The *trans*-inhibitory Rep78 protein of adeno-associated virus binds to TAR region DNA of the human immunodeficiency virus type 1 long terminal repeat

Ramesh B. Batchu, Paul L. Hermonat*

Departments of Obstetrics and Gynecology and Microbiology and Immunology, Slot 518, University of Arkansas for Medical Sciences, 4301 West Markham St., Little Rock, AR 72205, USA

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Abstract The large *rep* gene products, Rep78 and Rep68, of adeno-associated virus (AAV) are pleiotropic effector proteins which are required for AAV DNA replication and the *trans*-regulation of AAV gene expression. Apart from these essential functions prerequisite for the life cycle of AAV, these *rep* products are able to inhibit the replication and gene expression of human immunodeficiency virus type 1 (HIV-1) and a number of DNA viruses. Here, it is demonstrated that Rep78, as a chimeric with the maltose binding protein, directly binds the full-length HIV-1 long terminal repeat (LTR), and to a subset of these sequences containing the *trans*-activation response (TAR) sequence as DNA. These interactions, an effector protein physically binding a target promoter, suggest a direct mechanism of action for Rep78 inhibition. Furthermore, competitive binding studies between the TAR region and the full-length HIV-LTR, strongly suggested that another site(s) within the LTR was also bound by Rep78. Finally, as Rep78 binding is also believed to be affected by secondary structure within the DNA, it was found that Rep78 preferentially binds with HIV-LTR sequences with promoted secondary structure generated by heat denaturation and rapid cooling.

Key words: Human immunodeficiency virus; Adeno-associated virus; Rep78; *trans*-Activation response element

1. Introduction

AAV's life cycle as a human helper-dependent parvovirus requires that it interacts with other viruses, and it might generally be thought of as a parasite of its helpers, adeno- and herpes simplex virus [1,2]. AAV has been found to inhibit a variety of viruses, including, adenovirus [3,4], herpes simplex virus [5,6], papillomavirus [7–9], and HIV-1 [10–14]. The Rep78 and Rep68 proteins, encoded by the AAV *rep* gene, have been found to be responsible for these inhibitions [7–9]. Furthermore, Rep78/68 regulate the expression of cellular genes, including c-H-*ras* [15–17], c-*fos* [18,19], and c-*myc* [18,19]. These finding are consistent with the Rep78/68's function as regulators of AAV's own gene expresson.

Previous studies of AAV's Rep78/68 inhibition of HIV-1 have concluded that both HIV-1 gene expression and replication are inhibited [10–14]. During plasmid co-transfection and micro-injection experiments the wild type AAV genome will inhibit HIV-1 replication from 90 to 99%, depending upon the particular study. However, Rep78/68 inhibition of HIV-1 gene expression and replication is even stronger when Rep78/68 are expressed from the HIV-1 long terminal repeat itself (LTR) [12]. Although AAV's inhibition of HIV-1 is well documented, the mechanism by which Rep78/68 inhibit HIV-1 has not been determined. Rep78/68 are highly multifunctional proteins with activities which include covalent and non-covalent binding to DNA, ATP-dependent helicase activity, site-specific endonuclease activity, and transcriptional *trans*-activation and *trans*-repression [20–25]. It is likely that Rep78/68's function as transcriptional regulators are involved in the mechanism of inhibition of HIV-1.

Thus far, it is believed that the short DNA motif, GCTC, is involved in the recognition of DNA by Rep78/68. This sequence is present as a trimeric concatemer in the terminal repeats (TR) of AAV and near the site of AAV preferential integration on human chromosome 19 [22,23,26–28]. The number of GCTC motifs, in close proximity, appear to partially determine the affinity of Rep78/68 protein binding [29]. However, secondary structure within the DNA also plays a role in DNA recognition by Rep78/68. It has been noticed by others [11] that the *cis* TAR sequence of HIV-1, to which the HIV-1 encoded *tat trans*-activator binds as RNA, contains multiple GCTC motifs [30,31]. This laboratory has been studying Rep78 functions utilizing a maltose binding protein-Rep78 chimeric (MBP-Rep78), produced in bacteria, which has all the known biochemical functions of wild type Rep78 from eukaryotes [32]. In this study we demonstrate that MBP-Rep78 is able to bind to the TAR region of HIV-1 as DNA.

