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Human Immunodeficiency Virus Type 1 Rev-Responsive Element RNA Binds to Host Cell-Specific Proteins

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RNase protection-gel retention studies show human host cell-specific ribonucleoprotein complexes with human immunodeficiency virus type 1 Rev-responsive element (RRE) RNA. Nuclear proteins from rodent or murine cells appear to lack the ability to form these complexes. Human-mouse somatic cell hybrids retaining a single human chromosome, either 6 or 12, form the RRE–nuclear-protein complexes. One of the complexes requires the entire RRE RNA, while the other needs RRE RNA stem-loops 1 and 2 only. Two major proteins with molecular masses of 120 and 62 kDa specifically bind to RRE RNA. Rodent cells (CHO) either lack or contain small amounts of these RRE-binding proteins.

The human immunodeficiency virus type 1 (HIV-1) Rev protein is known to regulate the expression of viral structural genes *gag* and *env* (for a review, see references 14, 16, 30, 33, and 40). Among the proposed mechanisms of Rev function are its particular role in splice site selection, inhibition of pre-mRNA splicing, nucleocytoplasmic transport, and efficient translation of the Rev-responsive element (RRE)-containing viral mRNA (1–6, 8, 10, 11, 13, 18–21, 24). The issue is whether HIV-1 Rev accomplishes these goals by selective interaction with host cell proteins and, if so, what these host-specific Rev cofactors are.

A unique feature of Rev function is its requirement, in *cis*, of an RNA target sequence, RRE, a 220-nucleotide sequence element located within the *env* gene, which forms a complex secondary structure consisting of five stem loops. Deletion analysis shows that hairpin loops 1 and 2 of RRE RNA are required for both Rev binding and the transport of unspliced viral RNA (17, 25, 29, 37). The Rev-binding domain of RRE is essential but appears to be insufficient for *rev* function, since deletion of the non-Rev-binding domains of RRE results in a considerable loss of its biological activity (17, 25, 29, 37). These results suggest that interaction of RRE RNA with factors other than Rev may be required for *rev* gene function.

The Rev protein appears to be primarily localized in the nucleus (nucleolus). Amino acids 8 to 67 of Rev are essential for its binding to RRE RNA, nuclear localization, multimerization, and RNA transport (23, 25, 27, 31, 33). Although the C-terminal amino acids 67 to 83 are essential for Rev function in in vivo, they are not required for RRE binding (16, 23, 30). Mutations in this region generate a nonfunctional, *trans*-dominant Rev mutant with an RRE-binding affinity similar to that of the wild-type Rev, thus supporting the hypothesis that RRE RNA-Rev interaction is not sufficient for Rev function and that some accessory host cell factors may be required. The suggestion that host cell factors may be required for Rev function is also supported by the observation that Rev-mediated HIV-1 mRNA transport is host cell specific (38). Recently, it was demonstrated that Rev-mediated RNA transport can be achieved after exchanging the minimal Rev-binding domain of RRE (88-base element) with the MS2 operator RNA element or HIV-1 transactivation-response element (TAR) RNA in a heterologous expression system in which the Rev protein is tethered to the MS2 coat protein or to Tat (22, 26, 41). However, in a similar experiment, exchange of the entire 240-bp RRE with the MS2 element was only partially active in supporting Rev function (22, 26, 41). These experiments further support the functional requirement for the entire RRE (rather than the minimal Rev-binding domain) and suggest a role for a host factor(s) in Rev function. Here, we report the specific binding of two human cell proteins, with approximate molecular masses of 120 and 62 kDa, to the RRE RNA, which may play a significant role in the regulation of Rev function.

MATERIALS AND METHODS

HeLa, CHO, HOS, A9, Jurkat, and NIH 3T3 cells were maintained in cell culture as described earlier (28). Human mouse fibroblast (A9) hybrid cells were obtained from the laboratory of Eric J. Stanbridge. The experimental procedures for generating microcell hybrids containing single human chromosomes have been described earlier (35). Nuclear extract from these cells was prepared according to the protocol described by Dignam et al. (9). Radioactive RRE transcripts were prepared with a commercially available in vitro transcription kit (Promega Corp.). The RRE plasmid (obtained from Peter Shank, Brown University, Providence, R.I.) contained a 353-nucleotide *Mbo*II fragment of the *env* gene (from the SF2 isolate) cloned into the pBluescript vector, and it generated a 375-nucleotide transcript. The RRE deletion mutants, *dl*12S, *dl*345, *dl*45, and RRE 220 were obtained from George Pavlakis and Barbara Felber, National Cancer Institute, Frederick, Md. (2, 37).

