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The DNA-binding properties of an artificial 42-residue polypeptide derived from a natural repressor

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Bacteriophage 434 repressor recognizes the operator sequences ACAAG and ACAAT. As the same or similar sequences occur in the enhancer region of HIV-1, 434 repressor was a potential HIV enhancer-binding protein. We found that the interaction of the DNA-binding domain of 434 repressor with a 57-bp HIV enhancer DNA was very weak whereas a 42-residue construct, comprising the recognition helix and four copies of a positively charged segment of the repressor, bound strongly. The results of footprint and cell-free in vitro transcription studies showed that the 42-residue peptide bound preferably to the enhancer region of HIV-1 and acted as an artificial repressor. Replacement of an essential glutamine of the recognition helix by glutamic acid resulted in a partial shift of the sequence specificity of the 42-residue peptide.

Bacteriophage 434 operator; HIV-1 core enhancer; Recognition helix of 434 repressor; 434 repressor-derived HIV enhancer-binding polypeptide; DNase I footprint; Inhibition of HIV LTR-controlled in vitro transcription

1. INTRODUCTION

Designed sequence-specific DNA-binding polypeptides could be useful tools to study protein–DNA interactions at the molecular level and may even find practical application as repressors of the transcription of cellular genes or viral genomes including HIV-1. Embarking on the de novo design of an enhancer-specific HIV-inhibitory peptide would therefore be challenging but also very time-consuming [1]. The latter prompted us to base this work on a natural model.

In the operator region of bacteriophage 434 [2], the sequences ACAAG and ACAAT occur as nearly symmetric dimers on opposite strands and are recognized by the dimeric 434 repressor [2,3]. As the same or similar sequences, although monomeric, occur in the enhancer region of HIV-1 [4–7] (Fig. 1a, underlined sequences), we tried to prepare HIV enhancer-binding polypeptides based on the structure of the DNA-binding domain (residues 1–69, Fig. 1b) of 434 repressor [2,3].

Synthetic 434 repressor 1–69 (Fig. 1b) did not bind to HIV enhancer DNA (Fig. 1e) at salt concentrations >20 mM (Fig. 2, lane 2); probably, the interaction with a single ACAAG or ACTTT site was too weak. Likewise, a 26-residue repressor analogue (Fig. 1c, net charge +6) containing the recognition helix [3,8] in the center and the positively charged sequence 37–44 at each end showed no binding. Duplication of the repressor sequence 37–44 at both ends of the recognition helix, however, resulted in a 42-residue polypeptide (Fig. 1d, net charge +14, henceforth called R42) that bound strongly to the HIV enhancer DNA even at a cation concentration of ~250 mM at pH 7.4 (Fig. 2).

In the present work, the sequence specificity and in vitro activity of the artificial, 434 repressor-derived polypeptide and a mutant of its recognition helix were studied using band shift electrophoresis, DNase I footprinting, and cell-free in vitro transcription.

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Abbreviations: HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; R42, artificial 42-residue repressor; HPLC, high-performance liquid chromatography; bp, base pair(s); CD, circular dichroism.

2. EXPERIMENTAL

2.1. *Synthetic peptides and oligodeoxyribonucleotides*

Peptides were synthesized by the automated Merrifield procedure [9] and purified by gel filtration, ion exchange chromatography, and HPLC. Oligodeoxyribonucleotides were purchased and purified by HPLC or on denaturing 20% polyacrylamide gels.

2.2. *Band shift assay [10,11] of the R42-HIV enhancer DNA interaction (Figs. 2 and 5)*

Synthetic HIV enhancer DNA (Fig. 1e and analogues lacking either ACAAG or ACTTT) was labelled at the 5′-end by [γ-^{32}P]ATP and annealed with the unlabelled complementary strand following standard procedures [12]. R42 (Fig. 1d), R42 Arg^{32}, Glu^{33}, R42 Arg^{32}, and 434 repressor 1–69 (Fig. 1b) were incubated with 200 fmol each of labelled enhancer DNA in 20 μl buffer (100 mM potassium phosphate, pH 7.4, 60 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Nonidet P-40) for 15 min at 37°C and the mixtures applied to

