Herpesvirus saimiri small RNA and interleukin-4 mRNA AUUUA repeats compete for sequence-specific factors including a novel 70K protein

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A highly oncogenic strain of the lymphotropic tumour virus herpesvirus saimiri (HVS; strain 484-77) expresses four small RNAs (HSUR1 to 4) in high copy numbers in transformed T cells. In HSUR1 and HSUR2 the 5' terminal regions contain conserved AUUUA sequence repeats. The same AUUUA repeats occur in the 3' non-coding regions of growth factor, lymphokine and proto-oncogene mRNAs, and the sequence is involved in rapid mRNA degradation. We report here that by using a highly specific u.v. cross-linking method we identified a novel 70K binding factor with AUUUA sequence specificity. Non-radiolabelled competition and V8 protease analysis show that the protein can form a complex with the 3' non-coding region of interleukin-4 mRNA and bind the AUUUA repeats of a HVS small RNA. We also detected an AUUUA-specific minor 32K human protein with the same electrophoretic mobility as a marmoset factor implicated in growth factor mRNA destabilization. The findings are consistent with the hypothesis that the viral small RNAs can compete for factors involved in rapid degradation of growth factor mRNAs and may contribute to viral oncogenesis.

Introduction

Herpesvirus saimiri (HVS) is an oncogenic gammaherpesvirus, a close relative of Epstein–Barr virus (Gompels et al., 1988; for review see Fleckenstein & Desrosiers, 1982). In monkeys and rabbits the virus transforms CD8+ lymphocytes (Kiyotaki et al., 1986) and causes malignant lymphoproliferations (Daniel et al., 1974; Melendez et al., 1969). Several data indicate that lymphokine and growth factor upregulation is involved in the process and mediate autocrine cell growth (Medveczky & Medveczky, 1989). High-level interleukin (IL)-2 receptor expression (Medveczky & Medveczky, 1989) and IL-4 secretion was detected with transformed monkey cell lines (M. Medveczky, P. Geck, C.-S. Chou, A. Brown, J. Cus & P. G. Medveczky, unpublished results) and, in immortalized human CD4+ cell lines, upregulation of IL-3, IL-4, interferon-γ, and tumour necrosis factor-α and -β were reported (De Carli et al., 1993). Cell–cell contacts through CD2 and CD58 were also implicated in maintaining activation of transformed cells (Mittrucker et al., 1992). Cytolytic and killer cell activities were detected with transformed human lymphocytes (Medveczky et al., 1993; De Carli et al., 1993).

To study molecular mechanisms in transformation by HVS, we focused on a highly oncogenic strain (484-77) and sequenced 6-2 kb of the oncogenicity-associated region. Five transcripts were identified and mapped: a 1·2 kb mRNA (Geck et al., 1990b) and four nuclear small RNAs, 120, 110, 81 and 64 nucleotides in length, termed HSUR1, -2, -3 and -4 respectively (Geck et al., 1993).

The 1·2 kb mRNA has two open reading frames. The first (the analogue of STP-11, the HVS transforming protein in strain 11) codes for a polypeptide with a collagen-like domain (Geck et al., 1990b). The protein was localized in intracellular membranes (Jung et al., 1991) and in expression vectors the sequence can transform cells (Jung & Desrosiers, 1991). The second open reading frame has also been shown to be involved in transformation by deletion analyses (Medveczky et al., 1993).

The four small RNAs and their genes have regulatory elements and other structural features that indicate a close relationship to cellular U-type small nuclear RNAs (Geck et al., 1993), as with small RNAs of other HVS strains (Lee et al., 1988). The 5' terminal sequences of HSUR1 and HSUR2 are highly conserved and, in all three strains sequenced so far, AUUUA motifs occur repeatedly (Geck et al., 1990a) as shown in Fig. 1.