2. Materials and methods

2.1. Preparation of DNA substrates and MBP-Rep78 protein

The AAV TR and murine osteosarcoma virus (MSV)-LTR substrates (the positive and negative controls for Rep78 binding, respectively) have been described previously [32], and their sequences are shown in Fig. 1. The full-length HIV-LTR substrate (nt −454 to +42) was generated by PCR amplification with Taq DNA polymerase, using linearized pBennCAT plasmid [33] (*Bam*HI digested) as a template and the primers i and ii, shown in Fig. 1. This substrate is referred to as HIV-LTR (PCR) and was purified by Qiaquick spin column (Qiagen Inc.). Complementary strands of synthetic DNA oligonucleotides (oligo)(Operon) ii and iii of Fig. 1 were annealed to generate the TAR^A substrate (nt +23 to +42). A third HIV-1 DNA substrate was generated by PCR amplification utilizing primers iii and iv, and referred to as TAR^B (+23 to +86). When appropriate, the substrates were labeled utilizing polynucleotide kinase and [γ-^{32}P]ATP. The production and purification of the chimeric MBP-Rep78 protein used in the study has been described elsewhere [32].

2.2. PCR amplification

Amplification was performed in 50 μl reaction mixtures containing

*Corresponding author. Fax: (1) (501) 686 5784.

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50 mM KCl, 10 mM Tris-HCl pH 8.8, at 25°C, 2 mM $MgCl_2$, 150 mM each of dCTP, dATP, dGTP and dTTP, 100 pmol of both primers (shown in Fig. 1), 1 ng of template (*Bam*H I linearized pBennCAT), and 2.5 units of Taq DNA polymerase (Promega, Madison, WI) overlaid with mineral oil. The first denaturing step was performed at 94°C for 2 min. A total of 30 cycles were performed with a denaturing temperature at 92°C for 40 s, annealing at 60°C for 40 s and extension for 1.5 min at 75°C. Final extension was performed for 5 min. The PCR product was purified by Qaiquick spin column (Qiagen Inc.).

2.3. Electrophoretic mobility shift assay (EMSA)

EMSA assays were conducted as described previously with minor modifications [34]. Approximately 1 ng of 5′ end-labeled DNA substrate was incubated with increasing amounts of MBP-Rep78 for 10 min at room temperature in binding buffer [(25 mM HEPES KOH pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreiotol, 2% glycerol, 25 μg bovine serum albumin, 50 mM NaCl, 0.01% NP40 and 0.5 μg poly (dI–dC)]. Samples were electrophoresed in a 4% polyacrylamide gel (40:1 acrylamide and bis-acrylamide weight ratio) with 5% glycerol in 0.5 × TBE buffer at 100 V for about 3 h. Gels were dried and autoradiographed at −70°C. Addition of increasing amounts of MBP-Rep78 is indicated in the figure legend. To promote DNA secondary structure formation in the HIV-LTR (PCR) substrate was boiled for 2 min and chilled immediately on ice as described by others [35]. Experiments involving competition between radiolabeled substrate and unlabeled substrate were carried out as indicated in the specific figure.

3. Results

3.1. MBP-Rep78 binds to the full-length HIV-LTR

With the knowledge that Rep78 recognizes GCTC motifs (or its complement, GAGC) it was observed that the HIV-LTR contained 8 of these motifs. Fig. 1 shows the sequences of the HIV-LTR, the GCTC motifs, and the HIV-1 derived synthetic DNA oligonucleotides used in this study. To determine whether Rep78 bound anywhere within the full-length HIV-LTR, the sequences from nt −454 to nt +42 were amplified by polymerase chain reaction (PCR) and tested in an electrophoretic mobility shift assay (EMSA) for binding with a Rep78/maltose binding protein chimeric, MBP-Rep78. This chimeric MBP-Rep78 protein has identical biochemical activities to that of the fully wild type Rep78 in all assays so far attempted (DNA binding, helicase, endonuclease activity, etc.) [25,26]. As shown in Fig. 2 the MBP-Rep78 protein was able to bind the HIV-LTR (PCR) as indicated by the appearance of a new high shifted band which was dependent of the amount of MBP-Rep78 added to the reaction. This interaction of the effector molecule with the target promoter DNA suggests a direct mechanism of action for AAV Rep78 inhibition of HIV replication and gene expression. The MBP protein did not bind this or any other DNA (data not shown).

