THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 4, Issue of January 24, pp. 2307–2311, 1997
Printed in U.S.A.

Preferential Binding of Poly(A)-binding Protein 1 to an Inhibitory RNA Element in the Human Immunodeficiency Virus Type 1 *gag* mRNA*

(Received for publication, August 30, 1996)

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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^{gag} coding region. Here we show that poly(A)-binding protein 1 (PABP1) binds preferentially to INS-1 within the p17^{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)[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). Rev-dependent mRNAs are defective in expression, due to specific RNA regions scattered throughout the *gag*, *pol*, and *env* regions of HIV-1. These regions were named inhibitory sequences (INS/CRS/IN) and were shown to prevent efficient expression (10–15).[2]

We have previously reported the identification of a strong inhibitory RNA element (INS-1) within the coding region of the p17^{gag} matrix protein of HIV-1 (11). This element acts in *cis* and its inhibitory effect can be overcome by Rev-RRE. Inactivation of INS-1 was achieved by introducing multiple point mutations distributed primarily in the regions of high AU content without altering the amino acid sequence of the encoded p17^{gag} protein. This mutant displayed a Rev-independent, constitutively high level of expression of *gag* mRNA and protein, indicating that INS-1 had been inactivated.

Here we further characterize the mechanism of function of the inhibitory sequences. We studied the cellular factors interacting with INS-1 using UV-cross-linking of *in vitro* transcribed RNA to cellular extracts. We show that poly(A)-binding protein (PABP1) binds specifically to INS-1 within the p17^{gag} mRNA, but not to mutated RNA (p17M1234). Moreover, the ability of PABP1 to bind p17^{gag} RNA *in vitro* appears to be cell type-specific and correlates with inhibitory effects of INS-1 *in vivo*. We propose that binding of PABP1 to INS-1 within the *gag* mRNAs may contribute to the mechanisms regulating HIV-1 expression.

MATERIALS AND METHODS

Cell Culture and Preparation of Extracts—The human T cell line HPB-ALL (17), HLtat 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 $MgCl_2$, 4 mM KCl, 1 mM dithiothreitol, 1% Triton X-100, 2 mM Pefablock (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 of KCl. After nuclear lysis, the nuclear extract was cleared by centrifugation at 14,000 × *g* for 15 min. The protein concentration of cytoplasmic and nuclear extracts was determined by the Bio-Rad DC protein assay (Bio-Rad).

Transfections—HLtat cells, 85HG66, and U87-MG cells were transfected by the calcium coprecipitation technique (2, 22), with 5 μg of *gag*-expression plasmids (p17, p17R, and p17M1234) in the absence or presence of 1 μg of the Rev-expressing plasmid pBsrev (7). One microgram of tat-expressing plasmid, pL3tat, was added to the transfection mixtures for glial cells. Cotransfection with pL3luc, 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 μg/0.5 ml of precipitate/60-mm plate by using pBluescript (Stratagene). Transfected

* Research sponsored by the National Cancer Institute, DHHS, under contract with ABL. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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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.

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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^{gag} coding sequences (nucleotides 172–257 + 329–731 of the HXB2 HIV-1 sequence) was polymerase chain reaction amplified from pMcgag and cloned into the Bluescript KS(−) vector (10). pKS17M1234 containing p17M1234^{gag} was isogenic to pKS17 except it contained 28 point mutations introduced into *gag* sequence (11). pKS17 and pKS17M1234 were linearized by *Eco*RI 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 6% polyacrylamide with 8 M urea denaturing gels. [α-^{32}P]UTP was included for synthesis of radiolabeled RNAs.

UV Cross-linking—RNA binding reaction mixtures contained approximately 0.1 pmol of ^{32}P-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 mg/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 mg/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 CEAAQKAVNSATGVPTV (antiserum 39472) or CIPQTQNRAAYYPPSQVAQLRPS (antiserum 39473), derived from human PABP1 (26). For immunoprecipitations, three reaction mixtures were combined after UV irradiation, treated with RNase A, brought to a final concentration of 0.5 × radioimmune precipitation buffer using 5 × radioimmune precipitation buffer stock buffer, and incubated with rabbit antiserum against PABP1 or rabbit serum (5–10 μl) for 12–15 h at 4 °C, followed by addition of a 50% slurry of protein A-Sepharose. The samples were incubated for 3 h at 4 °C with rocking. The immunoprecipitate was prepared by low speed centrifugation and three washes with 0.5 × radioimmune precipitation buffer. Samples for SDS-polyacrylamide electrophoresis were prepared as described above.

