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Identification of Nuclear Factors that UV-crosslink to Rev-responsive Element RNA

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Abstract

HIV-1 Rev protein plays an important role in regulating the expression of viral structural proteins. It allows the nuclear export and accumulation of unspliced and partially spliced viral mRNA in the cytoplasm. The Rev-responsive element RNA, present in the env gene, forms a highly ordered RNA secondary structure and is required for the Rev-mediated mRNA export. For this process to complete, nuclear factor(s) are strongly suggested. From our experiments of electrophoretic mobility shift, UV-crosslinking and SDS/PAGE, RRE RNA was found to be recognized to several nuclear factors such as 36/37, 56, 41, 76, 150 kD proteins in the order of reactivity. Among them, 36/37 and 56 kD proteins are more reactive upon a brief UV treatment (5 min) and more persistent in the presence of high amount of nonspecific competitor, heparin. Certain nuclear protein(s) seemed to recognize the RRE RNA structure in competition with Rev according to gel mobility shift assay.

Key words : *UV-crosslinking, Electrophoretic mobility shift, Nuclear factors*

Introduction

Human immunodeficiency virus type 1 (HIV-1) gene expression is regulated at the posttranscriptional level by the virus-coded Rev protein that binds to a 244 nt viral mRNA sequences, RRE, embedded within the Env ORF of HIV RNA. HIV Rev facilitates the nuclear egress and subsequent cytoplasmic utilization of partially spliced and unspliced viral mRNAs that contain the RRE sequence[1,2,3,4]. A 63 nt subsequence within RRE which folds into a branched stem loop structure is necessary and sufficient to elicit the Rev response[5,6]. RRE RNA is not an intrinsically negative cis element and its major role appears to be to tether Rev. Rev/RRE interaction overcomes the effects of other cis negative elements (*crs*) in the HIV precursor. The Rev responsive phenotype of RRE is context sensitive to the RNA sequence the RRE is embedded in. With certain transcriptional units that place RRE within a sluggish intron, Rev can dissociate splicing complexes. In other instances, Rev enabled the extra-nuclear transport of the RRE containing transcripts irrespective of their splicing status[7,8,9]. Other studies have suggested that although partially spliced HIV mRNAs may accumulate in the cytoplasm in the absence of Rev, these mRNA are translated only when Rev is present[10,11]. These results suggest that the observed effects of Rev on the HIV mRNA complexity, transport and utilization result from interactions of cel-

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lular proteins with the RRE RNA, Rev or both.

Several studies have suggested that for optimal Rev function, RRE binding cellular proteins or Rev binding factors or ancillary factors may be required. For instance, the 5' most 132 nucleotide fragment of RRE lacked Rev response *in vivo*, although it bound Rev more avidly than RRE *in vitro*. It is possible that for optimal *in vivo* function, oligomeric forms of Rev have to interact with RRE and the above RRE sub-domain may not be recognized by Rev oligomers[12,13]. Alternatively, other subdomains in the RRE native structure may have to be recognized by cellular factors for Rev function[14,15,16]. In fact, a 56 kD cellular protein that bound to the 5' 90 nucleotides of RRE has been presumed to be a Rev helper factor. Certain RRE RNA mutants that were not Rev responsive, still facilitated HIV-Gag expression in the absence of Rev, implying the existence of cellular protein surrogate(s) for Rev. The restricted tropism of the Rev response suggests that some of these Rev helper factors may be tissue or species specific[17,18]. Candidate Rev binding proteins include 38-kDa B23 nucleolar shuttle protein that forms a tight complex with Rev, dissociate by RRE RNA is thought to be a carrier protein for migrating Rev to the nucleolus[19]. In this manuscript, we have identified several nuclear proteins on the basis of selective reactivity to RRE RNA on UV-irradiation and crosslinking. Reactivity of other RNA structures is also described.