3.2. MBP-Rep78 binds the HIV TAR DNA sequences

One region of the HIV-LTR was of particular interest as it contained three GCTC motifs (nt +25 to +38, motifs 1–3, Fig. 1B) in close proximity. This area corresponds to the *cis* TAR region, which is where the HIV-1 encoded *tat trans*-regulatory protein binds as an mRNA sequence, resulting in >100-fold

AAV TR: (positive control)

5′...G_{-143}AACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGC***GCTC*^{3}***GCTC*^{2}***GCTC*^{1}ACTGAGGC$_{-85}$

MSV-LTR: (negative control)

5′...C_{-58}TGTACCCGCGCTTATTGCTGCCCA***GCTC*^{1}TATAAAAAGGGTAAGAACCCCACACTCGGCG$_{+1}$

HIV-LTR:

5′ T$_{-454}$GGAAGGGCTAATTCACTCCC$_{-434}$AACGAAGACAAGATATCCTTGATCTGTGGATCTACCACA
CACAAGGCTACTTCCCTGATTAGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCT
TTGGATGGTGCTACAAGCTAGTACCAGTT***GAGC*^{8}CAGATAAGGTAGAAGAGGCCAATAAAGGAGAG
AA CACCAGCTTGTTACACCCTGT***GAGC*^{7}CTGCATGGGATGGATGACCCGGAGAGAGAAGTGTTAGA
GTGGAGGTTTGACAGCCGCCTAGCATTTCATCACGTGGCCCGA***GAGC*^{6}TGCATCGGGAGTACTTCAA
GAACTGCTGATATC***GAGC*^{5}TTGCTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGGCCT
GGGCGGGACTGGGGAGTGGC***GAGC*^{4}CCTCAGATCCTGCATATAAGCAGCTGCTTTTTGCCTGTACTG
↱ G_{+1}GTCTCTCTGGTTAGACCAGATC$_{+23}$T***GAGC*^{3}CTGG***GAGC*^{2}*TC*^{1}TCTG$_{+42}$GCTAGCTAGGGAACC
CACTGC$_{+63}$TTAAGCCTCAATAAAGCTGCCTT$_{+86}$ 3′

HIV-1 oligonucleotides:

i 5′ TGGAAGGGCTAATTCACTCCC
ii 5′ CAGAGAGCTCCCAGGCTCAG
iii 5′ CTGAGCCTGGGAGCTCTCTG
iv 5′ AAGGCAGCTTTATTGAGGCTTAAG

Fig. 1. Sequences of the DNA substrates and synthetic oligonucleotides used in this study. At the top of the figure are sequences from the AAV TR and murine osteosarcomavirus (MSV) LTR. The underlined bases represent substrate sequences. The motifs GCTC and GAGC are in italics, bolded and identified by superscript numbers. The AAV TR and MSV-LTR substrates were used as the positive and negative controls, respectively [29]. Also shown are the sequences of the HIV-1 LTR. The underlined bases in the HIV-LTR represent substrate sequences and/or PCR primers used to generate substrates. The nucleotide positions relative to the transcription initiation site (bent arrow) of the HIV-LTR promoter are denoted by subscript numbers. The oligonucleotides i and ii were used to PCR generate the HIV-LTR (PCR) substrate (nt −454 to +42). The oligonucleotides ii and iii were used together (complementary) as the TAR^{A} oligonucleotide substrate (nt +23 to +42), which encompasses the TAR sequences. The oligonucleotides iii and iv were used to PCR generate the TAR^{B} substrate (Nt +23 to +86). The plasmid pBennCAT was the PCR template.

