Human immunodeficiency virus type 1 (HIV-1) mRNAs encoding structural proteins contain multiple inhibitory/instability elements (INS), which decrease the efficiency of viral protein expression. We have previously identified a strong INS element (INS-1) within the p17\textsuperscript{gag} coding region. Here we show that poly(A)-binding protein 1 (PABP1) binds preferentially to INS-1 within the p17\textsuperscript{gag} mRNA, but not to a mutated mRNA in which INS-1 function is eliminated. Competition experiments performed in the presence of different nucleic acids and homoribopolymers demonstrated preferential binding of PABP1 to the INS-1-containing RNA. In contrast to HeLa cells and several lymphoid cell lines, certain human glioma cell lines exhibit high levels of gag expression in the absence of Rev upon transient transfection with wild type gag expression vectors. We analyzed extracts of different cell lines and found that the binding of PABP1 to INS-1 RNA is significantly diminished in glial cell extracts. The expression levels of gag correlate with the absence of binding of PABP1 to the INS-1 RNA in cellular extracts. These results suggest a role for PABP1 in the inhibition of gag expression mediated through INS-1.

The post-transcriptional regulation of human immunodeficiency virus type 1 (HIV-1)\textsuperscript{1} is mediated through the Rev protein. Rev functions by facilitating the transport, stability, and translation of partially spliced and unspliced HIV-1 mRNAs that contain a Rev-specific RNA-binding region (RRE) (1–5). The mechanism of Rev function has been the subject of intense study. It has been shown that Rev increases the half-life of RRE-containing HIV-1 mRNAs (2) and promotes their transport to the cytoplasm (2–4, 6) and efficient translation (5, 7–9). RRE-containing HIV-1 mRNAs (2) and promotes their trans-}

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**MATERIALS AND METHODS**

**Cell Culture and Preparation of Extracts**—The human T cell line HPB-ALL (17), HL\textsuperscript{tat} cells (18), and human astrocytoma cell line 85HG66 (19–21) have been previously described. The astrocytoma cell line U87-MG was obtained from the American Type Culture Collection (Rockville, MD). Nuclear and cytoplasmic extracts were prepared by the method of Moore and Fishel (17) with modifications. Briefly, cells were harvested by low speed centrifugation, washed three times with ice-cold phosphate-buffered saline, resuspended in hypotonic lysis buffer (10 mM Tris HCl, pH 7.8, 1 mM MgCl\textsubscript{2}, 4 mM KCl, 1 mM dithiothreitol, 1% Triton X-100, 2 mM Pefabloc (Boehringer Mannheim)), and incubated on ice for 12 min. Released nuclei were centrifuged for 5 min at low speed. The supernatant was used in the cross-linking reactions (cytoplasmic extract). The nuclei were washed twice with the same buffer and resuspended in the lysis buffer containing 400 mM KCl. After nuclear lysis, the nuclear extract was cleared by centrifugation at 14,000 \times g for 15 min. The protein concentration of cytoplasmic and nuclear extracts was determined by the Bio-Rad DC protein assay (Bio-Rad).

**Transfections**—HL\textsuperscript{tat} cells, 85HG66, and U87-MG cells were transfected by the calcium coprecipitation technique (2, 22), with 5 \mu g of gag-expression plasmids (p17, p17R, and p17M1234) in the absence or presence of 1 \mu g of the Rev-expressing plasmid pBluescript Rev (7). One microgram of tat-expressing plasmid, pl\textsuperscript{tat}, was added to the transfection mixtures for glial cell extracts. Cotransfection with pl\textsuperscript{3lac}, which contains the firefly luciferase gene linked to the HIV-1 long terminal repeat was used as an internal control for transfection efficiency. Luciferase activity was determined as described previously (23, 24). The total amount of DNA in the transfection mixtures was adjusted to 17 \mu g/0.5 ml of precipitate/60-mm plate by using pBluescript (Stratagene). Transfected

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1 The abbreviations used are: HIV-1, human immunodeficiency virus type 1; RRE, Rev-specific RNA-binding region; INS-1, inhibitory RNA element; PABP1, poly(A) binding protein 1.

2 R. Schneider, G. Nasioulas, M. Campbell, B. K. Felber, and G. N. Pavlakis, submitted for publication.
cells were harvested in 0.5 × radioimmune precipitation buffer 24 h post-transfection. gag protein production was analyzed by Western blotting using HIV-1 patient serum as described previously (11).

In Vitro RNA Synthesis—pKS17 containing the p17 coding sequence (nucleotides 172–257) was transcribed in vitro from the HXB2 HIV-1 sequence. A reverse transcription reaction amplified from pM gag and cloned into the Bluescript KS(−) vector (10). pKS17M1234 containing p17M1234 was isogenic to pKS17 except it contained 28 point mutations introduced into gag sequence (11). pKS17 and pKS17M1234 were linearized by EcoRI digestion and used as templates for in vitro synthesis of KS17 RNA and KS17MRNA by standard protocols (Promega). The in vitro transcribed RNAs (535 nucleotides) were used in cross-linking reactions after analysis in 8% polyacrylamide with 8 M urea denaturing gels.