HeLa cell nuclear extract (50 μg of protein) was preincubated with 2 mM $MgCl_2$ and 50 μg of tRNA for 15 min at room temperature before incubation with wild-type or deletion mutant ^{32}P-RRE transcripts (50,000 cpm). After digestion with RNases A (25 μg) and T_1 (50 U), the ribonucleoprotein (RNP) complexes were analyzed on a 6% nondenaturing polyacrylamide gel (60:1 [acrylamide–bis-acrylamide]) (2, 36).

For protein analysis, the RRE RNA-protein complexes were irradiated for 10 min with shortwave UV light (8 mW/cm^2) at room temperature, heated in sodium dodecyl sulfate (SDS)-

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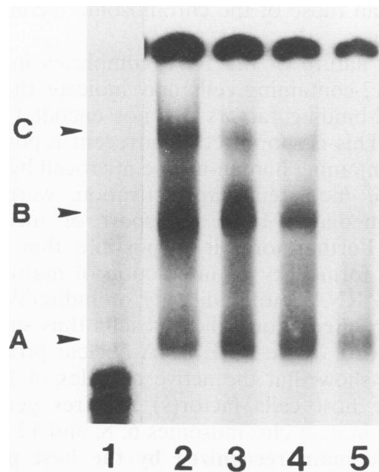

FIG. 1. Binding of host nuclear factor(s) to RRE RNA. Radioactive RRE transcripts, synthesized in vitro, were incubated with nuclear extract as described in Materials and Methods. Arrows on the left indicate the RNP complexes. Complexes are labelled A, B, and C in order of decreasing mobility in the gel. Lanes: 1, RRE RNA transcript alone; 2, NE preincubated with 50 μg of tRNA before the addition of radioactive RRE RNA; 3 to 5, NE preincubated with 50 μg of tRNA and a 15×, 30×, and 50× excess of cold RRE RNA, respectively, before the addition of radioactive RRE.

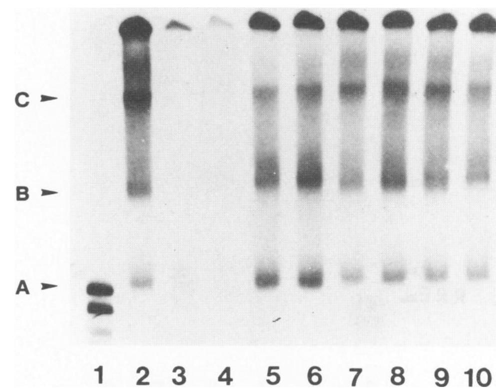

FIG. 2. Competition of RRE RNA-RNP formation by nonspecific RNAs. Unlabelled RRE RNA, antisense RRE RNA, HIV-1 TAR, and β-globin pre-mRNA transcripts were synthesized in vitro as described above. Nuclear extract was preincubated with a 150× or 300× excess of a particular cold RNA before the addition of radiolabelled RRE RNA to the binding reaction mixture. After digestion with RNase, the RNP complexes were resolved on native gel as described above. Lanes: 1, RRE RNA; 2, the RRE RNA-protein-binding reaction in the presence of tRNA; 3 and 4, the binding reaction in the presence of 150× and 300× cold RRE RNA, respectively; 5 and 6, binding reaction in the presence of 150× and 300× cold antisense RRE RNA, respectively; 7 and 8, the reaction in the presence of 150× and 300× cold HIV-1 TAR RNA; 9 and 10, the reaction in the presence of 150× and 300× cold β-globin pre-mRNA.

sample buffer, and analyzed on a 3 to 17% gradient polyacrylamide gel containing SDS (Jule, Inc.). The gel was stained and dried, and autoradiography was performed (34).