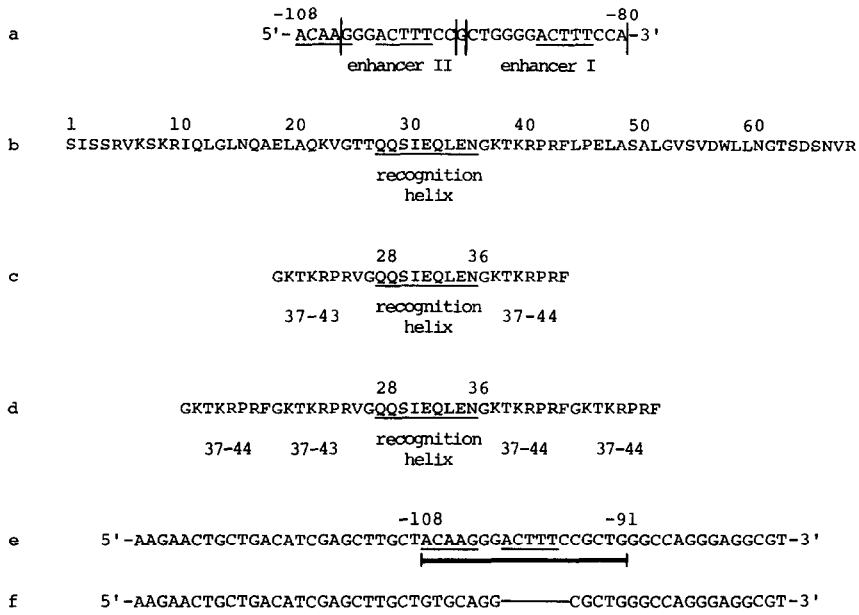

Fig. 1. Polypeptide and DNA sequences relevant to the present study. a, enhancer region of HIV-1, segments identical or similar to 434 operator sequences are underlined; b, DNA-binding domain of 434 repressor (recognition helix underlined); c, synthetic 26-residue analogue of 434 repressor composed of residues 37–43, valine, glycine, the recognition helix and residues 37–44; d, synthetic 42-residue analogue of 434 repressor (R42, composition as indicated); e, synthetic HIV enhancer DNA (57 bp); to avoid clustering of potential binding sites, one of the two ACTTT sequences (−88 to −83) was omitted; the heavy bar marks the region protected from DNase I digestion by R42; f, synthetic 51-bp analogue of the enhancer DNA shown in e lacking the ACAAG and ACTTT sequences.

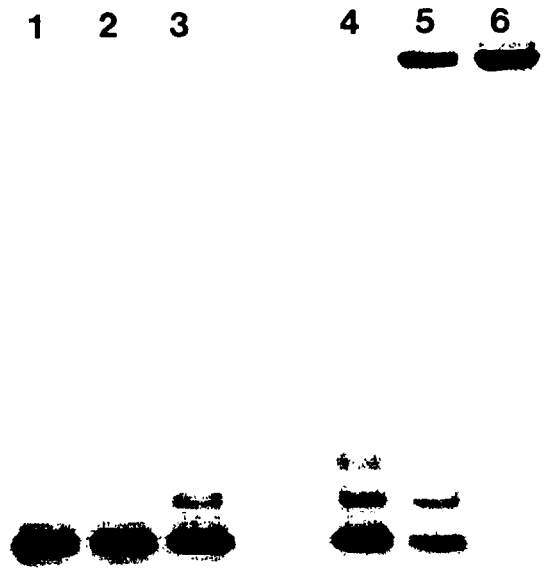

Fig. 2. Band shift assay of the complex formation between R42 and the labelled 57-bp HIV enhancer DNA on a 12% polyacrylamide gel. Lane 1, HIV enhancer DNA alone; lane 2, DNA-binding domain of 434 repressor (Fig. 1b, 35 pmol); lanes 3–6, R42 (Fig. 1d, 10, 15, 20, and 35 pmol, respectively).

polyacrylamide gels. Electrophoresis was run at 200 V for 4.5 h in 22.5 mM Tris-borate. Gels were analyzed by autoradiography.

2.3. *DNase I footprint analysis [13] of the R42-HIV enhancer DNA interaction (Fig. 3)*

Peptides were incubated with labelled 57-bp enhancer DNA (500 fmol each) in 100 μl buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.25 mM EDTA) for 15 min at 37°C. Each mixture was treated with DNase I (1 μg in 5 μl buffer) for 2 min at 22°C. The digests were precipitated and redissolved as described [12], heated to 90°C for 2 min, chilled, and applied to a denaturing 10% polyacrylamide gel (5 μl or ~12,000 cpm per lane). Electrophoresis was in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA at 2,000 V.