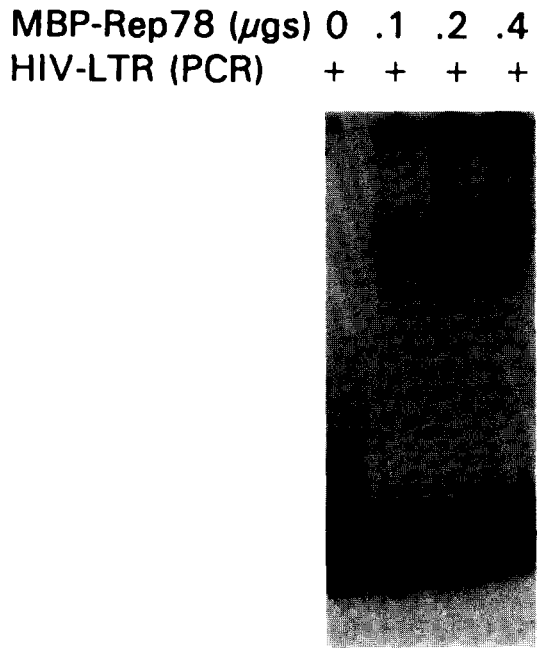


Fig. 2. Dosage dependent complex formation between HIV-LTR DNA sequences and MBP-Rep78 protein. Note that increasing amounts of the band shifted MBP-Rep78/HIV-LTR (PCR) complex appear with increasing amounts of MBP-Rep78. The HIV-LTR substrate (nt −454 to +42) was generated by PCR amplification with Taq DNA polymerase, using linearized pBennCAT plasmid (*Bam*HI digested) as a template and the primers i and ii, shown in Fig. 1. MBP-Rep78 protein production and purification has been described previously [32]. EMSA assays were conducted as described in section 2. Briefly, 1 ng of ^{32}P-5′-labeled HIV-LTR (PCR) was incubated with increasing amounts of MBP-Rep78 for 10 min at room temperature in binding buffer. Samples were electrophoresed in a 4% polyacrylamide gel, dried and autoradiographed. Addition of increasing amounts of MBP-Rep78 indicated above the panel.

higher expression levels [30,31]. This laboratory has previously demonstrated that a region of the c-H-*ras* promoter, with a similar three motif architecture, was able to bind an MBP-Rep protein chimeric [2]. To observe if sequences from the TAR region of the HIV-LTR were able to bind MBP-Rep78, a synthetic duplex DNA oligonucleotide substrate (Fig. 1A, ii and iii, and C from nt +23 to +42, referred to as TAR^A, was used as a substrate for MBP-Rep78 recognition in an EMSA (Fig. 3). The results show that MBP-Rep78 does bind the TAR^A sequences, as indicated by a shifted band. However, the affinity of MBP-Rep78 for TAR^A was at an intermediate level compared to the AAV TR (positive control, the natural substrate for Rep78, with 3 GCTC motifs in a concatemeric arrangement [29]) and a selected sequence from the MSV-LTR (negative control, one GCTC motif [29], also referred to as 'MSV').

In addition to the three GCTC motifs present in the AAV TR's stem, the secondary structure of the TRs, with multiple hairpin loops, are believed to play a role in the recognition and binding affinity of Rep78. Furthermore, this laboratory has demonstrated binding to a single duplex DNA GCTC motif within the context of a DNA hairpin [28]. The DNA sequence of the HIV-1 TAR region also shows the potential for significant secondary structure in the region from nt −1 to +82. To investigate whether Rep78 bound to this larger TAR sequence, a substrate larger than TAR^A was generated by PCR amplification and called TAR^B (from nt +23 to +86) utilizing oligonucleotides iii and iv (Fig. 1A). TAR^B did not contain any additional GCTC motifs over TAR^A. When assayed in the EMSA, the TAR^B sequence also was recognized by MBP-Rep78, and at a slightly elevated level compared to TAR^A (Fig. 3).