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 × 10^5 cells) for 2 h at 4 °C in phosphate-buffered saline. Poly(A)-Sepharose beads were washed twice with phosphate-buffered saline containing 1 M NaCl, and the presence of bound PABP1 was analyzed by SDS-polyacrylamide electrophoresis and immunoblotting. Immunoblots were quantitated for bound ^{125}I-labeled protein A on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Detection of Specific RNA-Protein Complexes—We prepared *in vitro* transcribed p17^{gag} RNA containing the region of INS-1 (KS17 RNA), and a mutant RNA transcribed from pKS17M1234 (11), which has inactivated INS-1 (KS17M RNA). 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 KS17M RNA alter RNA-protein interactions important for inhibition. To test this hypothesis, we compared the patterns of proteins interacting with these RNAs. ^{32}P-Labeled KS17 RNA and KS17M RNA 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

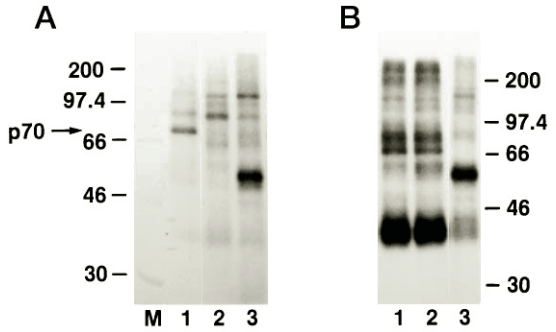


FIG. 1. **UV induced cross-linking of cellular proteins to KS17 RNA.** Cytoplasmic (*A*) or nuclear (*B*) extracts of HeLa cells were incubated with radiolabeled KS17 RNA (*lane 1*), KS17M RNA (*lane 2*) or RRE RNA (*lane 3*). After incubation, all probes were irradiated by UV light followed by RNase A treatment. Probes contained 2 μg of cytoplasmic extract (*A*), or ~3 μg of nuclear extract (*B*).

conditions used, since p70 was not present after incubations with KS17M RNA (*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 KS17M RNA (compare Fig. 1*B*, *lanes 1* and *2*), but these complexes were distinct from those obtained with RRE RNA (Fig. 1*B*, *lane 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 KS17M RNA (*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^{gag} mRNA.

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 ng 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

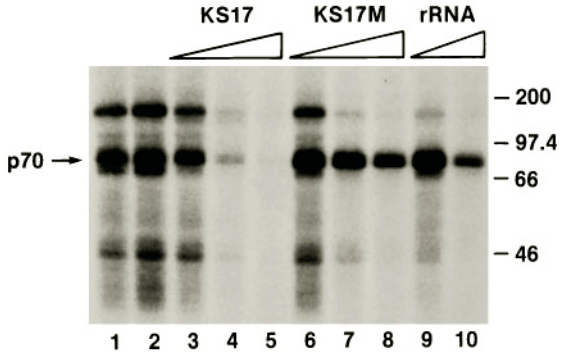


FIG. 2. **Specificity of KS17 RNA-p70 complex formation.** UV cross-linking experiments were performed in the presence of 10 pmol of DNA (*lane 1*); no competitor (*lane 2*); 0.379, 3.79, and 11.36 pmol of KS17 RNA, respectively (*lanes 3–5*); 0.4, 4, and 12 pmol of KS17M RNA, respectively (*lanes 6–8*); and 1.5 and 15 pmol of total ribosomal RNA, respectively (*lanes 9* and *10*).

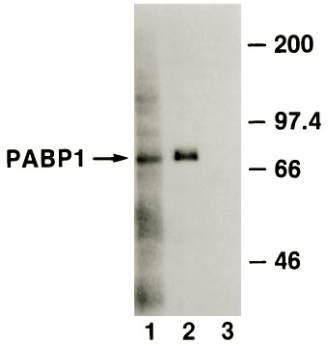


FIG. 4. **The complex KS17 RNA-p70 contains PABP1.** UV cross-linking experiments were performed as described for Fig. 1. The probe in *lane 1* represents an aliquot of UV cross-linked material used in immunoprecipitation. The probes in *lanes 2* and *3* were immunoprecipitated with antibodies against PABP1 (antiserum 39473) or normal serum, respectively.