2.4. *In vitro transcription of recombinant plasmids in the presence of R42*

Plasmid pCD12 [14,15] was isolated from pCD12-transformed *E. coli* AG1 using Qiagen anion-exchange columns. Cleavage by *Nco*I and isolation of the linearized plasmid followed standard procedures [12]. HeLa whole-cell extract was prepared by the method of Manley et al. [16]. The protein concentration of the dialyzed extract was 11 mg/ml. Standard in vitro transcription mixtures (50 μl) [14] contained HeLa cell extract and *Nco*I-cleaved pCD12 (1.2 μg each) preincubated for 15 min at 37°C with R42 or the DNA-binding domain of 434 repressor (Fig. 1b). Incubations were terminated after 2.5 h at 30°C by adding Promega stop solution and the transcripts isolated [12] and then separated on denaturing 6% polyacrylamide gels in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% SDS at 200 V. Control plasmid OVEC-1 [17] was isolated from OVEC-transformed *E. coli* HB101 and linearized by *Eco*RI cleavage. In vitro transcription and isolation and separation of the transcripts were as described for pCD12.

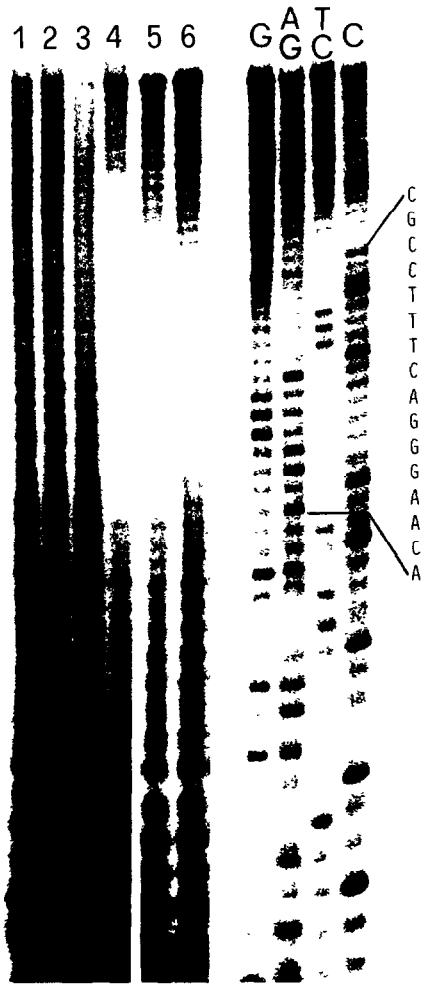

Fig. 3. DNase I footprint analysis of the interaction of R42 and two R42 analogues with the labelled 57-bp HIV enhancer DNA. Lane 1, DNase I digestion in the absence of protein; lanes 2 and 3, digestion in the presence of the DNA-binding domain of 434 repressor (Fig. 1b, 100 and 250 pmol resp.); lanes 4–6, digestion in the presence of R42 Arg^{32}, R42, and R42 Arg^{32}, Glu^{33} (100 pmol each resp.). Lanes G, A+G, T+C, and C show the sequence analysis of the 5'-labelled 57-bp HIV enhancer DNA. R42 Arg^{32} and its DNA-binding properties will be described elsewhere.

3. RESULTS AND DISCUSSION

3.1. *DNA binding specificity of R42*

The operator-binding domain of 434 repressor (Fig. 1b) did not interact with a 57-bp HIV enhancer DNA (Fig. 1e) whereas the 434 repressor-derived construct (R42, Fig. 1d) bound strongly. Gel electrophoresis showed that low and high molecular mass complexes were formed (Fig. 2) depending on the peptide concentration of the incubation mixture [11]. CD titration of enhancer DNA with R42 up to a fivefold molar excess of peptide indicated that the mode of binding was unique (manuscript in preparation).