3.3. *Competition studies between the full-length HIV-LTR (PCR) and the TAR^A oligonucleotide substrate suggest that MBP-Rep78 is binding to additional sites within the HIV-LTR*

As five other GCTC motifs are present within the HIV-LTR, in addition to those in the TAR region, the possibility that the TAR^A sequences were the major binding site for Rep78 within the HIV-LTR was investigated by EMSA competition studies. The small TAR^A duplex substrate was used as a unlabeled competitor against Rep78 binding with the full-length ^{32}P-labeled HIV-LTR (PCR generated). The AAV TR and MSV-LTR synthetic duplex DNAs were also used as control competitors (positive and negative controls, respectively). As expected the AAV TR was an effective competitor, while the MSV-LTR was ineffective (Fig. 4A). TAR^A was also a very weak competitor. TAR^A was next compared directly with the full-length HIV-LTR (PCR generated) for effectiveness as a competitior (Fig. 4B). As expected the HIV-LTR competed effectively against itself, while TAR^A did not. These data are consistent with the hypothesis that Rep78 is also binding to other regions of the HIV-LTR, possibly other GCTC motifs, and with higher infinity than to the TAR region.

3.4. *MBP-Rep78 preferentially binds to HIV-LTR substrates with promoted secondary structure*

As mentioned previously, it has been reported that Rep78/68 protein binding to DNA subtrates is accentuated if those substrates have significant secondary structure, as with the AAV TR which will form a cruciform structure [26]. To determine

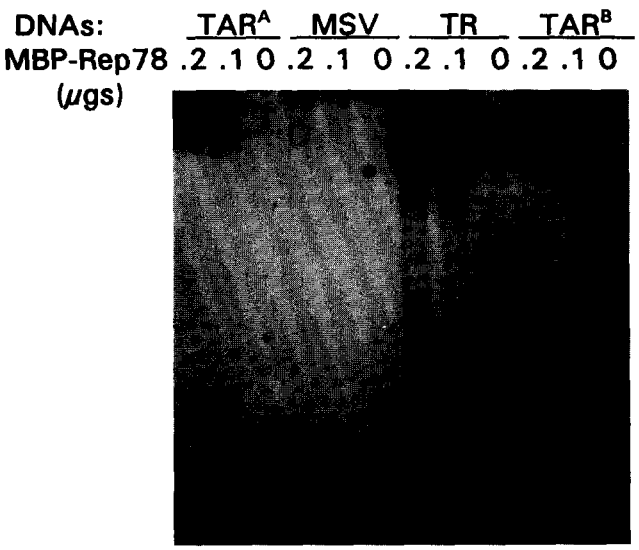
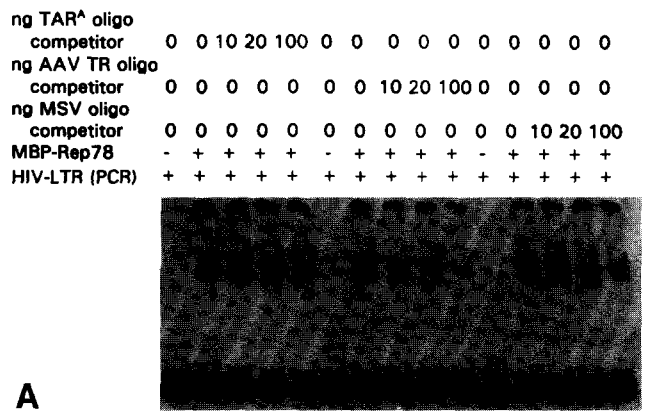


Fig. 3. Analysis of MBP-Rep78 binding to HIV TAR DNA. Note that a band shifted protein-DNA complex appears in both the TAR^A and TAR^B lanes indicating MBP-Rep78 binding to TAR. Approximately 1 ng of the indicated ^{32}P-5′-labeled substrate was incubated in the reaction. The TR and MSV-LTR are the positive and negative controls, respectively. EMSA assay was performed as mentioned in Fig. 2 with a 5% polyacrylamide gel.