UV Cross-linking—RNA binding reaction mixtures contained approximately 0.1 pmol of 32P-labeled RNA probe and 1–10 μl of cytoplasmic extracts in a final volume of 30 μl, containing 20 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 20 units of RNasin (Promega), and 0.5 μg/ml yeast tRNA (Boehringer Mannheim). The reaction mixtures were incubated at room temperature for 15 min and then irradiated by UV light (254 nm, Stratalinker 2400, Stratagene) while on ice. After digestion with RNase A (1 μg/ml) at 37°C for 20 min, the samples were heated at 65°C for 5 min in SDS sample buffer and electrophoresed on 10% polyacrylamide-SDS gels (Novex). Dried gels were exposed to Kodak XAR film.

Immunoprecipitation of Cross-linked Proteins—Antiserum 61925 against PABP1 was obtained from Dr. R. Moon, University of Washington, Seattle (25). Polyclonal antisera were made in rabbits after immunizations with synthetic peptides CEAQKAYNSATGVPYT (antiserum 39472) or CIPQTNRAAYPPSVQALRPS (antiserum 39473), derived from human PABP1 (26). Dried gels were exposed to Kodak XAR film.

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Quantitative Analyses—Determination of PABP1 by immunoblotting was done as described above for gag. Binding assays with PABP1 and poly(A) were performed using cytoplasmic extracts from HLTat, 85HG66, and U87-MG cells and poly(A)-Sepharose. Twenty microliters of a 50% slurry of poly(A)-Sepharose was incubated with 200 μl of cytoplasmic extract (corresponding to 5 × 106 cells) for 2 h at 4°C in phosphate-buffered saline. Poly(A)-Sepharose beads were washed twice with phosphate-buffered saline containing 1 mM NaCl, and the presence of bound PABP1 was analyzed by SDS-polyacrylamide electrophoresis and immunoblotting. Immunoblots were quantitated for bound 32P-labeled protein A on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Detection of Specific RNA-Protein Complexes—We prepared in vitro transcribed p17 RMRNA containing the region of INS-1 (KS17 RNA), and a mutant RNA transcribed from pKS17M1234 (11), which has inactivated INS-1 (KS17MRNA). We used UV cross-linking experiments to determine whether INS-1 in KS17 RNA interacts with cellular proteins. We hypothesized that elimination of inhibitory sequences in KS17 RNA alter RNA-protein interactions important for inhibition. To test this hypothesis, we compared the patterns of proteins interacting with these RNAs. 32P-labeled KS17 RNA and KS17MRNA were incubated with nuclear and cytoplasmic extracts from HPB-ALL cells. Binding reactions were performed in the presence of excess unlabeled yeast tRNA to eliminate nonspecific binding. After incubation, all reactions were irradiated by UV light followed by RNase A treatment. As shown in Fig. 1, a strong band corresponding to a cytoplasmic protein with an apparent molecular weight of approximately 70,000 (p70) was detected with KS17 RNA (lane 1). The complex appeared to be specific for KS17 RNA under the assay conditions used, since p70 was not present after incubations with KS17MRNA (lane 2) or RRE RNA (lane 3). Analysis of RNA-protein complexes obtained from nuclear extracts did not reveal any differences between KS17 RNA or KS17MRNA (compare Fig. 1B, lanes 1 and 2), but these complexes were distinct from those obtained with RRE RNA (Fig. 1B, lanes 3).

To examine the specificity of complex formation between p70 and KS17 RNA, we included an excess of various unlabeled nucleic acids in the binding reaction and examined their ability to affect p70-KS17 RNA complex formation (Figs. 2 and 3). Presence of up to 100-fold molar excess of competitor DNA did not affect binding of p70 to KS17 RNA (Fig. 2, compare lanes 1 and 2). On the contrary, inclusion of a 4-fold molar excess of unlabeled KS17 RNA decreased complex formation with the labeled probe (lane 3), whereas the presence of a 40-fold excess completely abolished the interaction of p70 with labeled p17 RNA (lane 4). The presence of rRNA (lanes 9 and 10) or unlabeled KS17 MRNA (lanes 6–8) did not prevent binding of p70 to the wild type KS17 RNA. Hence, formation of the p70-KS17 RNA complex appears to be specific and parallels the stability of p17 RNA.