A similar sequence with reiterated AUUUA motifs appears in the 3' non-coding mRNA regions of inducible growth regulatory proteins, growth factors and proto-oncogenes (for the IL-4 3' non-coding mRNA sequence see Methods; for a review of other sequences see Shaw & Kamen, 1986). These mRNAs are distinguished by
Fig. 1. Comparison of conserved 5'-terminal sequences of HSUR1 and HSUR2 between strain 484-77, strain 488 and strain 11. The conserved AUUUA elements are in upper case.

extremely rapid turnover (Raj & Pitha, 1983; Greenberg et al., 1986; Ryseck et al., 1988), which appears to be regulated (Levine et al., 1986; Wilson & Treisman, 1988; Wodnar-Filipowitz & Moroni, 1990). The 3' non-coding region and the AUUUA repeats were shown to signal rapid degradation (Caput et al., 1986; Shaw & Kamen, 1986; Vakalopoulou et al., 1991). Elements involved in the regulation of mRNA turnover have already been reported: for example endo- and exonucleases (Ross et al., 1987; Jochum et al., 1990) and various binding proteins from different cells with AUUUA specificities, like the Auf protein from an erythroleukemia line (Brewer, 1991), the AU-A protein from T lymphocytes (Bohjanen et al., 1991), the 32K protein from HeLa cells (Vakalopoulou et al., 1991), a lymphokine mRNA-specific factor (AU-B) in stimulated T cells (Bohjanen et al., 1991) and the AUBF factor in unstimulated T cells (Malter & Hong, 1991). A marmoset protein with similarities to the 32K HeLa cell protein was reported to bind some of the small RNAs of HVS strain 11 (Myer et al., 1992).

The finding that abundant viral small RNAs carry the key destabilization signals of growth factor mRNAs raised the intriguing possibility that their function may be related to mRNA turnover regulation (Geck et al., 1990a; Myer et al., 1992). In theory, by competing for binding or degradation factors, the small transcripts could interfere with the regulation of mRNA destabilization. Stabilized mRNAs could command growth factor overexpression and so contribute to viral oncogenesis.

Our studies were designed to address a key element of the hypothesis, to find the link between the viral and mRNA sequences. By applying a novel RNA–protein signal transfer method, we searched for highly specific AUUUA binding factor(s) present in both the viral RNA– and mRNA–protein complexes.

Methods

Tissue culture. Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 10 µg/ml gentamicin sulphate in 150 cm² flasks. Cells were harvested 6 to 12 h following addition of fresh medium.

Preparation of cellular extracts. Cells (5 x 10⁶) were disrupted in 1 ml of modified lysis buffer (25 mM-Tris–HCl pH 7.9, 0.5 mM-EDTA, 0.1 mM-phenylmethylsulphonyl fluoride, 0.6 µM-aprotinin, 1 µM- leupeptin and 1 mM-ATP) by freezing and thawing as described previously (Malter, 1989).

Subcloning. All enzymatic and DNA manipulations were performed with standard procedures (Sambrook et al., 1989) and enzymes were obtained from Promega. The full-length HSUR2 coding sequence (Geck et al., 1993) was generated by PCR using a 5' composite primer with the T3 RNA polymerase promoter sequence (in lower case), 5' aattaaccctcactaaagggagaACTCTACATATTTATTG 3'. The 3' primer used was 5' TCAAATGTACACCCAG 3'. The product was blunt-ended by the Klenow fragment of DNA polymerase and cloned into the Smol site of pUC19. To subclone the 3' non-coding AUUUA region from the human IL-4 cDNA (Yokota et al., 1986) an SpsI site at position 530 and KpnI cleavage downstream in the polylinker generated a 256 bp fragment that contained the 3' non-coding region, the poly(A) region and a short polylinker from the vector. Bluescript II SK vector (Stratagene) was linearized with SauII, blunt-ended by the Klenow fragment, cut by KpnI and the isolated IL-4 cDNA fragment was inserted using T4 ligase. For a template to synthesize RNA with five repetitions of the AUUUA motif, the sense and antisense oligonucleotides were designed with the added upstream promoter for T3 RNA polymerase (lower case), the sense strand being 5' aattaaccctcactaaagggagaATTTTTTATTATTATTTATTATA 3'. The two strands were annealed and cloned into the blunt-ended Xbal site of pUC19. Sequence and polarity were confirmed by DNA sequencing in each case.