In order to study how the sequence specificity of the recognition helix was affected by the positive charges at the N- and C-terminus of R42, the band shift assays were repeated in the presence of sonicated calf thymus DNA and HIV enhancer DNA, lacking the potential target sequences ACAAG and ACTTT (Fig. 1f), as competitors. It was found that a 100-fold excess of the competitor DNAs displaced 43% and 50%, respectively, of bound labelled HIV enhancer DNA (Fig. 1e) indicating that despite the many positive charges R42 had retained binding specificity. This was confirmed by DNA melting studies. In the presence of an equimolar amount of R42 (2×10^{-8} M) the melting temperature of the HIV enhancer DNA rose from 73.2 to 75.2°C whereas that of the enhancer DNA lacking ACAAG and ACTTT increased only from 74.4 to 74.6°C.

DNase footprinting [13] in connection with sequence analysis [18] of the synthetic HIV enhancer DNA revealed that the region that was protected from DNase I digestion by R42 (Fig. 3, lane 5, and sequence marked by heavy bar in Fig. 1e) extended several base pairs beyond the ACAAG and ACTTT segments. The operator-binding domain of 434 repressor (Fig. 1b) could not protect HIV enhancer DNA (Fig. 3, lanes 2 and 3).

3.2. *Inhibition of HIV LTR-controlled in vitro transcription by R42*

Okamoto and Wong-Staal [14] had shown that addition of *Nco*I-cleaved recombinant plasmid pCD12 [14,15] (Fig. 4a), containing the LTR region of HIV-1, to HeLa whole-cell extract [16] resulted in RNA polymerase II-catalyzed synthesis of a 633-nucleotide transcript (Fig. 4b, lane 2). In the presence of R42 the formation of this transcript was inhibited (Fig. 4b, lane 5). Complete suppression of transcription of 1.2 μg *Nco*I-cleaved pCD12 required 0.25 μg of R42 which probably interfered with the HIV enhancer binding of HeLa cell proteins such as EBP-1 [19] and NF-κB [6,20] and thus acted as artificial repressor. It did not block the transcription of the 18S rRNA gene [14] (Fig. 4b, lane 5). Suppression of the cell-free in vitro transcription of the recombinant control plasmid OVEC-1 [17] which has six upstream ACAA segments but no HIV enhancers required ~10 times the amount of R42 that inhibited the transcription of pCD12 (Fig. 4c).

3.3. *Characterization of the Arg^{32}, Glu^{33}-analogue of R42*

Gln^{33} of 434 repressor contributes to the specific recognition of $\frac{AT}{TA}$ and $\frac{AG}{TC}$ of the 434 operators through hydrogen bonds of its amide side-chain with $C^4{=}O$ of T and $C^6{-}NH_2$ of A or $C^4{-}NH_2$ of C [3]. In order to shift the binding specificity to $\frac{TT}{AA}$ of the ACTTT sequences of the HIV enhancers (Fig. 1a), Gln^{33} was replaced by glutamic acid because the side-chain of the latter would be a better acceptor of the two hydrogen bonds with the $C^6{-}NH_2$ groups of the adjacent adenines. To avoid clustering of negative charges in the recognition helix