We further analyzed the sequence specificity of p70-KS17 RNA complex formation by testing each of four homoribopolymers for the ability to compete with KS17 RNA (Fig. 3). The binding experiments were performed in the presence of increasing concentrations of poly(A), poly(C), poly(G), or poly(U). Only poly(A) was able to selectively inhibit p70-KS17 RNA complex formation. A 55% inhibition was achieved in the presence of 1 mg of poly(A) (Fig. 3, filled circles). The presence of poly(C) had no effect on the complex formation even in the presence of 4 μg of poly(C) (open circles). The sensitivity of complex formation to poly(G) was comparable to that of poly(C), while the presence of poly(U) increased the ability of KS17 RNA to interact with p70, probably due to elimination of unstable nonspecific interactions of KS17 RNA with other RNA-binding proteins present in the cytoplasmic extract (filled squares). The amount of unlabeled KS17 RNA necessary to inhibit complex formation of p70-KS17 RNA by 50% was 77 ng (filled triangles). These data suggest that the protein within the complex has strong preference for poly(A).

Antibodies against PABP1 Recognize Cross-linked p70—The observation that p70 has a high affinity for poly(A) and a similar molecular weight led to the hypothesis that p70 is the previously identified PABP1. To confirm this hypothesis, we tested polyclonal antibodies raised against PABP1 for their ability to react with labeled p70. UV cross-linking experiments were performed in the presence of KS17 RNA (Fig. 4, lane 1) followed by immunoprecipitation either with antibodies.
against PABP1 or with preimmune rabbit serum. As it shown in Fig. 4, antibodies against PABP1 (lane 2) recognized p70. We could not detect any reaction of preimmune rabbit serum with the complex (lane 3). This experiment was repeated with all three available types of antibodies against PABP1 (see “Materials and Methods”). Taken together, the strong competition of poly(A) for p70 binding to KS17 RNA and the ability of PABP1 antibodies to recognize labeled p70 support the conclusion that p70 is PABP1.

**Binding of PABP1 to KS17 RNA Correlates with Inhibitory Effect of INS-1 in Different Cell Lines**—We next examined whether the level of inhibition of gag expression by INS-1 after transfection in different cell lines correlates with the ability of PABP1 to bind KS17 RNA in vitro. To measure the effects of INS-1 in different cell lines, we transfected gag-expressing plasmids into three different cell lines, HeLa Tat, and human astrocytoma cell lines U87-MG and 85HG66. The plasmids used, p17, p17R, and p17M1234 have been previously described (27). p17 and p17R contain the wild type INS-1, p17R also contains a functional RRE, and p17M1234 contained inactivated INS-1.

**gag** expression by p17 was almost 10 times higher in U87-MG cells compared with HLtat cells. The levels of gag produced in the presence of Rev were similar to those produced by p17M1234 (Fig. 5A). Expression of the luciferase reporter gene after cotransfections in these cell lines were comparable (average values varied from 3 x 10^5 to 9 x 10^5 luciferase units). Examination of the second glial cell line, 85HG66, revealed that the overall level of protein expression upon transient transfection was lower because of less efficient transfection. Expression of luciferase was also lower (4–6 x 10^5 units). To compare these different cell lines, we expressed gag production in the absence of Rev as a percentage of gag production obtained from p17M1234 (Fig. 5B). Expression of gag protein by p17 in the absence of Rev was 48% of that obtained with p17M1234 for 85HG66 and 67.2% for U87-MG. This level is much higher than in HeLa cells (3.75% of the levels produced with p17M1234). These results suggest that inhibitory cellular factors are less functional in the astrocytoma cell lines compared with HeLa cells.

To examine whether the ability of PABP1 to bind KS17 RNA in vitro is different in extracts of different cell lines, we compared cytoplasmic extracts derived from a human T cell line (HPB-ALL), HeLa cells, and human astrocytoma cell lines U87-MG and 85HG66 by UV cross-linking. We performed UV cross-linking experiments using partially fractionated extracts from these cell lines (Fig. 6). We determined the total protein concentration in the extracts, and performed UV cross-linking experiments with different amounts of extracts. We found most of the specific binding in the 20–50% ammonium sulfate fraction of HPB-ALL and HeLa cell cytoplasmic extracts (lanes 1–3), whereas we could not detect significant binding in any fractions of U87-MG or 85HG66 cells (lanes 4–8). It is noteworthy that, although the amount of total proteins used in lanes 1, 3, 5, and 7 is comparable (approximately 7–12 μg), complex formation is significantly reduced in U87-MG and 85HG66. Therefore, binding of PABP1 to INS-1 RNA in cytoplasmic extracts correlates with the inhibition of expression of
the_p17M1234_Rev, and p17M1234. gag production was analyzed by
immunoblotting with serum from an HIV-1-infected patient. Gels were
scanned using PhosphorImager to quantify the band corresponding to
p17M1234 protein, A, gag protein production expressed in arbitrary PhosphorIm-
ager units. B, gag protein production expressed as a percentage of gag
production from p17M1234 (diagonal lined bars). Solid bars represent
gag production from p17, dotted bars, gag production from p17R + Rev.

