AU-rich mRNA instability elements on human papillomavirus type 1 late mRNAs and *c-fos* mRNAs interact with the same cellular factors

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Expression levels of human papillomavirus type 1 late genes are determined in part by an AU-rich inhibitory sequence in the 3' untranslated region on the late mRNAs. Fine mapping of the AU rich inhibitory sequence revealed that it mapped to a 57 nucleotide sequence, consisting of an AU-rich region containing two AUUUA motifs and a U-rich region containing three UUUUU motifs. An internal deletion showed that the U-rich region was required for inhibition. Point mutations in the AUUUA- and UUUUU-motifs inactivated the inhibitory sequence. Analysis of the stability of mRNAs containing the AU-rich sequence in sense or anti-sense orientation showed that mRNAs containing the AU-rich sequence in sense orientation had reduced half life. Analysis of RNA-protein interactions revealed that binding to the inhibitory sequence of three poly(U) binding proteins (38, 44 and 46 kDa) correlated with inhibition and that the same proteins bind to the AU-rich mRNA instability element in the 3' untranslated region on the human *c-fos* mRNA. We speculate that the human papillomavirus late mRNAs, produced from several hundred copies of the virus genome present in infected cells, compete with the *c-fos* mRNAs for destabilizing cellular factors and that this may lead to elevated Fos protein levels in human papillomavirus infected cells

Keywords: HPV-1; *c-fos*; RNA stability; hnRNP

Introduction

Human papillomaviruses (HPVs) are a heterogeneous group of epitheliotropic, small non-enveloped icosahedral viruses, containing a single circular double-stranded DNA molecule of about 8000 base pairs (bp) (Chow and Broker, 1994; Howley, 1991; Laimins, 1993; Lambert, 1991; Turek, 1994). Some HPV types infect cutaneous epithelium, while others infect mucosal epithelium. The former group includes HPV type 1 (HPV-1), the etiologic agent of warts on the plantar surface of the foot, and the latter group includes HPV type 16 (HPV-16), which is associated with cervical intraepithelial neoplasias and cervical cancer (zur Hausen, 1991). Following infection of cells in the basal layers of stratified epithelia, virus early (E) genes (Figure 1a) are expressed, whereas expression of the late viral genes (L1 and L2) (Figure 1a) and virus production, is restricted to terminally differentiated keratinocytes in the upper strata of squamous epithelium (Howley, 1991). It is likely that HPV late

gene expression is regulated both at the level of transcription and post-transcriptionally. A differentiation inducible promoter has been identified in HPV-16 (Grassmann *et al.*, 1996). In addition, the late mRNAs of HPV-1 and HPV-16 contain negative elements which decrease expression levels in proliferating cells (Kennedy *et al.*, 1990, 1991; Tan *et al.*, 1995; Tan and Schwartz, 1995). The distantly related bovine papillomavirus type 1 (BPV-1) also encodes an RNA element that reduces the levels of late mRNAs (Furth and Baker, 1991; Furth *et al.*, 1995), indicating that negative elements are conserved and important in the papillomavirus life cycle. In HPV-16, these elements are located in the L1 coding region (Tan *et al.*, 1995) and in the late 3' untranslated region (UTR) (Kennedy *et al.*, 1990, 1991; Tan *et al.*, 1995). In HPV-1, we have previously identified an AU-rich cis-acting inhibitory RNA sequence, located in the HPV-1 late 3' UTR (Tan and Schwartz, 1995) (Figure 1a and b). This element acts by reducing mRNA and protein levels (Tan and Schwartz, 1995). This effect is presumably dependent on nuclear factors since transcription of the HPV-1 mRNA sequence in the cytoplasm using the recombinant vaccinia virus T7 RNA polymerase expression system bypassed inhibition (Tan and Schwartz, 1995). Nuclear factors that interact with the HPV-1 RNA element to form specific RNA-protein complexes have been detected (Zhao *et al.*, 1996).

Results

Mapping of the minimal inhibitory RNA sequence

We have previously identified an AU-rich inhibitory sequence within the 3' UTR of HPV-1 late mRNAs (Tan and Schwartz, 1995) (Figure 1a and b). However, early experiments did not exclude the existence of more than one negative element in the HPV-1 late 3' UTR. Therefore, we first introduced internal deletions in the late 3' UTR, by digesting plasmid pBAX (Figure 1c) with combinations of restriction enzymes *Asp718*, *BssHII* and *XhoI* (Figure 1c). These sites were introduced in the late 3' UTR by site specific mutagenesis. Analysis of chloramphenicol acetyltransferase (CAT) production from the resulting plasmids pBAX, pΔKXb, pΔKB, pΔXX and pΔBX (Figure 1c) revealed that only pΔBX, in which the AU-rich inhibitory sequence was deleted, produced significantly higher CAT levels than plasmids in which sequences upstream or downstream of this element were deleted (Figure 1c), demonstrating that the AU-rich sequence was the major inhibitory sequence in the HPV-1 late 3' UTR. However, introduction of 10 nucleotide deletions in this region, as in pΔ13 and

pΔ15 (Figure 1c), did not show an effect on CAT expression (Figure 1c). Interestingly, analysis of the predicted RNA secondary structures suggested that the RNAs containing the 10 nucleotide (nt) deletions were differently folded (not shown). The RNA secondary structure of this element appeared to be less important for its activity than the primary RNA sequence.

We next investigated the activity of the inhibitory element in cells of different origin. pCCKH1, which contains inhibitory sequences, or pΔKXb in which the inhibitory sequences are deleted (Figure 1c) were transfected into six different cell lines of human

origin: HeLa, 293, Jurkat, Raji, U937-2, and K562. Figure 1d shows a six to 20-fold difference in CAT production between pCCKH1 and pΔKXb in the different cell lines, indicating that the inhibitory sequences are recognized in different cell types. In addition, transfections of cell lines from monkey, hamster, rat, and mouse, revealed that the HPV-1 AU-rich inhibitory sequence was recognized in cells from different species (Figure 1d), suggesting that this regulatory mechanism is active in different cell types and is conserved among different mammalian species.

To define the minimal inhibitory sequence, we inserted fragments of different lengths of the HPV-1 AU-rich sequence downstream of CAT in pNLCATW (Tan *et al.*, 1995) (Figure 2a). The resulting plasmids were transfected in triplicates into HLTat cells (Schwartz *et al.*, 1990) in the presence of the internal control plasmid pCS1X (Tan *et al.*, 1995), producing secreted alkaline phosphatase (SEAP). The results revealed that fragments B and C were only partially inhibitory (Figure 2b), whereas fragments L, D and F, inhibited CAT expression between 10- to 13-fold (Figure 2b) and predicted that the minimal inhibitory sequence was located between nt 6958 and nt 7014 (Danos *et al.*, 1982). To confirm this, fragment XB (nt 6958 to 7014) was inserted into pNLCATW, generating pXB (Figure 2a). This plasmid produced CAT levels comparable to those produced from pL, pD and pF (Figure 2b), demonstrating that the major HPV-1 AU-rich inhibitory sequence contained 57 nts and was located between nt 6958 and nt 7014. We have previously shown that the AU-rich region upstream of nt 6984 is required for inhibition (Tan and Schwartz, 1995). To confirm that the U-rich region between 6984 and 7009 (Figure 2a) was required for inhibitory activity, an internal deletion which removed the three UUUUU-stretches was introduced in the L fragment, resulting in plasmid pΔpU (Figure 2a). This plasmid produced higher levels of CAT, compared to the

parental pL plasmid (Figure 2b), establishing that the U-rich region was required for inhibitory activity.

