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A human Raf-responsive zinc-finger protein that binds to divergent sequences

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ABSTRACT

LZ321, a human liver cDNA, encodes a protein that bound to a *Drosophila* tramtrack binding site, GGTCCT. The sequence of LZ321 matched that of RREB1, a transcription factor that bound to a Ras responsive element (RRE) very different from the sequence with which we isolated LZ321. We therefore examined the binding of RREB1/LZ321 to different ligands. It bound to the GGTCCT-containing ligand and to the RRE with similar affinities (K_d 50–60 nM), but did not bind to a consensus RREB1 binding site. The RREB1/LZ321 protein contains four C_2H_2 zinc-fingers, the C-terminal two of which retained specific DNA binding to both ligands. A trimer of the GGTCCT site functioned as an enhancer in both CV-1 and H4IIE-C3 cells. Thus RREB1/LZ321 could function as a downstream activator in the Ras–Raf signaling pathway through different *cis*-acting elements. A longer human protein, Finb, contains RREB1/LZ321, and there are close homologs in both chicken and *Drosophila*, arguing that it plays important roles. The ability of transcription factors such as RREB1/LZ321 to bind diverse sequences gives them the potential to regulate previously unsuspected genes.

INTRODUCTION

Transcription is mediated by the specific interaction between *trans*-acting nuclear proteins and *cis*-acting DNA sequences. A large family of transcription factors is characterized by a tandemly repeated C_2H_2 zinc-finger, with the consensus sequence $CX_{2-4}CX_3FX_5LX_2HX_{3-4}H$ (where X is any amino acid) (1–3). A tetrahedrally coordinated zinc ion is bound by the conserved Cys and His residues; it organizes key amino acid residues into a structure capable of binding the target nucleotide residues (2,4–6). Binding occurs in the major groove of B-DNA, where each zinc-finger contacts a 3 bp, usually guanine-rich subsite (5,6). In a subset of these proteins, of which the *Drosophila* gap gene Kruppel is the prototype, the zinc-finger domains are separated by a short conserved motif called the H/C link (TGEKPYE/K) (7). The H/C link may play a key role in aligning zinc-finger domains within the major groove of the DNA helix (5). It has been used as a consensus sequence to isolate many human Kruppel-type zinc-finger proteins (3,8).

In an attempt to identify *trans*-acting transcription factors that regulate the human class I alcohol dehydrogenase genes (9–11), we screened (12,13) a human liver cDNA expression library using as probe a *cis*-element found in the *ADH3* promoter. We isolated a cDNA, LZ321, which bound specifically to the trimerized oligonucleotide. LZ321 encoded a Kruppel-type zinc-finger protein not represented in the database at that time. DNase I footprinting demonstrated that LZ321 did not bind to the *ADH3* promoter, but rather to the junction created by trimerization of the oligonucleotide. The trimerized oligonucleotide functioned as an enhancer. While we were characterizing this transcription factor, the Ras-responsive factor RREB1 was reported; its sequence matched LZ321.

RREB1 is a Kruppel-type zinc-finger protein, recently isolated from the human medullary thyroid carcinoma cell line TT, which binds to a Ras-responsive element (RRE) in the calcitonin gene promoter (14). Within cells with an activated Ras–Raf pathway, RREB1 stimulates the transcription of its downstream target genes (14). Ras proteins are GTP-binding proteins that play important regulatory roles in cell proliferation and differentiation (15,16). Ras regulates several classes of genes, including genes associated with the mitogenic response (e.g. c-fos and c-jun), growth factors (TGF-β), extracellular matrix proteases, tumorigenesis and metastasis. Among the RREs that have thus far been defined are binding sites for the transcription factors AP-1 (15,17–19), Ets family members (15,18) and GATA-2 (20).

The *Drosophila ttk* gene is a downstream target of the Ras/Raf pathway (21–25). Tramtrack protein (ttk) can repress the expression of a number of downstream target genes, including the segmentation gene *fushi tarazu* (*ftz*) and the pair-rule genes *even skipped*, *odd skipped*, *hairy* and *runt* (26–28). Ttk is required for cell fate determination during *Drosophila* eye development (29). Its DNA binding domain contains two Kruppel-type zinc-fingers that can bind to the sequence GGTCCT (25).

The binding site for LZ321 contained a GGTCCT sequence that differed greatly from the sites to which RREB1 bound.

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Here we compare the DNA binding affinity of this RREB1/LZ321 transcription factor to three distinct sequences: the original RRE with which RREB1 was isolated, a consensus sequence (14) derived by the method of cyclic amplification and selection of targets (30) and the GGTCCT site to which LZ321 bound. We mapped the DNA-binding domain of this protein. We also tested the transactivation activity of RREB1/LZ321.