FIG. 6. Comparison of the binding of PABP to KS17 RNA in
different cell lines. UV cross-linking experiments were performed in
the presence of radiolabeled KS17 RNA with different amounts of
cytoplasmic fractions from human T cell line HPB-ALL (lane 1), HeLa
(lanes 2 and 3), or glial cell lines U87-MG (lanes 4–6) and 85HG66
(lanes 7 and 8). Cytoplasmic fractions were prepared by precipitation
with 37% ammonium sulfate (lane 1) or 50% ammonium sulfate (all
other lanes). The total amount of protein in the probes was determined
as following: lane 1, 10 μg; lane 2, 6.9 μg; lane 3, 9.8 μg; lane 4, 3.6 μg;
lane 5, 12 μg; lane 6, 18 μg; lane 7, 7 μg; and lane 8, 14 μg.

these mRNAs in vivo.

By quantitative immunoblotting we also examined the levels
of expression of PABP1 in glial or HeLa cells and the ability of
PABP1 to bind poly(A) in vitro. The results showed that glial
cells produced 3–5 times less PABP1 as measured by Western
blots. PABP1 levels correlated with the binding of poly(A) to
cytoplasmic extracts of the different cell lines as well as with
the levels of UV cross-linking (Fig. 7), suggesting that differ-
ences in INS-1 binding might reflect different expression levels of
PABP1.

DISCUSSION

PABP1 is the major cytoplasmic poly(A)-binding protein and
is highly conserved among eukaryotic organisms (26, 28–30).
Deletion of the PABP1 gene in yeast (Saccharomyces cervisiae)
is lethal, indicating that PABP1 is an essential protein (31).
The role of PABP1 and poly(A) in mRNA metabolism remains
unclear. PABP1 might be a key factor in mediating regulation
of mRNA turnover through the inhibition of mRNA decapping
by the poly(A) tail or by influencing the rate of deadenylation
(32–35). Additionally, several lines of evidence argue that
PABP1 plays a role in stimulating translation initiation (36,
37), suggesting that the interaction of this protein with the 3’
poly(A) sequence can influence events at the 5’ end of an
mRNA. PABP1 has at least two distinct and separable activities:
specific poly(A) binding activity was found only in two amino-
terminal RNA binding domains, which could function in bind-
ing of PABP1 to the poly(A) tail, whereas two other RNA
binding domains do not have preference for poly(A) binding and
could function through binding either to a different part of the
same RNA or to other RNAs (38).

The results presented here suggest an interaction of PABP1
with the INS-1 region within p17gag mRNA. The p17gag coding
sequence has four regions with a high content of A and U
nucleotides. Point mutations resulting in elimination of the
inhibitory effect of INS-1 were introduced to interrupt mostly
A-rich stretches in these regions. INS-1 contains a maximum of
6 uninterrupted A nucleotides surrounded by AU-rich regions.
The ability of PABP1 to bind wild type p17gag mRNA and not
the mutant mRNA suggests that it binds to A-rich sequences
other than poly(A). Our results confirm previous findings (16,
38) that PABP1 is a multifunctional RNA-binding protein and
is able to interact with other sequences.

The observation that PABP1 binds less efficiently to INS-1...
regions in cytoplasmic extracts from astrocytoma cell lines, coupled with the increased expression of p17
mRNA in these cell lines, indicates a correlation between binding of PABP1 to INS-1-containing mRNA and expression of this mRNA in vivo. Therefore, binding of PABP1 to p17
mRNA might play a role in the inhibition of gag expression mediated by INS-1.

We propose that the interaction of PABP1 with INS-1 within p17
mRNA might prevent its efficient translation. Mutimerization of PABP1 on the 3' poly(A) tail of mRNA could lead to the formation of a ribonucleoprotein particle capable of interacting with the 5' end of the mRNA. This interaction may be necessary for efficient initiation of translation. The presence of additional sites for PABP1 binding within the INS-1 on the mRNA could result in the creation of cis-acting competitor sequences. Binding of PABP1 to these sites might alter or inhibit functionally important contacts on the 5' end of the mRNA and PABP1
occupying INS-1 sites might form, resulting in inefficient translation and/or higher degradation rate. In glial cells, lower levels of expression of PABP1 are sufficient for interaction with poly(A) tails, but there is no excess of unbound protein for interaction with additional targets such as INS-1; therefore, inhibition by INS-1 is significantly reduced. This model is consistent with our previous results that in the absence of Rev most HIV-1 mRNA is not efficiently translated and is associated with 40S ribosomal subunits, but not with polysomes (7). Rev might direct RRE-containing RNA through a different transport and utilization pathway, preventing binding of PABP1 and possibly of other inhibitory factors to INS-1, thus leading to efficient translation.

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