Oligonucleotide mutagenesis. A 2770 bp BamHI–HindIII fragment from the viral genome containing the gene for HSUR2 was cloned into the Bluescript II SK vector and ssDNA was prepared according to the manufacturer's instructions. A complementary oligonucleotide with two U to C mismatches (5' AAAGCAGCTATACATAACGATACATAGAGGTGCTG 3') was annealed to the template and extended and ligated by T4 DNA polymerase and ligase. After transformation, mutant clones were selected by colony hybridization using the mutant oligonucleotide as probe, and sequenced to confirm mutations. The mutant HSUR2 sequence was isolated by PCR and subcloned as described above.

In vitro transcription. To synthesize full-length viral small RNA, the HSUR2 recombinant plasmid was linearized by BamHI restriction endonuclease downstream of the HSUR2 sequence. For transcription of the 5' AU-rich region of HSUR2 (Fig. 1), a DraI site was used, 36 nucleotides downstream from the 5' end. For IL-4 mRNA 3' non-coding sequence synthesis, the IL-4 construct was digested with HindIII at nucleotide position 611 of the cDNA sequence, generating a template of 82 nucleotides at the 3' non-coding region (5' aattaATTATGTTAGTACGACG 3') was annealed to the template and extended and ligated by T4 DNA polymerase and ligase. After transformation, mutant clones were selected by colony hybridization using the mutant oligonucleotide as probe, and sequenced to confirm mutations. The mutant HSUR2 sequence was isolated by PCR and subcloned as described above.

In analytical binding reactions, 1 to 5 ng (3 x 10⁶ c.p.m.) of RNA was incubated with cytoplasmic extracts in various amounts. We found that some modifications in published
binding conditions (Malter, 1989) were necessary to enhance the resolution of minor bands. The extracts were preincubated with 400 units/ml of RNasin at 30 °C for 5 min and the binding reactions were incubated in 2.5 mM-Hapes pH 7.9, 3 mM-KCl, 0.05 mM-EDTA, 0.05 mM-dithiothreitol, 1 mM-MgCl₂, 400 units/ml RNasin, 2 μg of Escherichia coli tRNA and 10% glycerol at 34 °C for 10 min. Complexes were separated by electrophoresis at 70 V in 5% non-denaturing polyacrylamide gels with 0.25 x Tris-borate-EDTA running buffer.

U.v. cross-linking of RNA-protein complexes and protein analysis. After preparative electrophoresis in 5% polyacrylamide gels (5 mm spacer), one glass plate was removed and the gel was u.v.-irradiated for 3 min using a Stratalinker device (Stratagene). After autoradiography for 5 to 30 minutes, complexes were cut out, electroeluted into BSA-treated dialysis membranes and incubated with RNase A (100 μg/ml) and RNase T1 (10 μg/ml) at 22 °C for 30 min. The preparations were boiled in SDS sample buffer (Sambrook et al., 1989) with 100 mM-2-mercaptoethanol before separation by 12% SDS-PAGE. To isolate individual polypeptides, preparative gels were processed as described above and the electroeluted proteins were subjected to partial V8 protease analysis (Cleveland et al., 1977).

Results

The viral small RNA and IL-4 mRNA sequences bind proteins and compete for protein complexes

HSUR2 subclones and mutants established previously in this laboratory offered a convenient model to study viral small RNA-cellular binding factor interactions. Since viral transformation is T lymphocyte-specific and the virus can also immortalize human T cells (Biesinger et al., 1992; Medveczky et al., 1993), the human Jurkat T cell line was selected to study cellular binding factors. High-level expression of AUUUA binding factors has also been reported in Jurkat cells (Malter, 1989; Gillis & Malter, 1991).

We first determined whether the 5' end AUUUA region of the viral small RNA could bind proteins. As shown in Fig. 2(a), band-shift assays with the sequence revealed two major bands (complexes 3 and 4) and several minor bands (complexes 1, 2, 5 and 6) in standard binding conditions (Malter, 1989). An improved resolution of the same pattern is shown in Fig. 3, where the modifications described in Methods were used.