We next tested if sequences shorter than those present in pXB were inhibitory. Neither two AUUUA motifs, as in B2 (Figure 3a), nor three UUUUU motifs as in C1 (Figure 3a), were able to inhibit gene expression to the same extent as the XB fragment (Figure 3b), which essentially consisted of the sequences present in pB2 and pC1 (Figure 3a), suggesting that both AUUUA motifs and UUUUU motifs were required for efficient inhibition. However, pBC which encoded one AUUUA motif and one UUUUU motif (Figure 3a) produced higher CAT levels than pXB (Figure 3b), suggesting that multiple copies of AUUUA together with multiple copies of UUUUU were required for efficient inhibitory activity.

U to C substitutions in the AUUUA- and UUUUU-motifs abolished inhibitory activity

In order to investigate the importance of the AUUUA- and the UUUUU-motifs contained in the XB sequence, we substituted two Us in each AUUUA or UUUUU with two Cs, resulting in pAUM, pUM, and pAUM/UM (Figure 3c). These plasmids and pXB were separately transfected into HLTat cells (Schwartz *et al.*, 1990) and the CAT levels were compared to those produced from the parental plasmid pNLCATW. The

^d	<u>Cells</u>	<u>Origin</u>	<u>Fold inhibition</u>
	HeLa	(hu, epithelioid)	20 ± 3,4
	293	(hu, kidney)	8 ± 2,0
	Jurkat	(hu, T-cell)	11 ± 0,5
	Raji	(hu, B-cell)	10 ± 1,2
	U937-2	(hu, monocyte)	16 ± 4,0
	K562	(hu, undifferentiated blast)	6 ± 0,4
	CV-1	(monkey, kidney)	7 ± 0,2
	BHK-21	(hamster, kidney)	6 ± 1,0
	PC-12	(rat, pheochromocytoma)	7 ± 0,5
	PA-317	(mouse, fibroblast)	4 ± 1,5
	Balb/MK	(mouse, keratinocyte)	10 ± 1,4

Figure 1 (a) Genomic organization of HPV-1. Open boxes represent early (E) open reading frames (ORFs), light grey box represents the L2 ORF, dark grey box represents the L1 ORF and the black box represents the non-coding region (NCR), including the late 3' UTR. (b) AU content in the HPV-1 3' end of the L1 coding region and the late 3' UTR. The positions of the HPV-1 late poly(A) signals (pA1 and pA2) (Tan and Schwartz, 1995) are indicated. Numbers refer to nt positions in the genomic HPV-1 clone (Danos *et al.*, 1982). Discontinuous vertical lines mark the approximate location of the HPV-1 AU-rich sequence (Tan and Schwartz, 1995). (c) Internal deletions in the HPV-1 late 3' UTR. HeLa cells were transiently transfected and the CAT levels produced from each plasmid were determined by a CAT ELISA, and normalized to SEAP levels produced from the internal control plasmid pCS1X (Tan *et al.*, 1995). The human cytomegalovirus immediate-early (CMV) promoter, the CAT reporter gene, the HPV-1 sequences and the HPV-1 late poly(A) signals (pA1 and pA2) (Tan and Schwartz, 1995) are indicated. Brackets mark the limits of the deletions and numbers refer to positions on the HPV-1 genomic clone (Danos *et al.*, 1982). Positions of restriction enzyme recognition sites used for generation of the deletion mutants are indicated. Plasmid names are indicated on the left. The fold induction in CAT activity compared to those produced from pCCKH1 in a representative experiment are shown on the right. (d) The inhibitory HPV-1 sequence is active in cells of different origin. Fold inhibition represent the relative difference in CAT production from plasmids pCCKH1 and pΔKXb after transfection into cell lines of various origin as indicated. hu, human

Figure 2 Mapping of the minimal inhibitory sequence (a) Structures of the plasmids. The HIV-1 LTR and the CAT reporter gene are indicated. Capital letters represent the primary HPV-1 RNA sequence present in each plasmid, underlined sequences indicate the positions of the AUUUA motifs, doubly underlined sequences indicate the positions of the UUUUU motifs, and the brackets mark the limits of the deletion in p Δ pU. Plasmid names are indicated on the left. Numbers refer to nt positions on the HPV-1 genomic clone (Danos *et al.*, 1982). (b) HLTat cells (Schwartz *et al.*, 1990) were transiently transfected in triplicates and the CAT levels produced from each plasmid were determined by a CAT ELISA. Histograms show mean values and standard deviations of arbitrary CAT units produced from each plasmid, after normalization to SEAP levels produced from the internal control plasmid pCSIX (Tan *et al.*, 1995)

orientation (Figure 4d). Levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were similar through the course of the experiment (Figure 4b). In addition, we measured the mRNA levels in cells transfected with pXB or pAUM/UM which were either not treated or treated for 1 h with actinomycin D. The results revealed that mRNA levels produced from pXB were fourfold lower after 1 h actinomycin D treatment, whereas the mRNA levels produced from pAUM/UM remained unchanged (Figure 4c). This is consistent with a stabilization of the mRNA when the AUUUA and UUUUU motifs were mutated. To confirm these results, we next used plasmid pCHPV1pA which contained the complete HPV-1 late 3' UTR in its natural position upstream of the two late poly(A) signals pA1 and pA2 (Figure 5a) and pCHPV1ΔpA in which the *h1ARE* was deleted. Measurements of mRNA levels at different time points in transfected, actinomycin D treated cells revealed that deletion of the negative element markedly stabilized the produced mRNAs (Figure 5b and c). We concluded that the *h1ARE* acted by reducing mRNA stability.

Mapping of protein binding sites in the *h1ARE*

Previous experiments demonstrated that cellular factors bind to the *h1ARE* (Zhao *et al.*, 1996). Here we wished to map the binding sites of these proteins within the *h1ARE* and determine which of the proteins that failed to interact with the inactive, mutant *h1ARE*. Using RNA probe L (Figure 6a) in u.v. cross linking experiments, we detected six nuclear proteins of the following sizes: 38, 41, 44, 46, 52 and 80 kDa (Figure 6b) (Zhao *et al.*, 1996). The same pattern of bands was observed when radiolabelled XB RNA, which contains the minimal *h1ARE* (Figure 6a), was used in u.v. cross linking (Figure 6b). Fine mapping of the protein binding sites using RNA fragments B3, B2, BC, C1, C2 and C3 (Figure 6a), revealed that the 52 and 80 kDa proteins cross-linked efficiently to RNA B2 (Figure 6c), and less efficiently to B3 and BC, whereas RNA C1 detected five nuclear proteins (38, 41, 44, 46 and 80 kDa) (Figure 6c). These five proteins were also detected using probes BC or C3 (Figure 6c), which was consistent with binding of these proteins to the U-rich region, since the UUUUU motif was repeated three times in C1, but were present only once or twice in BC and C3, respectively (Figure 6a). The 52 kDa protein apparently interacted with sequences other than UUUUUU reiterations and the 80 kDa protein either contained multiple RNA binding domains with different specificities, recognized RNA sequences with a high AU content or bound to RNA in a non-sequence specific manner. None of the proteins cross linked to RNA probe C2 (Figure 6c).

The 38, 44 and 46 kDa proteins do not bind to mutant AUM/UM or UM which have reduced inhibitory activity

To investigate the ability of the various proteins to u.v. cross link to mutant *h1ARE* with impaired inhibitory function, we used the radiolabelled mutant HPV-1 RNA sequences named AUM, UM, CUC and AUM/UM depicted in Figure 3c, in u.v. cross linking assay. RNA AUM cross linked to all proteins detected using RNA XB (Figure 6d), demonstrating that intact

AUUUA motifs were not required for RNA binding of the proteins detected here. In contrast, mutant RNA UM and AUM/UM failed to detect the 38, 44 and 46 kDa proteins (Figure 6d), whereas all proteins bound efficiently to pCUC (Figure 6d), indicating that the 38, 44 and 46 kDa proteins recognized stretches of Us, consisting of more than three Us. Since UM and AUM/UM also showed lower inhibitory activity in transient transfection assay (Figure 3d), we concluded that binding of at least one of these three proteins was required for inhibitory activity. The 80 kDa protein binds to both B2 and C1 and binding appeared unaffected by the point mutations present in mutant RNA AUM/UM (Figure 6d). In conclusion, binding of the 38, 44 and 46 kDa proteins correlated with inhibitory activity of the *h1ARE*. The results from the mapping experiments are summarized in Figure 6e. u.v. cross linking of total cytoplasmic extract or ribosome associated proteins did not detect additional proteins that cross linked to wild type RNAs and mutant RNAs (data not shown).