Materials and Methods

RNA gel mobility retardation analysis

Reaction mixture containing protein samples and heparin (5 μg/25 μl of total reaction) in HEPES binding buffer (20 mM HEPES-KOH, pH 7.9, 62 mM KCl, 2 mM KCl, 0.15 mM DTT, 6% glycerol and RNAsin at 1000 U/ml as indicated) was incubated at 30℃ for 10 min followed by addition of labeled RNA probe (5×10^4 cpm) and further incubation for 10 min at 30℃. Samples were electrophoresed through non-denaturing 5 % polyacrylamide gel in 0.5×TBE and run at 30 mA at 4℃. The gel was dried and exposed to X-ray film for autoradiography at −70℃.

UV-Crosslinking

Nuclear proteins were extracted from HeLa monolayer cell cultures accoding to Dingnam's method[21]. After dialysis during the last step, precipitates were removed by centrifugation at 13,000×g for 2 min. Reaction mixture containing nuclear proteins, the labeled RNA, and heparinin HEPES binding buffer was irradiated with UV at 180 mjoules on ice. Samples were then digested with RNAse A (20 μg/25 μl reaction) for 30 min at 37℃ and resolved in 12% SDS-polyacrylamide gel. The gel was dried and exposed to X-ray film at −70℃.

Preparation of RNA probe

DNA templates for natural RNA transcripts HIV-1 Rev responsive element RNA (RRE), antisense RRE, adenovirus-2 VAI, HTLV-1 Rex responsive element RNA (RexRE)[22] or synthetic short RNAs in the reaction mixture containing the T7 promoter-tagged primer were amplified by polymerase chain reaction (30 cycles of reaction, each cycle constituting 95℃ for 30 sec, 55℃ for 30 sec and 72℃ for 90 sec). PCR products were electrophoresed in 2% agarose gel and harvested by Gene-Clean method (Bio101, USA). *In vitro* transcription was by use of a commercial T7 transcription kit (Stratagene, USA). [α−^{32}P]UMP labelled transcription products were extracted with phenol : chloroform and with chloroform : isoamylalcohol and purified by Sephadex G-50 chromatography. RNA purity was determined by polyacrylamide gel electrophoresis (PAGE) in 8 M urea, and the transcripts were purified from gels as necessary. The gel-purified RNAs were denatured by boiling and self-annealed by slow cooling to 50℃ in 0.2 M NaCl over a 60 min period.

Results and Discussion

With increasing amounts of purified cellular RNPs caused progressive decrease in the size of cellular RNPs and appearance of new RNP complexes containing Rev and nuclear proteins. In Fig. 1. A, free ^{32}P-UMP labeled RRE RNA molecule (lane 1) shifts near to the top in the presence of nuclear extracts implying that nuclear protein(s) : RNA complex is formed and retarded in movement (lane 2). When purified Rev protein alone is added to the free RRE RNA, the Rev : RRE complex moves slower than the free RRE RNA as expected but faster than RNP complexes (lane 3). In the case that both nuclear protein extracts and REV are mixed with RRE RNA (lane 4), gel retardation is less severe than RNP complex alone, suggesting that more specific recognition of Rev to RRE RNA replaces certain high molecular weight nuclear factor(s) to form a lighter complex.

When HeLa cell nuclear extracts were mixed with ^{32}P-UMP labeled RRE RNA in the presence of nonspecific competitors such as heparin and yeast tRNA, RNPs were readily visualized by electrophoretic mobility shift assay. The complex was stable in the presence of increasing amounts of 5, 25 and 50 μg of heparin in 25 μl of total reaction (Fig. 1. B, right panel) but was strongly inhibited by polyriboinosinic acid : polyribocytidilic acid [poly(rI) : poly(rC)] addition (Fig. 1. B, left panel). The specificity of nuclear protein binding to RRE