To investigate growth factor-mRNA interactions, IL-4 was selected, since previous data from this and other laboratories indicated IL-4 upregulation in the autocrine growth of HVS-transformed tumour cells. The 3' non-coding region of IL-4 mRNA carries three AUUUA repeats (Yokota et al., 1986) in an arrangement typically found in destabilization signal sequences. Gel retardation experiments with an RNA probe representing the sequence displayed complex band-shift patterns (Fig. 2b), demonstrating that the IL-4 mRNA 3' non-coding region (for sequence see Methods) also complexes with proteins.
associated with AUUUA-specific binding. In theory, whether bands in gel retardation patterns could be associated with AUUUA-specific binding. In theory, AUUUA-specific protein(s) occur, we first determined As a preliminary attempt to localize complexes where viral sequence) and indicates an overall higher binding affinity for the IL-4 3' non-coding region. In summary, the results suggest that the two RNAs compete for the same protein or protein complex. Therefore, AUUUA repeats in the viral sequence were changed to AUGUA by oligonucleotide-directed mutagenesis. Comparison of wild-type and mutant band-shift assays in Fig. 3(a) showed that complex 4 of the wild-type pattern did not form with the mutant sequence and complex 5 and complex 6 were also greatly reduced.

The change of complex 4 in the mutant pattern suggested that this complex only forms when intact AUUUA repeats are present. However, alternative explanations, other than a change in binding protein composition, were not excluded (for example different conformation or degradation of the mutant probe). To investigate further the association of this complex with AUUUA repeats, an RNA with five repetitions of the AUUUA element was synthesized and radiolabelled. Comparison of band-shift patterns in Fig. 3(b) demonstrates that the mobility of one complex with synthetic AUUUA repeats is identical to that of complex 4 of the wild-type viral sequence. Although identical mobilities do not directly indicate shared factors, these data represented strong circumstantial evidence that complex 4 might incorporate AUUUA sequence-associated component(s). Therefore, as a possible source from which to isolate AUUUA-specific proteins, we focused on complex 4 in subsequent studies.

Identification of AUUUA sequence-associated polypeptides in binding complexes
To characterize AUUUA-specific polypeptides in complexes, proteins were u.v.-cross-linked with radiolabelled RNA probes. Conventionally, u.v. cross-linking is performed with crude extracts in binding reactions. This method, however, is prone to non-specific signal transfer and degradation of RNA and proteins. We developed an alternative approach, where non-cross-linked complexes were electrophoretically separated first, immediately after brief incubation. Electrophoretic forces, running time and dilution in gel buffer allowed only highly specific proteins to remain RNA-associated and these were then u.v.-cross-linked in situ. After isolation and RNase A and T1 digestion, SDS-PAGE was performed.

First, we compared proteins of the viral wild-type complex 3 with wild-type complex 4 to investigate
whether there were additional proteins in complex 4 compared to complex 3, because complex 4 appeared to contain AUUUA sequence-associated factor(s). Identical patterns were observed, with an additional 70K protein in complex 4, as shown in Fig. 4(a). Since complex 3 formed with the mutant sequence, but complex 4 appeared only when intact AUUUA repeats were present, the result suggested that the 70K protein was probably involved in the association of complex 4 with the AUUUA repeat.

To confirm this role of the 70K polypeptide, protein patterns were also compared between the mutant and wild-type sequences (Fig. 4b). The 70K protein was present in the wild-type complex but absent in the mutant pattern, and a minor 32K polypeptide also showed AUUUA specificity. In addition, a new protein band appeared at the 55K range with the mutant probe.

Complexes 5 and 6 were also isolated and their polypeptide patterns compared. As shown in Fig. 4(c), the 70K factor is a major binding protein in the wild-type pattern and absent with the mutant probe in a similar way to the minor 32K protein. The 55K new band was also observed in the mutant pattern.