Poly(U) competes with the *h1ARE* for all proteins detected here

To characterize the HPV-1 RNA binding proteins further, competition experiments using homoribopolymers were performed. Figure 7 shows that poly(U) competed efficiently with RNA XB for the 38, 44 and 46 kDa proteins and to a lower extent for the 80 and 52 kDa proteins. The 41 kDa protein appeared to have the lowest affinity for poly(U) (Figure 7). Interestingly, the 52 kDa protein also appeared to bind to poly(C), while poly(A) and poly(G) failed to compete with RNA XB for the proteins detected here (Figure 7). In conclusion, the 38, 41, 44, 46 and 80 kDa proteins were poly(U) binding proteins whereas the 52 kDa protein had affinity for polypyrimidine sequences.

The *h1ARE* and the *c-fos ARE* interact with the same cellular proteins

The ARE in the *c-fos* 3' UTR shows structural homology to the *h1ARE* (Tan and Schwartz, 1995). For example, both elements contain AUUUA motifs and a U-rich region. We speculated that nuclear proteins that interact specifically with the *h1ARE* may bind specifically also to the *c-fos ARE*. Radiolabelled riboprobe L and FOS were incubated with nuclear extracts from HeLa cells, respectively. RNA gel shift assay revealed that RNA-protein complexes were formed (data not shown). Competition experiments using unlabelled RNA FOS or tRNA as competitors showed that unlabelled RNA FOS, but not tRNA inhibited complex formation between cellular factors and the *h1ARE*, indicating that at least some cellular proteins that interact with the *h1ARE*, bind also to the *c-fos ARE* (Figure 8a).

To investigate whether the *c-fos ARE* cross linked to nuclear proteins from HeLa cells, radiolabelled riboprobes FOS and L were analysed in parallel using u.v. cross linking. Five nuclear proteins with molecular masses of approximately 80, 52, 46, 44 and 38 kDa were observed to cross link efficiently to probe FOS and L (Figure 8b), further indicating that probe FOS and L interacted with the same proteins. Competition

experiments were performed followed by u.v. cross linking (Figure 8c). The results showed that unlabelled RNA FOS competed efficiently with the radiolabelled riboprobe FOS for binding to 80, 52, 46, 44 and 38 kDa proteins, whereas tRNA did not (Figure 8c). Therefore, these results demonstrated that the interactions between RNA FOS and the nuclear proteins from HeLa cells detected here were specific.

We next tested if the *hIARE* competed with FOS RNA for binding to the detected nuclear proteins by using HPV-1 RNA transcripts containing various portions of the *hIARE* as competitors. As shown in Figure 8c, HPV-1 RNA L and C competed efficiently with probe FOS for all nuclear proteins that were detected using the FOS probe, indicating that all proteins binding to the RNA FOS also bind to the HPV-1 RNA. Poly(U) competed with probe FOS for all the detectable proteins (Figure 8c). Using the two subfragments of C, C1 and C2, as competitors,

demonstrated that only C1 competed with the *c-fos* RNA (Figure 8c), confirming that the proteins detected here bind primarily to the U-rich region. In contrast, HPV-1 RNA B did not compete significantly with the *c-fos* RNA, except for the 80 kDa protein.

To confirm that the *hIARE* and the *c-fos* ARE interacted with the same proteins, we partially purified these proteins on mono Q and heparin columns and then tested their ability to bind to the *hIARE* and the *c-fos* ARE. Fractions with *hIARE* binding activity were identified by RNA gel shift and u.v. cross linking (not shown). Partially purified proteins in selected fractions from the heparin columns (10KH₂₂, 50KH₂₀ and 200KH₂₈₋₃₆) were subjected to u.v. cross linking using HPV-1 probe L or FOS. The results showed that the 80 kDa protein in fraction 10KH₂₂, the 38 kDa protein in fraction 50KH₂₀, and the 44 and 46 kDa proteins in fraction 200KH₂₈₋₃₆ cross linked to both HPV-1 probe L and probe FOS (Figure 9a). The

52 kDa protein was purified on mono S- and heparin-columns and was shown to cross link to both HPV-1 RNA L and FOS RNA (not shown). Therefore, the results confirmed that the *hIARE* and the *c-fos ARE* interact with the same cellular proteins.

We next wished to identify the 38, 44 and 46 kDa proteins that cross linked to the *hIARE* but not to the mutant RNAs and to the *c-fos ARE*. The u.v. cross

linked proteins were then subjected to immunoprecipitation using antibodies or sera against different RNA-binding proteins that interact with AU-rich, U-rich or pyrimidine-rich RNA sequences: hnRNP A1, hnRNP I, hnRNP C1/C2, U2AF65, U170K La and Ro60 and SR proteins. The antibodies used are indicated above the gel (Figure 9b). The 44 and 46 kDa proteins could be immunoprecipitated by the anti-hnRNP C1/C2

Figure 3 The inhibitory sequence requires two AUUUA motifs and three UUUUU motifs. (a, c) The HIV-1 LTR and the CAT reporter gene are indicated. Capital letters represent the primary HPV-1 RNA sequence present in each plasmid, underlined sequences indicate the positions of the AUUUA motifs, and doubly underlined sequences indicate the nt positions of the UUUUU motifs. The positions of uridine to cytosine base substitutions are shown and are indicated by X below the sequence. Plasmid names are indicated on the left, and numbers refer to positions on the HPV-1 genomic clone (Danos *et al.*, 1982). (b, d) HLTat cells (Schwartz *et al.*, 1990) were transiently transfected in triplicates and the CAT levels produced from each plasmid were determined by a CAT ELISA. Histograms show mean values and standard deviations of arbitrary CAT units produced from each plasmid after normalization to SEAP levels produced from the internal control plasmid pCS1X (Tan *et al.*, 1995)

antibody, but not by any of the other antibodies or sera (Figure 9b). The 38, 41, 52 and 80 kDa proteins were not recognized by the antibodies or sera tested here.

To obtain further proof that the 44 and 46 kDa proteins were indeed hnRNP C1 and C2, we separated the 44 and the 46 kDa proteins from the other *hIARE* binding proteins by using mono Q and heparin

Figure 4 The *hIARE* acts as an mRNA instability determinant. (a) Structures of the plasmids. The HIV-1 LTRs, the CAT reporter gene, and the HPV-1 sequences are indicated. Plasmid names are indicated on the left. Numbers refer to positions on the HPV-1 genomic clone (Danos *et al.*, 1982). (b) Total cytoplasmic RNA was harvested from cells transfected with p167(S) or p167(A) after treatment with actinomycin D (10 μ g/ml) for 0, 1, 2 or 4 h, as indicated. RNA levels were analysed by primer extension, and normalized to the GAPDH levels as indicated. (c) Total cytoplasmic RNA was harvested from cells transfected with pXB or pAUM/UM after treatment with actinomycin D (10 μ g/ml) for 0 and 1 h, as indicated. RNA levels were analysed by primer extension, and normalized to the GAPDH levels as indicated. (d) RNA decay plot for p167(S) and p167(A), as quantified by using a phosphorimager (Molecular Dynamics). The background levels, monitored immediately above the CAT mRNA signal in the gel, were subtracted from the CAT mRNA levels

columns. The results presented above (Figure 9b) predicted that the anti-hnRNP C1/C2 monoclonal antibody should react with the 44 and 46 kDa proteins in fractions 200KH28–36 (Figure 9a) from the heparin column. These proteins should not be present in the other fractions. Western blot analysis of the fractions using anti-hnRNP C1/C2 monoclonal antibody as a primary antibody, revealed that the nuclear extract and 200KH28–36 contained the 44 and 46 kDa proteins recognized by the anti-hnRNP C1/C2 monoclonal antibody (Figure 9c). We concluded that the 44 and the 46 kDa proteins were hnRNP C1 and C2.