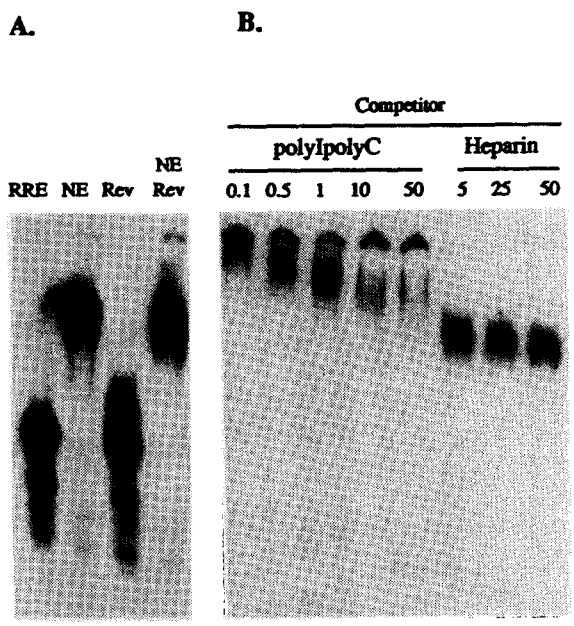

Fig. 1. Electrophoretic mobility shift of the RNP complex.
A. Gel mobility shift of RRE RNA as a free form (RRE) or in the presence of nuclear extracts (NE), Rev protein (Rev) or both (NE/Rev).
B. Gel mobility shift of the RNP complex in the presence of competitors such as polyIpolyC and heparin at the concentration indicated (μg unit per 25 μl of total reaction).

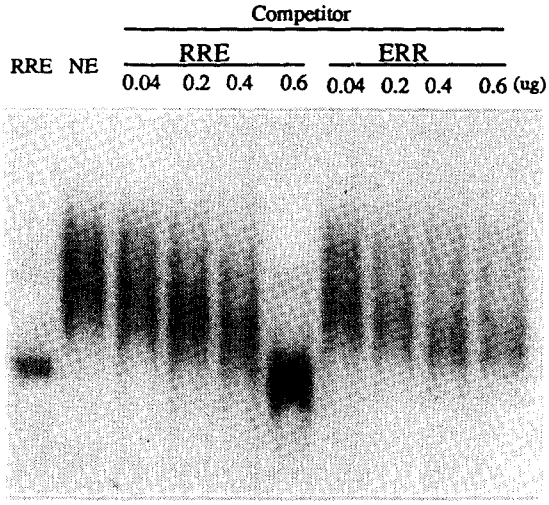

Fig. 2. Electrophoretic mobility shift of the RNP by unlabeled RNAs as competitors.
Lane 1 and 2 : RRE, free labeled RRE RNA ; NE, RNP complexes
The other lanes : RNP complexes are challenged by competitor/RRE (unlabeled RRE RNA at the concentrations indicated) or competitor/ERR (unlabled antisense RRE RNA).

RNA structure was examined by challenging the RNP complex with antisense RRE RNA molecule and monitored in gel mobility shift assay. In Fig. 2, RRE (lane 1) forms the RNP complex (lane 2) as observed in Fig. 1. When increasing amount of unlabeled RRE RNA (lane 3,4,5,6) was added as a competitor to the labeled RRE from the RNP complex, the complex was gradually dissociated to finally leave the labeled RRE RNA free when 0.6 μg of unlabeld RRE RNA in 25 μl of total reaction (lane 6) was added. The addition of the equal amount of the antisense RRE RNA (ERR, lane 7,8,9,10) structure showed less competitive replacement suggesting an existence of more RRE reactive nuclear factor(s).

To enumerate the various cellular proteins binding to RRE RNA, the RNPs complexes were cross-linked and digested with RNAse to remound unbound RNA and the resulting products were resolved by SDS/PAGE. Although prolonged UV irradiation (30 min) cross-linked many RRE-nuclear protein adducts as shown in Fig. 3. A, a subset of these species ; ca. 150, 76, 56, 47, 41, 37 kDa proteins were observed after 5 min UV treatment. Among these, the 36/37 and 56 kDa proteins were most reactive for RRE binding. By limiting UV irradiation to 5 min, RNP formation was still observed with other HIV and non HIV RNAs, including ERR (Fig. 3. B, lane 3,4), 44 nt HIV-1 TAR RNA (lane 5,6), adenovirus-2 VAI RNA (lane 7,8) and the HTLV1 REX responsive element (RXRE) (lane 9,10) RNA. With a ten fold increase in the heparin concentration (5 to 50 g), RRE RNA binding to many of the proteins was still preserved ; however, only the 56 kDa protein and to a lesser degree the 36/37 kDa protein bound the other RNAs. However, at lower heparin concentration, and particulaly with higher UV dose (not shown), the other proteins reacted with ERR, TAR, VAI RNA and RXRE. RNA blotting to nuclear proteins blotted from SDS gels (Northwestern blotting) demonstrated that while less abundant 47 kDa protein was mostly restricted to RRE binding, the more abundant the 36/37 and 56 kDa species had high affinity for RRE RNA, but lacked specificity (not shown).