RESULTS

HeLa cell proteins form RNP complexes with RRE RNA. The RNP complexes formed between RRE RNA and HeLa cell nuclear proteins are shown in Fig. 1. RRE RNA alone migrates as a doublet in the nondenaturing condition (Fig. 1, lane 1), presumably because of its complex secondary structure. Incubation of the HeLa nuclear extract with labelled RRE RNA resulted in the formation of three RNP complexes; however, resolution of the complexes was very poor (results not shown). Preincubation of the nuclear extract with 50 μg of tRNA accentuated the formation of these complexes (referred to as A, B, and C in order of decreasing mobility; Fig. 1, lane 2), presumably by sequestering the nonspecific RNA-binding proteins. In subsequent experiments, therefore, nuclear extract was preincubated with unlabelled tRNA before the addition of radioactive RRE RNA.

The specificity of the RRE RNA nuclear protein binding was demonstrated by competition with homologous transcripts and a variety of heterologous transcripts. In such competition assays, the nuclear extract was preincubated with 50 μg of tRNA and an excess of unlabelled wild-type RRE RNA, antisense RRE RNA, HIV-1 TAR RNA (34), or β-globin pre-mRNA (36) prior to incubation with the radiolabelled RRE transcripts. As shown in Fig. 1, preincubation of nuclear extract with unlabelled RRE transcripts results in the gradual loss of RRE-RNP complexes. The more slowly migrating RNP complexes B and C are largely inhibited by a 50-fold excess of unlabelled RRE RNA (Fig. 1, lane 5). However, inhibition (Fig. 1, lanes 3 to 5) requires a 300-fold excess of unlabelled RRE transcripts (Fig. 2, lane 4). The results of the experiments with nonspecific RNA transcripts are shown in Fig. 2. Competition with up to a 300-fold excess of unlabelled antisense RRE RNA (lanes 5 to 6), TAR RNA (lanes 7 to 8), or β-globin pre-mRNA (lanes 9 to 10) did not affect the formation of RRE-RNP complexes A, B, and C (Fig. 2). These results suggest that the RRE-RNP complexes are formed by a specific interaction between RRE RNA and HeLa cell nuclear proteins.

Host cell specificity of RRE RNA-protein interactions. The host cell specificity of HIV-1 *tat*-mediated *trans* activation, as well as Rev-mediated transport of unspliced RNA, has been reported elsewhere (15, 28, 38). The issue is whether Rev function requires the cooperation of relevant cellular proteins. To determine whether the binding of nuclear proteins to the RRE RNA could be responsible for such host cell-specific *rev* function, we studied the binding of nuclear proteins to RRE RNA in several human and nonhuman cell lines. Nuclear extracts from CHO (rodent), A9 (murine), HOS (human osteosarcoma), Jurkat (human T-cell line), and NIH 3T3 cells were prepared as described elsewhere (9). Equal amounts of nuclear proteins from various cell types were incubated with radioactive RRE RNA in the presence of tRNA, and the RNP complexes were resolved on a 6% native gel as described previously. The results are shown in Fig. 3. As is evident in lanes 2, 4, and 5, the human cells HeLa, HOS, and Jurkat formed similar RNP complexes in the native gel. However, nuclear extracts from CHO, NIH 3T3, and A9 cells did not produce the characteristic RRE-RNP complexes (Fig. 3, lanes 3 and 6, and Fig. 4, lane 3). To ensure that the lack of binding was specific to RRE RNA and was not due to an inactive nuclear extract, the CHO, NIH 3T3, and A9 cell extracts were also tested for their abilities to bind to HIV-1 TAR RNA. Indeed, these extracts formed normal RNP complexes with HIV-1 TAR RNA (results not shown). These results suggest that the host cell specificity of *rev* function may be mediated by the specific binding of human proteins to the RRE RNA.

To further demonstrate the human cell-specific nature of the nuclear proteins binding to RRE RNA, we prepared nuclear

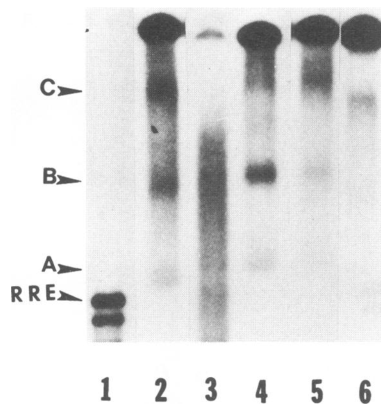

FIG. 3. Host-specific binding of nuclear proteins to RRE RNA. Nuclear extracts from various cell lines were incubated with radiolabelled RRE RNA transcripts, and the binding reaction was carried out as described above. A, B, and C represent the RNP complexes in order of mobility on the native gel. Lanes: 1, RRE RNA transcript; 2, HeLa cells; 3, CHO cells; 4, Jurkat cells; 5, HOS cells; 6, NIH 3T3 cells.