Discussion

We have shown that (i) the *hIARE* spans 57 nt and acts as an mRNA instability element, (ii) efficient inhibitory activity requires two AUUUA repeats and three UUUUU repeats, (iii) binding to hnRNPC1/C2 and the 38 kDa poly(U) binding protein correlates with inhibitory activity and (iv) that the *hIARE* interacts with the same cellular factors as the *c-fos* ARE.

The *c-fos* ARE can be divided into an AU-rich region containing three AUUUA motifs in an AU-rich context, and a U-rich region. Interestingly some of the properties of the HPV-1 AU-rich element are similar to

those previously described for the *c-fos* ARE (reviewed in (Chen and Shyu, 1995)). The *c-fos* mRNAs are very unstable with a half-life of less than 30 min, and are usually present at low levels in cells. It is generally believed that the natural expression of the *c-fos* gene does not transform cells, whereas inappropriate or overexpression of the *c-fos* gene may induce transformation of primary or established cell lines (Schiavi *et al.*, 1992). It has been shown that the presence of the *c-fos* ARE renders the *c-fos* mRNA short-lived (Shyu *et al.*, 1989) and that the *c-fos* gene can be converted into a transforming oncogene when the ARE in the 3' UTR of *c-fos* mRNA is deleted (Lee *et al.*, 1988; Meijlink *et al.*, 1985; Raymond *et al.*, 1989). Stabilization of *c-fos* mRNAs during oncogenesis has been demonstrated in tissue culture cells (Lee *et al.*, 1988; Raymond *et al.*, 1989) and in transgenic mice (Rüther *et al.*, 1989). Factors that influence the stability of the *c-fos* mRNA may therefore affect expression levels of the Fos protein. Since the HPV-1 inhibitory element shares sequence homology with the *c-fos* ARE (Tan and Schwartz, 1995) and both sequences bind to the same nuclear proteins, one may speculate that the production of high levels of the HPV-1 late mRNAs may sequester RNA binding proteins on the HPV-1 inhibitory element. This is not unreasonable since an HPV-1 infected cell may contain up to 1000 copies of

Figure 5 The *hIARE* reduces mRNA stability. (a) Structures of the plasmids. The CMV promoter, the p17gag open reading frame, the HPV-1 late poly(A) signals (pA1 and pA2) and the HPV-1 late 3' UTR-containing sequences are indicated. Brackets mark the limits of the deletion and numbers indicate nt positions in the HPV-1 genomic clone (Danos *et al.*, 1982). Plasmid names are indicated on the left. (b) Total cytoplasmic RNA was harvested from cells transfected with pCHPV1pA or pCHPV1ΔpA after treatment with actinomycin D (10 $\mu\text{g}/\text{ml}$) for 1, 2 or 4 h, as indicated. RNA levels were analysed by Northern RNA blotting. The mRNA produced from pCHPV1pA is larger than the mRNA produced from pCHPV1ΔpA, since the latter plasmid has a deletion in the HPV-1 sequence. (c) RNA decay plot for pCHPV1pA and pCHPV1ΔpA, as quantified by using densitometry (Molecular Dynamics)

That the *c-fos* ARE and the *h1*ARE interact with the same cellular proteins suggests that expression of the HPV-1 late mRNAs and the *c-fos* mRNAs are processed by the same cellular pathway. The binding of several proteins to the *h1*ARE may reflect the complexity of the regulated expression of the HPV-1 late mRNAs and *c-fos* mRNAs. Some of these proteins may target the mRNAs for degradation while others may have opposite functions. The ultimate effect may be dependent on the balance between mRNA protective and destabilizing factors. The life cycle of HPV-1 is strictly linked to the differentiation stage of the epithelial cell (Chow and Broker, 1994; Howley, 1990; Laimins, 1993; zur Hausen and de Villiers, 1994). As the cell differentiates, HPV-1 late gene expression is

Our results established that hnRNP C1 and C2 interacted specifically with the *h1*ARE and the *c-fos* ARE. hnRNP C1/C2 proteins have been shown to interact with the *c-fos* ARE (Vakalopoulou *et al.*, 1991). In another study, Katz *et al.* reported that a 41 kDa RNA-binding activity from Jurkat cells cross linked to an AUUUA-containing sequence, UA(UUUA)₃CUCG, and was immunoprecipitated with monoclonal antibody specific for hnRNP C1/C2 proteins (Katz *et al.*, 1994). Nakamaki *et al.* showed

The hnRNP family hosts several abundant nuclear proteins, some of which appear to be involved in pre-mRNA polyadenylation and splicing (Dreyfuss, 1986). The hnRNP C1/C2 differ by only 13 amino acids and are produced from two different mRNAs generated by alternative splicing (Burd *et al.*, 1989). hnRNP C1/C2 have been shown to cross link to the polypyrimidine tract in the 3' end of the pre mRNA intron (Swanson and Dreyfuss, 1988) and immunodepletion of hnRNP C1/C2 from *in vitro* splicing reactions has suggested a

role for hnRNP in splicing. However, a direct binding to the pre-mRNA of hnRNP C1/C2 does not seem to be important for splicing (Staknis and Reed, 1994). It was previously reported that hnRNP C1/C2 proteins cross linked efficiently to a UUUUU tract downstream of the simian virus 40 late poly(A) signal (Wilusz and Shenk, 1990), suggesting a role for hnRNP C1/C2 in polyadenylation. It was later shown that the efficiency of *in vitro* 3' end processing of mutant RNAs which fail to bind to hnRNP C1/C2 was not impaired in *in vitro* 3' end processing (Chou *et al.*, 1994). Several investigators have reported that hnRNP C1/C2 interact with AU-rich RNA instability determinants on labile mRNAs (Ross, 1995). In summary, hnRNP C1/C2 have been shown to interact with rapidly degraded RNA sequences such as cleavage products from the mRNA 3' end processing and pre-mRNA splicing reactions, and to AREs of labile mRNAs. It is tempting to speculate that the binding to an mRNA of hnRNP C1/C2, earmark these mRNAs for rapid degradation. However, hnRNP C1/C2 is an abundant cell protein with apparently simple RNA binding requirements and it may therefore interact with

Figure 6 Mapping of nuclear protein binding sites in the *hIARE*. **(a)** Primary sequences of the HPV-1 RNA probes used in u.v. cross linking. AUUUA motifs are underlined and UUUUU motifs are doubly underlined. Names of the probes are indicated on the left. Numbers refer to nt positions on the HPV-1 genomic clone (Danos *et al.*, 1982). **(b)** u.v. cross linking of nuclear proteins from HeLa cells to radiolabelled L and XB probes, as indicated. Numbers refer to molecular weights in kilodaltons of the detected proteins. **(c)** u.v. cross linking of nuclear proteins from HeLa cells to radiolabelled RNA L, B3, B2, BC, C1, C3, and C2, as indicated. Numbers refer to molecular weights in kDa of the detected proteins. **(d)** u.v. cross linking of nuclear proteins from HeLa cells to radiolabelled RNA probes CUC, UM, AUM, XB, and AUM/UM, as indicated. The primary RNA sequences of the probes are depicted in Figure 3c. Numbers refer to molecular weights in kilodaltons of detected proteins. **(e)** Schematic illustration of binding of proteins to various parts within the wild type (XB) or mutant (AUM/UM) *hIARE*.

various U-rich RNAs *in vitro*. The functional importance of these interactions remains to be addressed.