The appearance of the 55K protein raised the possibility that it might arise from the 70K factor. Different RNase degradation of the mutant probe may result in fewer u.v.-cross-linked nucleotides and that might affect electrophoretic mobility. The following considerations, however, argue against this possibility. Firstly, the difference in nucleotides cannot account for the 15K shift in protein mobility. Secondly, the original sequence and the mutant are equally susceptible to degradation by RNase A or T1. Thirdly, the amount of protein missing from the 70K bands should reappear in the 55K range, which was not observed. Consequently, the 55K protein is probably an AUGUA sequence-associated factor and needs to be characterized in future studies.

To confirm the AUUUA specificity of the 70K protein, polypeptides complexing with the synthetic AUUUA repeats were compared with proteins binding to the 5' end region of HSUR2 (and also with those binding to the IL-4 3' non-coding AUUUA region; see below). Polypeptide patterns showed that the synthetic AUUUA repeats bind the 70K protein (Fig. 4d). It should be noted that the actual M, values of polypeptides may be somewhat different from the ones measured owing to nucleotide residue(s) being covalently linked to proteins after signal transfer.

The 70K protein can also complex with the IL-4 mRNA 3' non-coding region

To investigate whether the 70K protein can form a complex with the 3' non-coding region of IL-4 mRNA, polypeptides of complex 4 with the viral wild-type and IL-4 AUUUA regions were characterized. As shown in Fig. 4(d) and Fig. 5(a), polypeptide patterns of viral and IL-4 RNA-binding proteins were similar, but not identical. A protein in the 70K electrophoretic mobility range, however, was consistently present in the IL-4
RNA-binding complex. In order to investigate whether the 70K polypeptides from the viral and IL-4 complexes were identical, the proteins were electroeluted from SDS-polyacrylamide gels and characterized by V8 protease analysis. The results in Fig. 5(b) demonstrate that although the IL-4 RNA-associated polypeptide was somewhat degraded, the patterns of the 70K proteins from the viral and IL-4 RNA complexes were identical.

Discussion

The results presented in this work on a small nuclear RNA (HSUR2) of HVS demonstrate that the conserved 5'-terminal AU-rich region binds proteins with high specificity. We report a novel 70K binding factor and show that it is specific for the conserved AUUUA repeats in the viral sequence. Partial proteolytic analysis demonstrated that the same protein can complex with the IL-4 mRNA 3' non-coding region, which contains three AUUUA motifs. Non-radiolabelled competition experiments showed that the viral sequence competes with the IL-4 mRNA for binding, and indicated approximately fivefold higher affinity of the IL-4 mRNA sequence over the viral probe. We also observed a minor AUUUA-specific polypeptide in human lymphocyte extracts with the same electrophoretic mobility as a 32K marmoset protein reported to bind the viral sequence (Myer et al., 1992) and implicated in growth factor mRNA destabilization (Myer et al., 1992; Vakalopoulou et al., 1991).

The AUUUA repeats of the nuclear RNAs HSUR1 and HSUR2 are highly conserved in different strains of HVS, indicating a biologically important function for the sequence. In a search for this function we found that the same sequence motif occurs in the 3' end non-coding mRNA regions of growth factors and proto-oncogenes and functions as a destabilization signal. Recent data indicate that the instability of short half-life mRNAs is predetermined in the nucleus (Vakalopoulou et al., 1991). The conserved AUUUA repeats tag mRNAs for immediate degradation in the nucleus, or when they enter the cytoplasm. In post-transcriptional regulation of growth factor expression, protein factors bind and protect these sequences from rapid decay.

Since the viral small RNAs are abundantly expressed in the nucleus (2 x 10^4/cell; Lee & Steitz, 1990), we determined whether they could interact with factors engaged in the nuclear regulation of mRNA destabilization. The results of this report demonstrate that the viral AUUUA repeats can bind multiple factors from protein complexes associated with mRNA destabilization regions. The function of the 70K factor has yet to be determined, but it clearly establishes a novel link between the AUUUA motifs of HSUR RNAs and growth factor mRNA turnover regulation.