extract from human-mouse somatic cell hybrids retaining single human chromosomes. The hybridomas containing human chromosome 6, 8, 11, or 12 were obtained from the laboratory of Eric Stanbridge (University of California at Irvine). Nuclear extracts from the hybridomas and A9 cells (the parent cells) were prepared, and the RRE-binding studies were performed as described above. Nuclear proteins from the parent cells (A9) did not form distinct RRE-RNP complexes B and C; instead, a smear was observed (Fig. 4, lane 3). A9 nuclear proteins formed complex A, albeit weakly (Fig. 4, lane 3). Nuclear extract from the hybrid cells containing human chromosome 6 formed all three of the RNP complexes (Fig. 4, lane 4). Chromosome 11 cells did not form any complexes, while chromosome 8-containing cells produced one complex that migrated more slowly than complex C (Fig. 4, lanes 6 and 5 respectively). Cells containing chromosome 12 also formed complexes A, B, and C, although less efficiently, and they were

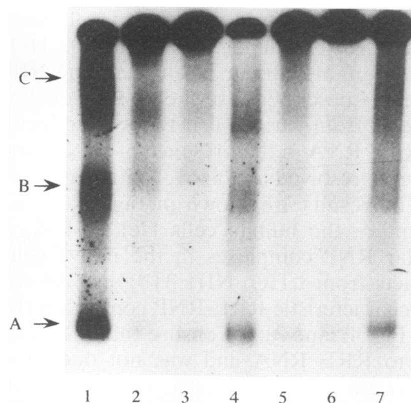

FIG. 4. Binding of nuclear proteins from human-mouse hybrid cells containing single human chromosomes to RRE RNA. Nuclear extracts from the A9 cells (parent cell line) or the microcell hybrids were prepared, and the binding reactions were carried out as described above. A, B, and C represent the RNP complexes in order of mobility on the native gel. Lanes: 1, HeLa cells; 2, CHO cells; 3, A9 cells; 4, CH6 cells; 5, CH8 cells; 6, CH11 cells; 7, CH12 cells.

less distinct than those of the chromosome 6-containing cells (Fig. 4, lane 7).

The smeary nature of the RNP complexes in the human chromosome 12-containing cells may indicate that all of the required RRE-binding factors are not encoded by a single chromosome. This is supported by a recent report that chromosome 12-containing human-mouse microcell hybrids, which can support *tat*-mediated *trans* activation, were unable to support Rev-mediated RNA transport of unspliced viral mRNA (42). Furthermore, it is possible that these RNP complexes are formed by an interaction of multiple proteins with the RRE RNA, either directly or indirectly through a protein-protein interaction. These results thus show the host cell-specific nature of the RRE RNA-nuclear protein interaction, and they show that the active complex of HIV-1 RRE RNA and the host cells factor(s) requires gene products contributed by human chromosomes 6, 8, and 12.

RRE RNA domain recognized by the host proteins. To ascertain that the observed RNP complexes are formed by essential RRE RNA domains and are not due to the extra sequences at the 5′ or 3′ ends, we synthesized minimal RRE transcripts from the plasmid RRE_{220} (derived from an HIV HXB2 clone). The RRE_{220} transcripts formed RNP complexes similar to those seen with the extended RRE (results not shown).