The 41, 52 and 80 kDa proteins bind to mutant AUM/UM which lacks inhibitory activity, indicating that binding of these proteins to the *hIARE* is not sufficient for inhibition. However, it is not known if their binding is required for inhibitory function. In contrast, the 38, 44, 46 kDa proteins failed to interact with AUM/UM, demonstrating that at least one of these proteins is required for inhibition. The 38, 44, and 46 kDa proteins failed to bind to UM, as well as AUM/UM, indicating that they recognized the UUUUU motifs but not UUU sequences. This is consistent with the high affinity of these proteins for poly(U). We have previously shown that the 38 kDa protein binds specifically to the HPV-1 late 3' UTR and that it does not interact with the 3' UTR of early HPV-1 mRNAs which lacks inhibitory activity (Zhao *et al.*, 1996), indicating its importance for the inhibitory function of the *hIARE*. While proteins with molecular weights between 40 and 50 kDa cross linked to the 3' UTR of early HPV-1 mRNAs (Zhao *et al.*, 1996), it is not likely that these proteins correspond to the 44 and 46 kDa proteins cross linking to the *hIARE* and identified here as hnRNP C1 and C2. These two proteins bind preferentially to penta-U nucleotides (Görlach *et al.*, 1994) and such sequence motifs are not present in the HPV-1 early 3' UTR. Taken together, our previous results together with those presented here, support a role for hnRNP C1/C2 and the 38 kDa poly(U) binding protein in the regulation of HPV-1 late gene expression.

The protein with estimated molecular weight of 80 kDa, consists of two related proteins with similar molecular masses and binding properties (not shown). Both proteins bind to all mutant HPV-1 RNAs as well as to the non-overlapping B2 and C1 halves of the XB sequence. These proteins may have two different RNA binding domains, with affinity for

different sequences. Alternatively, the 80 kDa protein recognizes RNA without sequence specificity. Since the 80 kDa protein did not bind to poly(A), this is not the poly(A) binding protein. However, it has some properties in common with the AU-rich RNA binding protein nucleolin (Zaidi and Malter, 1995), e.g. it is a nuclear phosphoprotein and may have multiple RNA binding domains. Furthermore, the 80 kDa protein is stable at 37°C when the nuclear extract was incubated in the absence of protease inhibitors (not shown). Proteolysis of nucleolin is inhibited by a factor in nuclear extract (Chen *et al.*, 1991). However, we were unable to immunoprecipitate the 80 kDa protein using monoclonal antibodies to nucleolin (not shown). The 41 kDa protein, which remains bound to AUM/UM and does not bind efficiently to homoribopolymers, is not sufficient for inhibition. Since this protein cross linked to HPV-1 RNA with higher efficiency in the presence of poly(U), it is likely that it binds with lower affinity to the HPV-1 RNA than the other detected proteins.

Several groups identified AU-rich RNA binding proteins based on their ability to interact with reiterated AUUUAs (Chen and Shyu, 1995; Ross, 1995, 1996). In contrast, we were unable to demonstrate a specific interaction between the proteins identified here and the AUUUA motifs. Our results are in agreement with those reported by You *et al.* (1992). These investigators identified four proteins that interacted specifically with the U-rich sequence in the *c-fos* ARE and observed that point mutations in the AUUUA motifs did not alter the number of u.v. cross linked proteins. This is similar to what we observed with mutant AUM, which cross linked to all proteins detected with the wild type HPV-1 RNA sequence. We could not detect proteins that interacted with the AUUUA motifs, but substitutions of two Us for two Cs in both motifs substantially affected the inhibitory activity of the *hIARE*, indicating that these sequence motifs play an important role in the function of the

Figure 7 u.v. cross linking of nuclear proteins from HeLa cells to probe XB in the absence (—) or presence of decreasing amounts (1000, 250, 62.5, 15.6 and 3.9 ng) of poly(U), poly(A), poly(G) or poly(C). u.v. cross linking in the presence of 1000 ng poly(U) is not shown. Numbers refer to molecular weights in kDa of the detected proteins

Figure 8 (a) FOS RNA competes specifically with radiolabelled probe L (P_L) for binding to nuclear proteins. Competitions were performed by incubating nuclear extract in the absence (–) or presence of 50-, 100-, 200-, or 400-fold molar excess of cold FOS RNA competitor, or 200- or 400-fold molar excess of tRNA. (b) Nuclear extracts from HeLa cells were incubated with radiolabelled probe L or FOS, followed by u.v. cross linking. The sizes and positions of the u.v. cross linked proteins are indicated on the left. (c) Nuclear extracts were preincubated with a 200-fold molar excess of each nonradiolabelled RNA competitor (tRNA, FOS, poly(U), L, C, B, C1 or C2) or no competitor (–) at room temperature for 15 min prior to the addition of radiolabelled probe FOS. The reaction mixtures were then subjected to u.v. cross linking. The sizes and positions of the u.v. cross linked proteins are indicated on the left

hIARE. We favor a model in which nuclear proteins interact with the U-rich region of the element, thereby acting at an early stage in the mRNA processing pathway. This is consistent with our earlier results which demonstrated that the human immunodeficiency type 1 (HIV-1) Rev/RRE regulatory axis could overcome the inhibitory effect of the *hIARE* (Tan and Schwartz, 1995). The HIV-1 Rev protein binds to its target RNA element in the nucleus and acts by promoting export of nuclear mRNAs (Emerman *et al.*, 1989; Felber *et al.*, 1989; Hammarström *et al.*, 1989; Malim *et al.*, 1989) through an alternative, productive route (Bogerd *et al.*, 1995; Fisher *et al.*, 1995; Fritz *et al.*, 1995), thereby presumably preventing the *hIARE* from interacting with nuclear proteins that mediate inhibition as the mRNA enters the cytoplasm. Recent reports have described the identification of an RNaseE like activity in mammalian cells and have shown that partially purified mammalian RNaseE cleaves AUUUA containing RNAs *in vitro* (Wennborg *et al.*, 1995). The AUUUA motifs may be targets for RNases in the cytoplasm, explaining the absence of cross linking of nuclear proteins to these sequences.

HPV-16 and BPV-1 late mRNAs also contain negative elements in their 3' UTRs (Furth and Baker, 1991; Kennedy *et al.*, 1990, 1991). A cellular 65 kDa protein recognizes a GU-rich negative regulatory element on HPV-16 late mRNAs (Dietrich-Goetz *et al.*, 1997). This protein was identified as U2AF65. In BPV-1, an unutilized 5' splice site interacts with U1 snRNA to inhibit late gene expression (Furth *et al.*, 1995). Here we show that two AUUUA- and three UUUUU-motifs are required for the function of the *hIARE*. Therefore, different papillomaviruses use different strategies to inhibit late gene expression in proliferating cells. This may reflect the adaptation of HPV-1, HPV-16 and BPV-1 to cutaneous and mucosal epithelial cells and fibroblasts, respectively and may help the virus to escape the immune surveillance of the host.