Involvement of two proteins (the 70K and 32K factors) reflects the complexity of the regulation that directs
mRNA stability (Schiavi et al., 1992). Multiple proteins may represent the balance between protective and destabilizing forces. Consequently, viral small RNAs may stabilize mRNAs in two possible ways. They could sequester destabilizing factors, or they could activate protective factors.

Destabilization factors can be directly nucleolytic. Our data do not rule out the possibility that the detected factor(s) are nucleases. The hallmarks of mRNA turnover-related ribonucleases are the requirement for a divalent cation and RNase inhibitor-resistant specific probe degradation (Ross et al., 1987; Wagner & Assosian, 1990). These were both observed with the protein reported here. In addition, u.v. crosslinking can identify endoribonucleases (D. C. Eichler, personal communication). It has yet to be determined whether nuclease activity is a direct function of the AUUUA binding factors.

Alternatively, if the identified proteins are protective factors, the viral U-type small RNAs may facilitate their transport to the nucleus. Primary transcripts of U-type RNAs are exported to the cytoplasm, where they complex with ribonucleoproteins and return to the nucleus (Zeller et al., 1983; Madore et al., 1984). AUUUA elements of HSUR RNAs would take up AUUUA-binding factors in the cytoplasm and, by releasing them in the nucleus, the accumulation of protective factors could stabilize mRNAs. Notably, the IL-4 3' non-coding region exhibits fivefold higher affinity for binding than the viral 5' AU-rich region (Fig. 2) indicating that the IL-4 sequence could easily take up proteins from the HSUR complex. Further experiments are required to explore this alternative.

Although the mechanism of mRNA destabilization is not well understood, some factors have already been characterized. These proteins, however, are smaller in size than the 70K polypeptide and probably unrelated to this factor. On the other hand, several proteins in the 68K to 75K range have been shown to complex with primary transcripts or mRNAs. A 71K factor was reported to bind U-rich sequences (U-rich binding protein; URBp) in the c-fos mRNA 3' non-coding region (You et al., 1992). This factor complexes with stretches of five to seven U residues implicated in poly(A) degradation but, unlike the protein of the present report, no binding was observed with AUUUA elements and mutations of the AUUUA motifs had no effect on binding.

Recent data indicate that mRNA destabilization is preceded by poly(A) decay (Brewer & Ross, 1988) under the regulation of the 3' non-coding mRNA region (Wilson & Treisman, 1988). The poly(A) binding protein (PABP) is a 72K factor (Bernstein et al., 1989), and one domain binds U-rich sequences (Burd et al., 1991) although no AUUUA sequence binding has been reported.

Several other proteins in the 70K M, range are involved in mRNA processing and have poly(U) or polypyrimidine binding affinity [for example the 68K L hnrNRP protein (Pinol-Roma et al., 1989), the 70K intron binding protein or IBP (Gerke & Steitz, 1986; Tazi et al., 1986), the 65K U2 auxiliary factor U2AF (Zamore & Green, 1991) and the 70K polypeptide of U1 snRNP (Theissen et al., 1986)]. No AUUUA binding has been reported with these proteins.

Although very little is known about the actual molecular mechanisms leading to HVS malignancy, circumstantial evidence also suggests that the viral small RNAs are involved in oncogenesis, and that growth factor mRNA stabilization may be part of the process. Expression of viral small RNAs is consistently observed in all HVS-transformed T cell lines and deletion experiments have demonstrated that the loss of HSUR1 and HSUR2 in strain 11 has a negative effect on cell proliferation (Murthy et al., 1989). Preliminary data show that HSUR1 and HSUR2 deletion mutants of HVS strain 484-77 are non-oncogenic in New Zealand white rabbits. Changes in growth factor, growth factor receptor and lymphokine expression in HVS-transformed tumour cells have already been observed, although no solid evidence exists to relate them to HSUR expression.

In summary, this report further explores the possibility that HVS small RNAs have the potential to interfere with the transport, delivery or distribution of mRNA turnover regulatory factors in the nucleus and may ultimately contribute to viral oncogenesis. Characterization of the 70K factor, its function in mRNA stability, and its putative role in HVS transformation await further investigations.

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