To define the region of RRE RNA recognized by the host cell proteins, we used three deletion mutants of RRE, *dl*12S, *dl*45, and *dl*345. The secondary structures of RRE RNA and of the deletion mutants were determined by MacDNASIS, version 2. The predicted secondary structures of the RRE RNA and its mutants are diagrammed in Fig. 5. The binding of HeLa nuclear proteins to the mutant RRE transcripts shows that the deletion mutant *dl*45 (lacking hairpin loops 4 and 5) forms complexes A and C efficiently, while complex B is not formed (Fig. 6, lane 3). It should be noted that *dl*45 is 55% as active as the wild-type RRE in *rev* function as assayed in vivo (2, 37). The deletion of hairpin loops 3, 4, and 5 (*dl*345) results in complete loss of complex B and the partial loss of complex C (Fig. 6, lane 2). As noted before (2, 37), the RRE mutant *dl*345 is only 26% active compared with wild-type RRE in in vivo assays. The deletion mutant *dl*12S, which lacks the Rev-binding domain and is functionally inactive (2, 37), shows no binding to the nuclear factors (Fig. 6, lane 4). These results are further substantiated by the competition experiments with unlabelled RNA transcripts of the selected RRE mutants. Competition using up to 300-fold excess of the unlabelled RRE mutant *dl*12S did not affect formation of the specific RRE RNA-nuclear protein complexes (Fig. 7, lanes 2 to 4). However, unlabelled *dl*345 and *dl*45 RNAs inhibited the formation of complexes B and C (Fig. 7, lanes 5 to 10). These results further suggest that the binding of host proteins to RRE RNA requires hairpin loops 1 and 2. Loss of host protein RNP complexes upon deletion of hairpin loops 4 and 5 or 3, 4, and 5 suggests the requirement of these loops in the direct binding of host proteins. Alternatively, these non-Rev-binding RRE RNA stem-loops may stabilize the RRE-host protein RNP complex formation. The fact that *dl*345 and *dl*45 do not form complex C but inhibit its formation by wild-type RRE RNA suggests that these non-Rev-binding RRE RNA stem-loops help in the nucleation of the RNP complexes.

The proteins in the RRE RNA-RNP complex. To characterize the proteins which form the RNP complex with the RRE RNA, the complexes were stabilized by UV irradiation and analyzed on a 3 to 17% gradient polyacrylamide gel containing SDS (34). A heterogeneous group of nuclear proteins appear to bind to the RRE RNA even in the presence of nonspecific

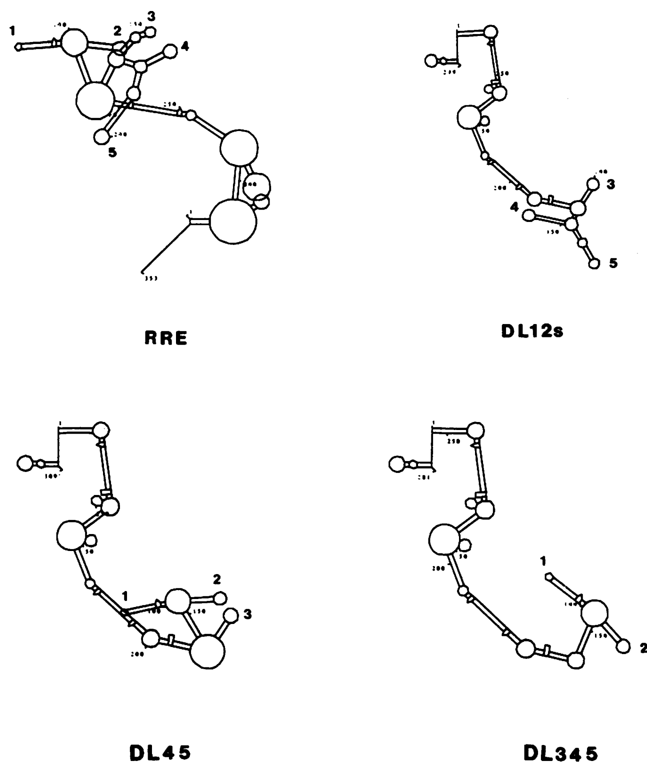

FIG. 5. Predicted secondary structures of the RRE RNA and various deletion mutants. Secondary structures were determined by using MacDNASIS, version 2. Numbers represent the stem-loops of RRE RNA, double lines represent the stems, and circles represent the bulges. We have followed the pattern described by Solomin et al. (37) for numbering various RRE RNA stem-loops.

tRNA (Fig. 8, lane 1). Prominent among these are proteins with apparent molecular masses of 120 and 62 kDa. The specificities of their interactions were demonstrated by efficient competition with unlabelled RRE RNA and the lack of competition by TAR RNA transcripts (Fig. 8, lanes 2 and 3, respectively). To determine whether these proteins were host