Materials and methods

Plasmid constructions

The plasmid constructs were generated by using standard procedures (Sambrook *et al.*, 1989). The following plasmids have been described previously: pCS1X (Tan *et al.*, 1995), pCHPV1pA (Tan and Schwartz, 1995), pCHPV1ΔpA (Tan and Schwartz, 1995), pCCKH1 (Zhao *et al.*, 1996), pBAX (described as pCCKH1BAX) (Zhao *et al.*, 1996) and pΔKXb (Zhao *et al.*, 1996). pΔBX was generated by excising a *Bss*HII–*Xho*I fragment from pBAX, followed by T4 DNA polymerase treatment and religation. To construct pΔKB, pBAX was digested with *Kpn*I and *Bss*HII, followed by T4 DNA polymerase treatment and religation. pΔXX was generated by digestion of pBAX with *Xho*I and *Xba*I, followed by T4 DNA polymerase treatment and religation. Deletions Δ13 (nucleotide [nt] 6946–6955; numbers refer to positions in the HPV-1 genomic clone (Danos *et al.*, 1982)) and Δ15 (nt 6966–6975) were generated by site-directed mutagenesis on uracil-containing single-stranded DNA (U-ssDNA) of pKSHPV1pA (Tan and Schwartz, 1995) (as described in Luukkonen *et al.*, 1995), by using oligonucleotides: MASP (5'-GTTTTCGCTTTGTGGTGGGTA CCGTAGCAGTACGTTGTG-3') and M13 (5'-TAATCTACTAATAAA-

Figure 9 Identification of partially purified nuclear proteins that interact with the hIARE. (a) u.v. cross linking of partially purified nuclear proteins with radiolabelled probe L or FOS. After elution from Heparin columns, selected fractions (10KH₂₂, 50KH₂₀ and 200KH₂₈₋₃₆) were subjected to u.v. cross linking with radiolabelled probe L or FOS. The cross linked products were separated on 10% polyacrylamide-SDS gels under reducing conditions. The positions and sizes of the u.v. cross linked proteins are indicated on the left. (b) Immunoprecipitations of u.v. cross linked proteins. Proteins in the nuclear extract from HeLa cells were first u.v. cross linked to radiolabelled probe L, then immunoprecipitated with the different monoclonal antibodies or sera indicated on the top of the gel. Immunoprecipitations were performed as described in Materials and methods. (c) Western immunoblotting of partially purified nuclear proteins with anti-hnRNP C1/C2 specific monoclonal antibody. The positions and sizes (in kDa) of hnRNP C1/C2 are indicated

TATAGTATATACTAAGCCTTACGC-3') or M15 (5'-ATATAAAAAATATATAATAAAATATAGTTATATA-3'). The mutated HPV-1 sequences were transferred as *KpnI*-*XbaI* fragments to pCCKH1, digested with *KpnI* and *XbaI*, generating plasmids pΔ13 and pΔ15.

pL was generated by PCR-amplification of a 109 bp fragment (L; nt 6926-7035 (Danos *et al.*, 1982)) by using oligonucleotides 1S-2a (Tan and Schwartz, 1995) and 1A-4 (Tan and Schwartz, 1995), followed by blunt end ligation to pNLCAW, digested with *KpnI* and treated with CIAP and T4 DNA polymerase. pC, pB, and pD were generated by first excising HPV-1 containing *XhoI*-*BamHI* fragments from pKSC (Zhao *et al.*, 1996), pKSB (Zhao *et al.*, 1996), and pKSD (Zhao *et al.*, 1996), respectively, followed by directional subcloning into pNLCAW, digested with *XhoI* and *BamHI*. In pNLCAW, the unique *Asp718* site downstream of CAT has been replaced by *XhoI*- and *BamHI*-sites. To generate pF, U-ssDNA of pKSL (Zhao *et*

al., 1996) was first subjected to site-directed mutagenesis by using oligonucleotide BAMHI-C (5'-CTTTTATACCTTGATCCTTCTAAATAG-3'), which introduced a *BamHI* site at nt 7015 (Danos *et al.*, 1982), generating pKSL-*BamHI*. pF was constructed by transferring an *XhoI*-*BamHI* fragment excised from pKSL-*BamHI* (nt 6926-7009 (Danos *et al.*, 1982)), to pNLCAW digested with *XhoI* and *BamHI*. To generate pXB, U-ssDNA of pKSL was first subjected to site-directed mutagenesis by using oligonucleotides BAMHI-C and XHOI (5'-GTATATATTATATCTC-GAGATATTTATTAG-3'), introducing an *XhoI*- and *BamHI*-site at nt 6952 and at nt 7015 (Danos *et al.*, 1982), respectively, resulting in pKSL-XB. pKSL-XB was digested with *XhoI* and *BamHI*, and the HPV-1 fragment (nt 6958-7014 (Danos *et al.*, 1982)) was inserted into pNLCAW, digested with *XhoI* and *BamHI*, resulting in pXB. Digestion of pKSL-XB with *XhoI* and *BamHI*, followed by religation, resulted in pKSXB. Site-directed mutagenesis on U-ssDNA

of pKSL, by using oligonucleotide Δ pU (5'-GTAGATTA-TTTATTA TCTTG TTTAG TTCTAAAT AG-3'), deleted nt 6984–7011, resulting in pKSL- Δ pU. pKSL- Δ pU was digested with *Bam*HI and *Xho*I, and the deleted fragment (L Δ pU; nt 6926–7035 (Danos *et al.*, 1982)) was transferred to pNLCATM, digested with *Xho*I and *Bam*HI, generating p Δ pU. pKSB3, pKSC2, and pKSC3 were constructed by annealing oligonucleotides: B3-S (5'-AGCTTATATATTA-TATATAACTATATTTATTAG-3') and B3-A (5'-AATTC-TAATAAATATAGTTATATATAATATATA-3'), 7035S (5'-AGCTTCTTGTTTTGTCTAAATAGTCATGG-3') and 7035A (5'-AATTCCATGACTATTTAGAACTAAAACAA-GA-3'), C3-S (5'-AGCTTTTATACCTTTTATCTTGT-TAGTTG-3') and C3-A (5'-AATTCACCTAAACAAAGTA-AATAAGTATAAAA-3'), respectively, followed by ligation to pBluescript, digested with *Eco*RI and *Hind*III. Annealing of oligonucleotides: B2-S (5'-AGCTTACTATATTTATTAG-TAGATTATTTATTATAG-3') and B2-A (5'-AATTCTA-TAATAAATCTACTAATAAATATAGTA-3'), BC-S (5'-AGCTTAGA TTATTTA TTA TATATTT TTA TAG-3') and BC-A (5'-AATTCTATAAAAAATATATAAATAA-TCTA-3'), or 7011-S (5'-AGCTTATATTTTATATTTT-TACTTTTATAG-3') and 7011-A (5'-AATTCTATAAA-AAGTATAAAAAATATAAAAAATATA-3'), followed by ligation to pBluescript digested with *Hind*III and *Eco*RI, resulted in pKSB2, pKSB3, pKSC1, pB2, pBC, and pC1 were constructed by transferring *Xho*I–*Bam*HI fragments excised from pKSB2, pKSB3, and pKSC1, to pNLCATM digested with *Xho*I and *Bam*HI. Annealing of oligonucleotides: AUMS (5'-AGCTTATACCTATTAGTAGATTACCTAT-TA-3'), AUA (5'-AAAAATATAAAAAATATAAATAA-TAATCTACTAATAAATATA-3'), UMS (5'-TATAT-TCCTATATTCCTATACTTCCTATACTTG-3'), and UA (5'-AATTCAAGTATAAAAAAGTAT-3'), oligonucleotides AUMS, AUA, UM2S (5'-TATATTTTCTCTTTTCT-CCTTTTCTCCTT-3'), and UA, oligonucleotides AUMS, AUA, US (5'-TATATTTTATATTTTATATCTTTT-TACTT-3'), and UA, or oligonucleotides AUS (5'-AGCT-TATATTTATTAGTAGATTATTTATTA-3'), AUA, UMS, and UA, followed by insertion into *Hind*III- and *Eco*RI-digested pBluescript, resulted in pKSAUM/UM, pKSCUC, pKSAUM, and pKSUM. pAUM/UM, pCUC, pAUM, and pUM were constructed by transferring *Xho*I–*Bam*HI fragments from pKSAUM/UM, pKSCUC, pKSAUM, and pKSUM, respectively, to pNLCATM digested with *Xho*I and *Bam*HI. To construct p167(S) and p167(A), a 167 bp fragment (nt 6868–7035 (Danos *et al.*, 1982)) was PCR amplified by using oligonucleotides 1S-2 (Tan and Schwartz, 1995) and 1A-4 (Tan and Schwartz, 1995), followed by blunt end ligation to pNLCATW (Tan *et al.*, 1995), digested with *Asp*718 and subjected to calf intestinal alkaline phosphatase (CIAP)- and T4 DNA polymerase-treatment. To construct plasmid pKSFOS, the four *c-fos* oligonucleotides: FOSS (5'-AGCTTTTATTTATTTATTTAT-3'), FOSS-1 (5'-TAA-GATGGATT CTCAGATATTTATA TTTT ATTT ATTT-TTG-3'), FOSSA (5'-AATTCAAAAATAAAAAATAA-TAAAT-3'), and FOSSA-1 (5'-ATCTGAGAATCCATCT-TAATAAATAAATTAATAAAAA-3') were mixed and heated at 65°C for 5 min, and then annealed at room temperature for 30 min. The annealed oligonucleotides containing 57-nt of the *c-fos* ARE were ligated to the *Hind*III and *Eco*RI digested pBluescript (Stratagene) plasmid, generating plasmid pKSFOS. Plasmids were verified by sequencing of double stranded plasmid-DNA.