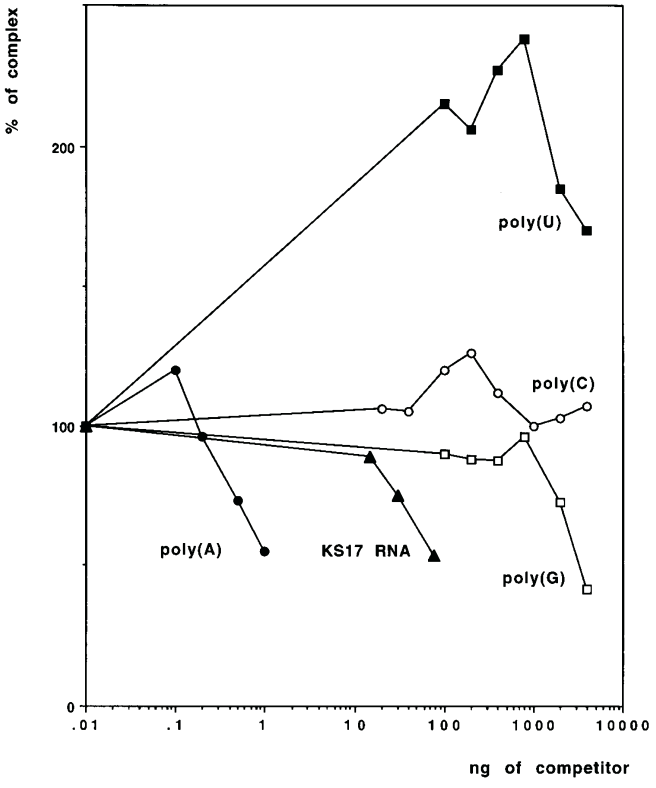


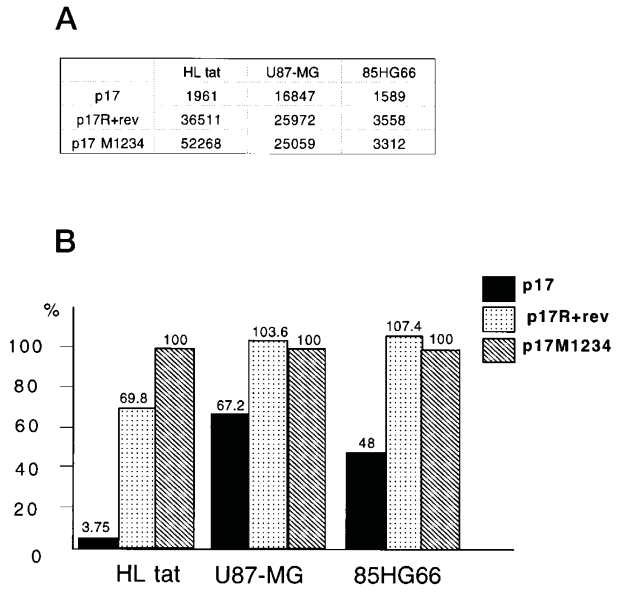
FIG. 3. **Sequence preference of KS17 RNA-p70 complex.** UV cross-linking experiments were performed in the presence of labeled KS17 RNA. The indicated cold polyribonucleotide was added to the probe prior to incubation of labeled KS17 RNA with the cytoplasmic extract: *filled squares*, poly(U); *open squares*, poly(G); *filled circles*, poly(A), *open circles*, poly(C); *filled triangles*, unlabeled KS17 RNA. The cross-linking products were analyzed on the polyacrylamide-SDS gels and the gels were scanned by a PhosphorImager to quantify the band corresponding to the KS17 RNA-p70 complex.