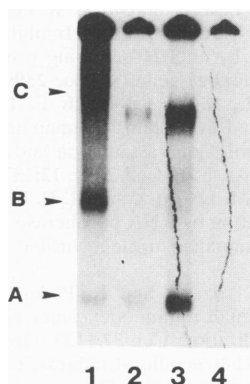

FIG. 6. Host factor binding site in the RRE RNA. Binding reactions with wild-type and various mutant RRE RNAs were carried as described previously. Lanes: 1, binding of wild-type RRE RNA; 2, binding of mutant *dl*345; 3, binding of mutant *dl*45; and 4, binding of mutant *dl*12S.

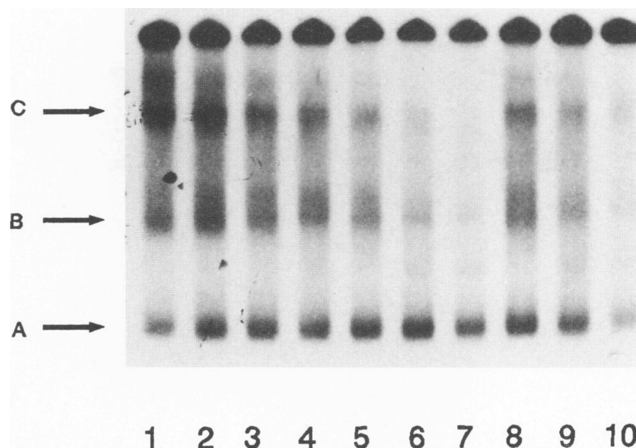

FIG. 7. Competition of RRE RNP formation by cold RRE deletion mutant RNAs. Unlabelled deletion mutant RRE RNA transcripts were synthesized in vitro as described above. Nuclear extract was preincubated with a 75×, 150×, or 300× excess of a particular unlabelled RNA before the addition of radiolabelled RRE RNA to the binding reaction mixture. After digestion with RNase, the RNP complexes were resolved on native gels as described above. Lanes: 1, RRE RNA-protein binding reaction in the presence of tRNA; 2, 3, and 4, the binding reaction in the presence of 75×, 150×, and 300× cold *dl*12S RNA, respectively; 5, 6, and 7, the binding reaction in the presence of 75×, 150×, and 300× cold *dl*345 RNA, respectively; 8, 9, and 10, the binding reaction in the presence of 75×, 150×, and 300× cold *dl*45 RNA.

cell specific, we UV cross-linked the RRE RNA-nuclear protein complexes formed from CHO and HOS cells and analyzed them using SDS-polyacrylamide gel electrophoresis. It was reported earlier that CHO cells did not support *tat*-mediated *trans* activation as well as Rev-mediated transport of unspliced HIV-1 mRNA (15, 28, 38, 42). CHO nuclear extract, which does not form characteristic RRE-RNP complexes, does not contain a significant amount of the 120- and 62-kDa RRE RNA-binding nuclear proteins (Fig. 8, lane 4). Conversely, the levels of these proteins in complexes formed from HOS cells were comparable to those seen in HeLa cells (Fig. 8, lane 5).

The 62- and 120-kDa protein bands were detected in A9 cells and in all of the human-mouse hybrid cell lines, although the intensity among cells containing different chromosomes varied (results not shown). It is important to note that the 62-kDa RRE RNA-binding protein described here is different from the 56-kDa RRE RNA-binding protein reported by Vaishnav et al. (39). The 56-kDa protein was present in CHO cells, whereas our 62-kDa protein is not. Furthermore, Western blot (immunoblot) analysis of a partially purified nuclear fraction enriched for the 62-kDa protein shows that it is different from double-stranded RNA-activated kinase (data not shown) (antibody for double-stranded RNA-activated kinase was kindly provided by Charles Samuel, University of California at Santa Barbara).