Cells and transfections

For transfection of adherant cells (HeLa, HLTat (Schwartz *et al.*, 1990), 293, PA-317, BHK-21, CV-1, and PC-12), 3×10^5 cells were seeded per 60 mm plate 24 h prior to transfection. Transient transfections were carried out by using the calcium-phosphate coprecipitation technique

(Graham and van der Eb, 1973), as described (Tan and Schwartz, 1995). For suspension cells (Raji, Jurkat, K562 and U937-2) and Balb/MK cells, 10^6 cells were transfected with Lipofectamine (Life technologies) according to the manufacturers instructions. The cells were harvested 20–48 h post-transfection, and the amount of CAT protein was quantified in a CAT antigen capture enzyme-linked immunosorbent assay (ELISA: Boehringer Mannheim GmbH). The SEAP producing plasmid pCS1X (Tan *et al.*, 1995) was included in each transfection to control for transfection efficiency. The SEAP-assay was performed as described previously (Tan *et al.*, 1995). The relative CAT levels produced from the various plasmids were similar in at least three independent experiments.

Cellular extracts, in vitro transcription, RNA band shift assay and u.v. cross linking

Nuclear extracts were prepared as described (Dignam *et al.*, 1983). *In vitro* synthesis of radiolabelled RNA was performed as described previously (Zhao *et al.*, 1996), by using linearized pKSL (Zhao *et al.*, 1996), pKSB3, pKSB2, pKSB3, pKSC1, pKSC2, pKSC3, pKSB3, pKSAUM, pKSUM, pKSCUC, pKSAUM/UM or pKSFOS as DNA-templates. RNA gel shift assay and u.v. cross linking of α^{32} P-UTP-labelled RNA to proteins in nuclear extracts were performed as described previously (Zhao *et al.*, 1996). In competition experiments, nuclear extract was preincubated with poly(A), poly(U), poly(G) or poly(C) (Pharmacia) at room temperature for 5 min prior to the addition of radiolabelled RNA probe.

RNA extraction, primer extension and Northern RNA blotting

Total cytoplasmic RNA was prepared from transfected HeLa or HLTat cells, as previously described (Tan and Schwartz, 1995). Cells were treated with 10 μ g/ml of actinomycin D. Primer extension was carried out by first co-precipitating 50 μ g of total cytoplasmic RNA with 100 000 c.p.m. of γ^{32} P-ATP-labelled oligonucleotides NL-PX2 (5'-GGGCACACACTACTTTGAG-3') and GAPDH-PX5 (5'-CTGGCGACGCAAAAGAAGA-3'). Oligonucleotide NL-PX2 hybridizes to the 5' end of mRNA transcribed from the HIV-1 long terminal repeat (LTR) promoter, whereas oligonucleotide GAPDH-PX5 hybridizes to the 5' end of the GAPDH transcript. Annealing was performed by resuspending the RNA pellet in 10 μ l 1 $\times$ HYB buffer (10 mM Tris-Cl (pH 8.3), 75 mM KCl, 3 mM MgCl₂), followed by incubation at 65°C for 1 min, at 37°C for 1 min, and on ice for 1 min. cDNA synthesis was carried out by adding 40 μ l 1 $\times$ RT mix (50 mM Tris-Cl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM dNTPs, 5 U/ μ l M-MLV RT (Life Technologies)), 0.25 U/ μ l RNA guard (Pharmacia), followed by incubation at 42°C for 60 min. The reaction was stopped by RNase-treatment (1 μ g RNase A and 20 U RNase T1 (Ambion)) at 37°C for 10 min, followed by ethanol precipitation. The pellet was resuspended in formamide loading buffer (80% deionized formamide, 1 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) and boiled for 2 min. The reaction products were separated on 6% polyacrylamide-UREA gels. Gels were analysed by autoradiography and RNA bands were quantified by using a phosphorimager (Molecular Dynamics) or densitometry (Molecular Dynamics). Northern blotting was performed as described previously (Tan and Schwartz, 1995).

Western blot analysis and immunoprecipitations

Western immunoblotting was carried out essentially as previously described except that monoclonal antibody 4F4 (anti-hnRNP C) was used as a primary antibody at a

dilution of 1:1000, and horseradish peroxidase-conjugated goat anti-mouse antibody was used as a secondary antibody, diluted 1:20 000. For immunoprecipitations, the u.v. cross linking reaction mixture was first diluted 1:50 in 0.5×radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl, pH 7.4, 75 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS). Various monoclonal antibodies or sera (mAb104 (anti-SR proteins), 9H10 (anti-hnRNP A1), 4F4 (anti-hnRNP C), 7G12 (anti-hnRNP 1), 2.73 (anti-U1-70K), #22 and #36 (patient sera reacting with La and Ro60) and serum U2AF (anti-U2AF65)) were then added to the samples that were incubated for 2 h. Protein A-Sepharose beads were added and the incubation was continued for 30 min. Prior to the addition of Protein A-Sepharose to u.v. cross linked reactions incubated with monoclonal antibody 104, the samples were incubated for 2 h with rabbit anti-mouse IgM antiserum (Dako). After brief centrifugation, the Sepharose beads were washed three times in 0.5×RIPA buffer, and denatured by boiling in 5×SDS-loading buffer (625 mM Tris-HCl, pH 6.8, 6.25% SDS, 50% glycerol, 20% β-mercaptoethanol) and loaded onto 10% polyacrylamide-SDS gels under reducing conditions. Immunoprecipitated proteins were visualized by autoradiography (Agfa film) at −70°C.

Partial purification of RNA-binding proteins

10 mg of nuclear extract of HeLa cells were dialysed overnight at 4°C against buffer S (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM DDT, 20% Glycerol v/v) containing 10 mM KCl, prior to loading onto a 1 ml HiTrap mono Q column (Pharmacia) equilibrated with buffer S containing 10 mM KCl. The column was washed with buffer S containing 10 mM KCl and the washing fractions were collected. The bound proteins were eluted

respectively with three different elution buffers: buffer S containing 50 mM, 200 mM or 1000 mM KCl. The protein concentration of each fraction was determined using the Bio-Rad Bradford Micro assay as described by the manufacturer. The protein-containing samples from flow through or from each elution step were pooled and dialysed against 0.01 mM sodium phosphate buffer (pH 7.0) at 4°C overnight. The dialysed solutions were loaded onto 1 ml HiTrap Heparin columns (Pharmacia) equilibrated with 0.01 M sodium phosphate buffer (pH 7.0) and the flow through fractions were collected. After washing with 0.01 mM sodium phosphate buffer (pH 7.0), the bound proteins were eluted with linear gradients of 0.01–1 M of KCl in sodium phosphate buffer (pH 7.0) at a flow rate of 1 ml/min. Protein concentration of each fraction was determined using the Bio-Rad Bradford Micro assay and protein-positive fractions were subjected to RNA gel shift assay and u.v. cross linking to identify positive fractions.