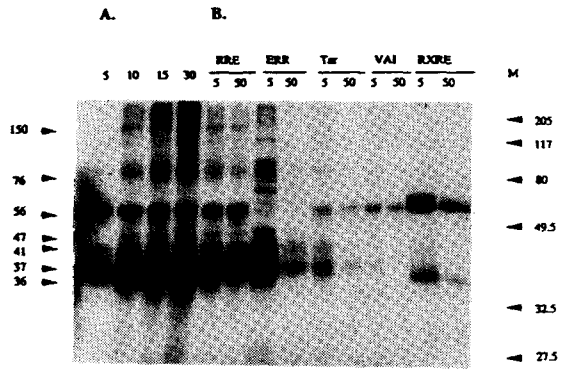

Fig. 3. UV-crosslinking assay of RRE RNA/nuclear proteins.
A. RNP complex formation during the time course of UV-irradiation (5 to 30 min as indicated). Numeric values at left mean molecular weight of each nuclear protein reactive to RRE RNA.
B. RNP complex formation with RRE, antisense RRE RNA (ERR), HIV-1 Tar RNA (TAR), adenovirus-2 VAI RNA (VAI), HTLV1 RXRE RNA (RXRE) in the presence of nonspecific competitor, heparin at the concentrations as indicated (5 or 50 μg/25 μl of total reaction). Molecular weight standards (M) are marked at the right.

Several distinct RRE binding nuclear factors have been identified including the previously reported 56 kDa protein[17], RBFI[21,22,23], B23 nucleolar protein[19] and a human cellular factor[18]. These are known to be in association with the RRE RNA or its derivative mutants, the RRE/Rev complex, or Rev transacting factor, by the manner of direct or transacting mode in human cellular system. However, in the case of RBFI, it was revealed to be reactive rather to the cytoplasmic secondary RNA structures and inhibitory to double-stranded RNA dependent kinase regulating translation initiation step. The

other factors were suggested to be critical for RRE/Rev-mediated mRNA expression. In this study, especially 36 /37 kDa proteins are presumed to be novel nuclear factors which are thought to be of value for elucidating still unclarified mechanism of REV/RRE-mediated unspliced and partially spliced RNA transport to the cytoplasm.

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초록 : UV조사에 의해 Rev-responsive element RNA와 결합하는 핵단백질인자의 확인

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HIV-1 Rev단백질은 바이러스의 구조단백질의 발현조절에 매우 중요한 역할을 하며 RNA가 1차발현된 원형대로 또는 일부만이 절단/재조합된 상태로 세포질에 집적되는 것을 가능하게 한다. *env gene*에 존재하는 Rev-responsive element RNA는 복잡한 RNA구조를 지니면서 Rev의 기능을 위하여 필수적으로 필요시 된다. 그러나 이러한 절차의 완전한 진행을 위해서는 핵단백질이 요구되는 것으로 추정된다. 본 연구에서는 electrophoretic mobility shift, UV-crosslinking 또는 SDS/PAGE등의 실험을 통하여 36/37, 56, 41, 76, 150 kD등의 핵단백질등이 RRE RNA와 결합반응하는 것을 발견하였으며 특히 36/37과 56 kD 단백질을 5분간의 자외선조사에 의해 특히 RRE RNA와 반응적이었으며 고농도의 heparin 존재하에서도 그 반응성이 그대로 유지됨이 관찰되었다. RRE RNA에 결합하는 핵단백질들은 gel mobility shift assay에서 Rev과 RRE RNA인식결합에 경쟁적인 특징을 나타내고 있다.