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. 5*A*). Expression of the luciferase reporter gene after cotransfections in these cell lines were comparable (average values varied from 3×10^5 to 9×10^4 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 ($4–6 \times 10^3$ 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. 5*B*). 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



	HL tat	U87-MG	85HG66
p17	1961	16847	1589
p17R+rev	36511	25972	3558
p17 M1234	52268	25059	3312

FIG. 5. **Quantitation of p17^{gag} protein production upon transient transfection of different cell lines.** HLtat cells and two human glial cell lines (U87-MG, 85HG66) were transfected with plasmids p17, p17R + 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 p17^{gag} protein. *A*, *gag* production expressed in arbitrary PhosphorImager 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.

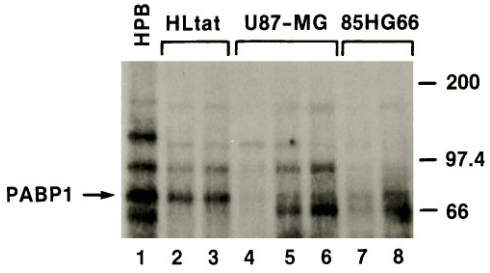


FIG. 6. **Comparison of the binding of PABP to KS17 RNA in different cell lines.** UV cross-linking experiments were performed in the presence of radiolabeled KS17 RNA with different amounts of cytoplasmic fractions from human T cell line HPB-ALL (*lane 1*), HeLa (*lanes 2* and *3*), or glial cell lines 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.

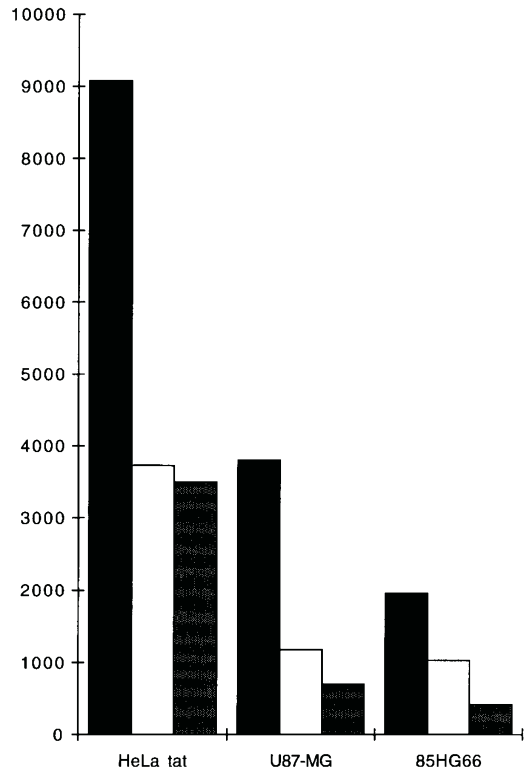


FIG. 7. **Comparison of PABP1 expression, poly(A) binding and KS17 RNA interaction in different cell lines.** Quantitation of PABP1 in cytoplasmic extracts of HeLa, U87-MG, and 85HG66 cells was done by immunoblotting (■). Affinity of PABP1 to poly(A) was determined as an amount of PABP1 from cytoplasmic extracts of different cell lines bound to poly(A)-Sepharose (□). The amount of PABP1 interacting with KS17 RNA was determined by UV cross-linking experiments as described under "Materials and Methods" (▨). Films were scanned by an UltroScan XL laser densitometer (Pharmacia Biotech Inc.). Data are presented in PhosphorImager or densitometer units, normalized to the total protein concentration. ■, PABP1 (Western blot)/μg of protein; □, poly(A) binding/100 ng of protein; and ▨, UV cross-linking/ng of protein.

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 differences 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 cerevisiae*) 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. PABP 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 binding 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 p17^{gag} mRNA. The p17^{gag} 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 p17^{gag} 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 $p17^{gag}$ 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 $p17^{gag}$ 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 $p17^{gag}$ mRNA might prevent its efficient expression. 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 with the 3′ poly(A)-PABP1 ribonucleoprotein particle. Instead, nonfunctional complexes of 5′ end 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.

Acknowledgments—We thank B. K. Felber for encouragement, helpful discussions, and critical reading of the manuscript. We are grateful to S. Gulnik for support and help with experiments, to A. Gragerov, A. Zolotukhin, and R. Schnider for suggestions and discussions, to P. Carney and J. Harrison for technical assistance, R. Moon and T. Copeland for rabbit anti-PABP1 polyclonal antisera, and A. Arthur for editing.

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