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References

- Boger HP, Fridell RA, Madore S and Cullen BR. (1995). *Cell*, **82**, 485–494.
- Burd CG, Swanson MS, Görlach M and Dreyfuss G. (1989). *Proc. Natl. Acad. Sci. USA*, **86**, 9788–9792.
- Chen C-M, Chiang S-Y and Yeh N-H. (1991). *J. Biol. Chem.*, **266**, 7754–7758.
- Chen C-YA and Shyu A-B. (1995). *Trends Biochem. Sci.*, **20**, 465–470.
- Chou Z-F, Chen F and Wilusz J. (1994). *Nucleic Acid Res.*, **22**, 2525–2531.
- Chow LT and Broker TR. (1994). *Intervirology*, **37**, 150–158.
- Danos O, Katinka M and Yaniv M. (1982). *EMBO J.*, **1**, 231–236.
- Dietrich-Goetz W, Kennedy IM, Levins B, Stanley MA and Clements JB. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 163–168.
- Dignam JD, Lebovitz RM and Roeder RG. (1983). *Nucleic Acids Res.*, **11**, 1475–1489.
- Dreyfuss G. (1986). *Annu. Rev. Cell Biol.*, **2**, 459–498.
- Emerman M, Vazeux R and Peden K. (1989). *Cell*, **57**, 1155–1165.
- Eng AM, Jin Y-T, Matsuoka LY, Grgurich CV, Robinson J and Armin A. (1985). *J. Cut. Pathol.*, **12**, 46–54.
- Felber BK, Hadzopoulou-Cladaras M, Cladaras C, Copeland T and Pavlakis GN. (1989). *Proc. Natl. Acad. Sci. USA*, **86**, 1495–1499.
- Fisher C, Byers MR, Iadarola MJ and Powers EA. (1991). *Dev.*, **111**, 253–258.
- Fisher U, Huber J, Boelens WC, Mattaj IW and Lührmann R. (1995). *Cell*, **82**, 475–483.
- Fritz CC, Zapp ML and Green MR. (1995). *Nature*, **376**, 530–533.
- Furth PA and Baker CC. (1991). *J. Virol.*, **65**, 5806–5812.
- Furth PA, Choe W-T, Rex JH, Byrne JC and Baker CC. (1995). *Mol. Cell. Biol.*, **14**, 5278–5289.
- Görlach M, Burd CG and Dreyfuss G. (1994). *J. Biol. Chem.*, **269**, 23074–23078.
- Graham FJ and van der Eb AJ. (1973). *Virology*, **52**, 456–460.
- Grassmann K, Rapp B, Maschek H, Petry KU and Iftner T. (1996). *J. Virol.*, **70**, 2339–2349.
- Grussendorf E-I and zur Hausen H. (1979). *Arch. Derm. Res.*, **264**, 55–63.
- Hammarskjöld ML, Heimer J, Hammarskjöld B, Sangwan I, Albert L and Rekosh D. (1989). *J. Virol.*, **63**, 1959–1966.
- Howley PM. (1990). *Fields virology*, Vol. Second edition. Field BN, Knipe DM, Chanock RM, Hirsch MS, Melnick JL, Monath TP and Roizman B. (eds). Raven Press: New York, pp. 1625–1650.
- Howley PM. (1991). *Fundamental virology*, Vol. 2, Fields BN and Knipe DM. (eds). Raven Press: New York, pp. 743–768.
- Jenson AB, Rosenthal JD, Olson C, Pass F, Lancaster WD and Shah K. (1980). *J. Natl. Cancer Inst.*, **64**, 495–500.
- Katz DA, Theodorakis NG, Cleveland DW, Lindsten T and Thompson CB. (1994). *Nucleic Acids Res.*, **22**, 238–246.
- Kennedy IM, Haddow JK and Clements JB. (1990). *J. Virol.*, **64**, 1825–1829.
- Kennedy IM, Haddow JK and Clements JB. (1991). *J. Virol.*, **65**, 2093–2097.
- Laimins LA. (1993). *Infectious Agents and Disease*, **2**, 74–86.

- Lambert PF. (1991). *J. Virol.*, **65**, 3417–3420.
- Lee WMF, Lin C and Curran T. (1988). *Mol. Cell. Biol.*, **8**, 5521–5527.
- Luukkonen BGM, Tan W and Schwartz S. (1995). *J. Virol.*, **69**, 4086–4094.
- Malim MH, Hauber J, Le S, Maizel JV and Cullen BR. (1989). *Nature*, **338**, 254–257.
- Meijlink F, Curran T, Miller AD and Verma IM. (1985). *Proc. Natl. Acad. Sci. USA*, **82**, 4987–4991.
- Nakamaki T, Imamura J, Brewer G, Tsuruoka N and Koeffler HP. (1995). *J. Cellular Physiology*, **165**, 484–492.
- Raymond V, Atwater JA and Verma IM. (1989). *Oncogene Res.*, **4**, 861–865.
- Ross J. (1995). *Micobiol. Rev.*, **59**, 15–95.
- Ross J. (1996). *Trends. Biochem. Sci.*, **12**, 171–176.
- Rüther U, Komitowski D, Schubert FR and Wagner EF. (1989). *Oncogene*, **4**, 861–865.
- Sambrook J, Fritsch EF and Maniatis T. (1989). *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York.
- Schiavi SC, Belasco JG and Greenberg MF. (1992). *Biochim. Biophys. Acta*, **1114**, 95–106.
- Schwartz S, Felber BK, Benko DM, Fenyö EM and Pavlakis GN. (1990). *J. Virol.*, **64**, 2519–2529.
- Shyu A-B, Greenberg ME and Belasco JG. (1989). *Genes Dev.*, **3**, 60–72.
- Staknis D and Reed R. (1994). *Mol. Cell. Biol.*, **14**, 2994–3005.
- Swanson MS and Dreyfuss G. (1988). *EMBO J.*, **11**, 3519–3529.
- Tan W, Felber BK, Zolotukhin AS, Pavlakis GN and Schwartz S. (1995). *J. Virol.*, **69**, 5607–5620.
- Tan W and Schwartz S. (1995). *J. Virol.*, **69**, 2932–2945.
- Turek LP. (1994). *Adv. Virus Res.*, **44**, 305–356.
- Vakalopoulou E, Schaack J and Shenk T. (1991). *Mol. Cell. Biol.*, **11**, 3355–3364.
- Wennborg A, Sohlberg B, Angerer D, Klein G and von Gabin A. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 7322–7326.
- Wilusz J and Shenk T. (1990). *Mol. Cell. Biol.*, **10**, 6397–6407.
- You Y, Chen C-YA and Shyu A-B. (1992). *Mol. Cell. Biol.*, **12**, 2931–2940.
- Zaidi SHE and Malter JS. (1995). *J. Biol. Chem.*, **270**, 17292–17298.
- Zhao C, Tan W, Sokolowski M and Schwartz S. (1996). *J. Virol.*, **70**, 3659–3667.
- zur Hausen H. (1991). *Science*, **254**, 1167–1173.
- zur Hausen H and de Villiers E-M. (1994). *Annu. Rev. Microbiol.*, **48**, 427–447.