation (30). Deadenylation was not observed here. It has
also been proposed that the shuttling elav-like proteins are involved in mRNA translation directly (31, 32). Similarly, redistribution of HuR protein from the nucleus to the cytoplasm is associated with increased protein production from mRNAs containing AREs with HuR binding sites (33, 34). Therefore, HuR may act similarly to HuB and promote polysomal loading of ARE-containing mRNAs. HIV-1 Rev protein that overcomes the inhibitory effect of the h1ARE in HeLa cells also induces polysomal loading of target mRNAs (35, 36). Perhaps elav-like proteins such as HuR lead mRNAs onto a productive pathway that includes efficient nuclear export and polysomal loading.

The role of the h1ARE, HuR, and the PABP in the HPV-1 life cycle remains to be determined.

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