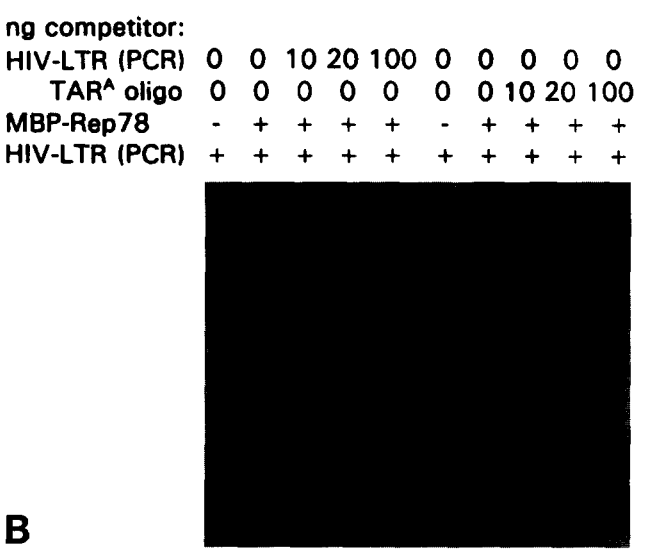


Fig. 4. Competition studies between the HIV-LTR and other DNA substrates for Rep78 binding. (A) Competition studies between ^{32}P 5′-labeled and unlabeled HIV-LTR (PCR) and with unlabeled TAR^A, for MBP-Rep78 binding activity. Note that the HIV-LTR (PCR) is a better competitor than TAR^A. Increasing amounts of unlabeled oligonucleotide competitors were added in the EMSA assay as indicated with equal amounts of MBP-Rep78 protein (500 ng). Approximately 1 ng of ^{32}P-5′-labeled HIV-LTR (PCR) substrate was incubated in every reaction. (B) Competition studies between ^{32}P-5′-labeled HIV-LTR (PCR) and unlabeled AAV terminal repeat (TR) or the MSV-LTR oligonucleotides for MBP-Rep78 binding. Note that the AAV TR oligonucleotide is an effective competitor when compared to the MSV-LTR oligonucleotides or the TAR^A oligonucleotide. Increasing amounts of unlabeled oligonucleotide competitors were added in the EMSA assay as indicated with equal amounts of MBP-Rep78 protein (500 ng). Approximately 1 ng of ^{32}P-5′ end-labeled HIV-LTR (PCR) substrate was incubated in every reaction.

if secondary structure had an affect on MBP-Rep78 recognition of the full-length HIV-LTR, the HIV-LTR (PCR) substrate was heat denatured and quick-chilled on ice to generate 'snap back' secondary hairpin structures [35]. This 'snap back' HIV-LTR substrate was compared to normal duplex HIV-LTR (PCR) for Rep78 binding in an EMSA (Fig. 5). As shown in Fig. 5, the normal duplex and 'snap back' HIV-LTR (PCR) substrates migrated at different positions in the gel so that it was possible to distinguish between them. In one set of reactions equal amounts of the 'snap back' substrate and normal duplex substrate were added together in the EMSA. Both the normal duplex and the 'snap back' HIV-LTR (PCR) substrates were able to bind MBP-Rep78. However, when the duplex and 'snap back' substrates were together in the same reaction tube the 'snap back' substrate was preferentially band shifted by MBP-Rep78 demonstrating a higher affinity for this substrate.

4. Discussion

HIV infection is a global problem with no effective treatment. The inhibitory properties of AAV (Rep78) on HIV-1 replication and gene expression have been well documented, however, the specific mechanism of action is unknown. Our results demonstrate that the Rep78 *trans*-effector protein physically binds to target promoter sequences. Although the functional implications of Rep78 binding to TAR DNA (not TAR RNA to which *tat* binds) of the HIV-LTR are still unknown, these results suggest a direct mechanism of action for Rep78 *trans*-regulation of HIV. Rittner et al. (1992) have previously predicted that the Rep78/68 proteins may bind to specific sequences of the HIV-LTR [11]. Our data supports their hypothesis. The sequences of the HIV TAR oligonucleotides (Fig. 1) which we have shown binding to MBP-Rep78 are nearly identical to those predicted sequences. Possible detailed mechanisms for Rep78 inhibition may include the following: (i) the steric hindrance by Rep78 of the binding of important positive *trans*-regulators (e.g. *tat*), (ii) the signalling of other, more direct, effectors of negative *trans*-regulation, (iii) the direct interaction and effect of Rep78 with the RNA polymerase II complex of proteins, or (iv) the physical disruption of the HIV-1 DNA sequences by the Rep78 endonuclease function.