DISCUSSION

HIV-1 *rev* gene function has been implicated in the regulation of a complex series of host cell functions, such as RNA splicing and transport, which the virus must evade to express its structural genes. A crucial role of RNP complexes in mRNA splicing and nucleocytoplasmic transport has been reported for a variety of systems (12). Several studies have suggested a

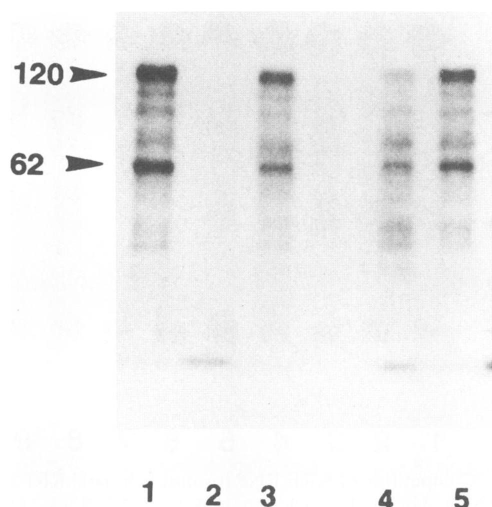

FIG. 8. UV cross-linking of host proteins to RRE RNA. The binding of nuclear proteins to RRE RNA, RNase digestion of the unprotected RNA transcripts, and competition of the binding with unlabelled RRE RNA were carried out as in the gel shift assays. After RNase digestion, samples were exposed to UV rays, heated with SDS sample buffer, and analyzed on a 3 to 17% gradient polyacrylamide gel containing SDS. The molecular masses of the major RRE RNA-binding proteins are listed on the left. Lanes: 1, control binding reaction; 2, competition with cold RRE RNA; 3, competition with cold TAR RNA; 4, CHO nuclear extract; 5, HOS nuclear extract.

regulatory role of host cell factors in HIV-1 gene expression, particularly that of *tat*-mediated *trans* activation. The role of host factors in HIV-1 Rev function has been proposed, but the evidence which corroborates this hypothesis is not sufficient. Possible roles of the host factors which bind to RRE RNA may be to allow the RRE-containing mRNA to escape the host cell splicing machinery and facilitate the transport of unspliced or singly spliced viral mRNA. It is likely that the host factor(s) also facilitates the multimerization of Rev for optimal Rev function in vivo. Additionally, host proteins interacting with RRE RNA may contribute to the function of Rev in the translation of RRE-containing mRNA. We have taken the initial steps in characterizing such human cell-specific proteins that are likely to regulate HIV-1 Rev function.

A strong suggestion for the regulatory role of host proteins in Rev function emerged from results showing the loss of Rev function, i.e., the transport of unspliced RNA, when the non-RRE-RNA-binding domains of Rev were deleted (2, 23, 27). Such *trans*-dominant Rev mutants efficiently bind to RRE RNA but cannot support its function (2, 23, 27). Vaishnav et al. (39) reported the binding of a 56-kDa nuclear protein to the Rev-binding domain of RRE. The precise role of this protein is not known. Recently, Constantoulakis et al. (7) reported the binding of a 17-kDa protein to RRE RNA. This protein is encoded by the interferon-inducible *9-27* gene and can inhibit Rev-mediated HIV-1 gene expression, presumably at the post-transcriptional level. However, the mechanism of this inhibition is still unknown. Trono and Baltimore (38) demonstrated the host dependence of Rev function, since nonhuman cells, such as rodent or murine cells, did not show Rev function. Our results substantiate a host cell dependence for Rev function, since cells which do not support Rev function cannot efficiently form specific RRE RNA-nuclear protein complexes. It is possible that rodent or murine cells lack certain RRE RNA-binding proteins. Alternatively, they may be deficient in a crucial posttranslational step which may be essential for the nucleation of RRE RNA-host protein complexes. Such a supposition will predict that even though some nonhuman cells may contain RRE RNA-binding proteins, a proper posttranslational modification may be required for the formation of a functional RNP complex made up of RRE RNA, host proteins, and Rev. It is likely that the interaction of a host protein(s) with RRE RNA is an essential step toward the formation of such functional RNP complexes. The formation of similar RNP complexes between RRE RNA and nuclear proteins from murine hybridoma cells containing a single human chromosome further substantiates the role of human cell cofactors in Rev function. Two HeLa cell nuclear proteins in particular, with apparent molecular masses of 120 and 62 kDa, bind to the RRE RNA in a host cell-specific manner and may be candidates for such host cell regulators of HIV-1 Rev function. Defining the precise role(s) of these proteins must await their further purification and the cloning of the cDNA sequences which encode them.

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