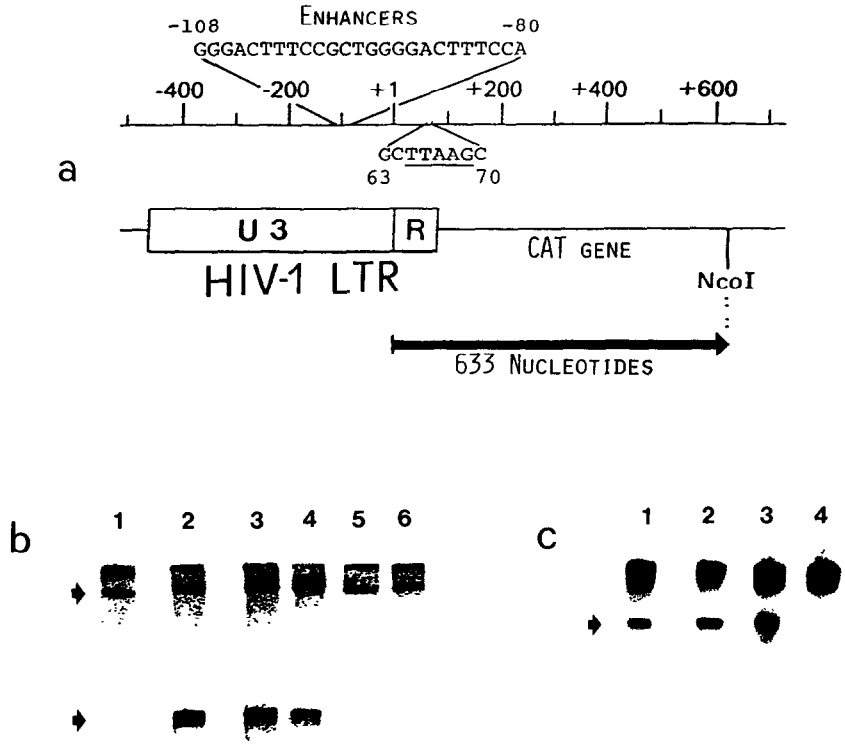

Fig. 4. (a) control region and single *Nco*I site of recombinant pCD12. (b) gel electrophoretic analysis of the effect of R42 on in vitro transcription of *Nco*I-cleaved pCD12 in HeLa whole-cell extract [14]. Lane 1, transcription in the absence of plasmid; lane 2, transcription in the presence of plasmid; lane 3, plasmid and DNA-binding domain of 434 repressor (500 pmol); lane 4, plasmid and R42 (25 pmol); lane 5, plasmid and R42 (50 pmol); lane 6, plasmid and α-amanitin (0.5 μg/ml) which inhibits RNA polymerase II-catalyzed transcription. Upper arrow, position of 18S rRNA [14]; lower arrow, position of run-off transcript of *Nco*I-cleaved pCD12. (c) effect of R42 on in vitro transcription of *Eco*RI-cleaved OVEC-1 [17] in HeLa cell extract. Lane 1, transcription in the presence of plasmid and R42 (250 pmol); lane 2, plasmid and R42 (100 pmol); lane 3, plasmid without peptide; lane 4, transcription in the absence of plasmid. The arrow marks the position of the 989-nucleotide run-off transcript of *Eco*RI-cleaved OVEC-1. In b and c, the isolated transcripts were dissolved in 40 μl formamide buffer each and 5 μl applied per lane.

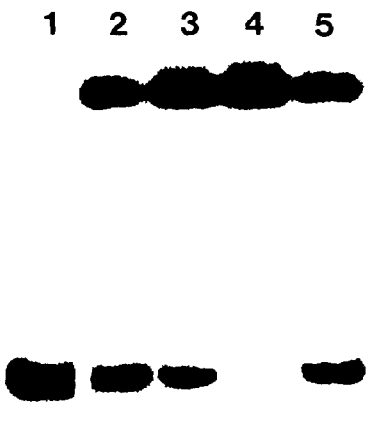

Fig. 5. Band shift assay of the complex formation of R42, R42 $Arg^{32}Glu^{33}$, and R42 Arg^{32} (10 pmol each) with labelled HIV enhancer DNAs on an 8% polyacrylamide gel. Lane 1, HIV enhancer DNA lacking ACAAG; lane 2, HIV enhancer DNA lacking ACAAG + R42; lane 3, HIV enhancer DNA lacking ACTTT + R42 $Arg^{32}Glu^{33}$; lane 4, HIV enhancer DNA lacking ACAAG + R42 $Arg^{32}Glu^{33}$; lane 5, HIV enhancer DNA lacking ACAAG + R42 Arg^{32}.

$(Glu^{32,33,35})$, Glu^{32} was changed to arginine yielding the Arg^{32},Glu^{33}-analogue.

Fig. 5 shows that the binding specificity of R42 Arg^{32},Glu^{33} was indeed shifted toward ACTTT (lanes 3 and 4) and that the R42 analogue bound twice as much of an enhancer DNA lacking ACAAG than R42 (lanes 2 and 4). These results confirm the importance of Gln^{33} of 434 repressor for the specific recognition of the target DNA and represent a first step toward improving the binding specificity of our R42 construct.

4. OUTLOOK

Elucidation of the structure in solution and in the crystal of the complex of R42 with HIV enhancer DNA is a realistic goal and may give us useful hints for the design of R42 analogues with improved HIV enhancer binding specificity. The genes of these analogues could be expressed in HIV-infected cells using a recombinant virus [21] and the gene products might suppress the transcription of HIV-1 and thus provide intracellular immunity [22]. The major problem would be that we do not know if the expression of cellular genes would also

be affected. In this respect, it might be of advantage that R42 has intermediate affinity for its target sequence(s).

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