The inability of TAR^A to effectively compete against the full-length HIV-LTR (PCR) suggests that Rep78 is binding to additional sites within the HIV-LTR and with higher affinity than Rep78 recognition of TAR. Preliminary experiments in this laboratory are consistent with Rep78 strongly binding to another site within the HIV-1 LTR, near TAR (Batchu and Hermonat, unpublished). Furthermore, this study demonstrates that the HIV-LTR, with promoted secondary structure generated by rapid denaturation/renaturation, is preferentially bound by Rep78. These finding are consistent with studies of Rep78/68 binding to the AAV TR and to sequences from the c-H-*ras* promoter [20–23,29]. To what extent these secondary stuctures actually form in vivo is unknown and predicting the extent and importance of the these structures would be highly spectulative. However, the 'snap back' form may have relevance to HIV-1 gene expression as some transcription factors are known to recognize interrupted palindromes which have the potential to generate secondary structures (e.g. the papillomavirus E2 *trans*-activator which recognizes the sequence ACCN$_6$GGT [36].

Continuing these studies we intend to uncover Rep78's mechanism of action for inhibiting HIV-1. Ultimately, it is hoped that AAV Rep78 may be used as a treatment for HIV infection. As a gene therapy vector, another interesting area of AAV research [37], AAV is able to transduce bone marrow progenitor cells [38]. By placing the Rep78 gene under the regulation of an HIV responsive promoter and then transducing hematopoietic progenitor cells, it may be possible to construct an HIV resistant immune system, by generating intracellular immunization.

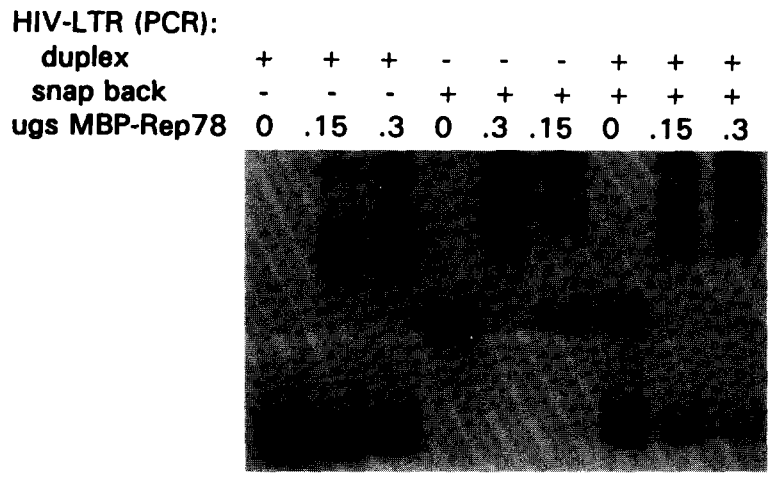


Fig. 5. Preferential binding of MBP-Rep78 with promoted secondary structure of HIV-LTR DNA compared to the same substrate without such promoted structures. Note that the HIV-LTR (PCR) with promoted secondary structure, the higher band compared to the normal duplex, disappears first with the addition of MBP-Rep78 protein. The HIV-LTR (PCR) was subjected to over 100°C for 2 min and quickly chilled on ice to form a 'snap back' secondary structure prior to the EMSA assay. Approximately 1 ng of labeled HIV-LTR (PCR) was used in the study. When normal duplex and promoted secondary structure HIV-LTR (PCR) were both added into one reaction, 0.5 ng of each substrate was used.

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