MATERIALS AND METHODS

Screening of the library

The probe was a trimer of annealed oligonucleotides HE321 (5'-ccgaAGAGAAGCAGGAACTTGAGAGAGGA) and HE322 (5'-tcggTCCTCTCTCAAGTTCCTGCTTCTCT), containing the sequence from bp –245 to –262 of the *ADH3* promoter (10), with CCGA and TCGG overhangs added to facilitate unidirectional ligation. Ligation products were cloned into the *Ava*I site of pUCHE-AvaI, a modified pUCHinEco1 (31) with an *Ava*I linker inserted (unpublished data). The HE321/2 trimer was isolated from the pUCHE-AvaI construct by digesting with *Bam*HI and *Bgl*II, followed by gel purification. It was radioactively labeled with [α-^{32}P]dCTP by nick translation (Boehringer Mannheim, Indianapolis, IN). The labeling mixture was passed through a Sephadex G25 column to remove unlabeled [α-^{32}P]dCTP.

The λgt11 liver cDNA expression library (Clontech, Palo Alto, CA) was constructed from poly(A)$^+$ RNA from a 40 year-old Caucasian female liver by oligo(dT)- plus random-priming. This library was screened by the methods of Singh *et al*. (12) and Vinson *et al*. (13) with some modifications (32,33). One positive clone, called λc0, was isolated from 10^6 plaques screened, and was purified through four rounds of re-screening. This 1.2 kb partial cDNA was subcloned into pUCHinEco1 (31) after PCR amplification from the original clone using λgt11 flanking primers. To obtain the full-length cDNA, the same library was screened by hybridization using radioactively labeled c0 DNA as the probe. Nine positive clones were isolated (λc1–λc4 and λc6–λc10). The clones were plaque-purified and inserts ranging from 0.8 to 2.7 kb were excised with *Eco*RI and subcloned into pUC19. The nucleotide sequence of each clone was determined on both strands using the Sequenase protocol (US Biochemical Corp., Cleveland, OH) or by cycle sequencing (Perkin Elmer/Applied Biosystems Division, Foster City, CA).

Plasmid constructs

pTATA was constructed by moving a *Hin*dIII/*Sst*I fragment containing bp –55 to +33 of the *ADH2* promoter from pTE2$\delta\delta$-TATA (34) into the pXP2 vector (35). p321F and p321R were derived by inserting a trimer of annealed oligonucleotides HE321 and HE322 (above) into the *Bam*HI site of pTATA just upstream of the *ADH2* sequence. Note that HE321 and HE443 are circular permutations of the same sequence; therefore the trimer of HE321 is substantially similar to one of HE443, and contains three GGTCCT sites. pLJZ-1 contains bp –326 to +69 of the *ADH3* promoter, from pG390 (10), inserted between the *Sac*I and *Hin*dIII sites of pXP2. pMV7p1 is a mammalian retroviral expression vector with the Moloney Murine Leukemia Virus long terminal repeat as promoter (36). pMV7p1-371 contains the full-length RREB1 cDNA inserted into pMV7p1 between the *Xba*I and *Eco*RI sites (14). pMV7p1 and pMV7p1-371 were generously provided by Dr Barry Nelkin (The Johns Hopkins Medical Institutions, Baltimore, MD). pcEXV-3 is a mammalian expression vector (37), and pcEXV-myc-Raf-Cx is a modified human Raf-1 construct with the 17 amino acid Ki-Ras membrane localization sequence at the C-terminus and a myc-tag at the N-terminus (38); both were generous gifts from Dr Mark Marshall (Indiana University School of Medicine, Indianapolis, IN).

Expression and purification of GST-fusion proteins in *Escherichia coli*

The c0 fragment was excised from pUCHinEco1 with *Eco*RI and *Sal*I and subcloned in frame into pGEX-4T-1 (Pharmacia Biotech, Piscataway, NJ), an *E.coli* expression vector that produces GST-fusion proteins (39). The c4 fragment was excised from pUC19 with *Eco*RI and cloned in frame into pGEX-4T-1. The c9 fragment was similarly cloned in frame into pGEX-3X (Pharmacia Biotech). pGEX-3-RREB1 contains the full-length coding sequence of the RREB1 protein (GenBank accession no. U26914), and pGEX-2-δRREB1 encodes the 705 C-terminal amino acids of RREB1; the RREB1 constructs were generously provided by Dr Barry Nelkin (The Johns Hopkins Medical Institutions, Baltimore, MD).

The plasmids were introduced into *E.coli* BL21 cells. Clones were cultured to an OD_{600} between 0.6 and 1.0 before fusion proteins were induced with 1 mM IPTG for 4 h. Bacteria were lysed by sonication. Fusion proteins were affinity purified (39) using glutathione agarose beads as instructed by the manufacturer (Sigma, St Louis, MO). Protein concentrations were measured by the Bradford method (Bio-Rad, Richmond, CA) with BSA as standard. The size and purity of the proteins were checked by electrophoresis in 7.5, 12 or 4–15% SDS gels (Pharmacia LKB Phast System; Pharmacia, Uppsala, Sweden). The purified fusion proteins were stored at –80°C after adding 0.5 mM PMSF, 0.05 µg/µl Leupeptin, and glycerol to 20%.

Gel mobility shift assays and DNase I footprinting

DNase I footprinting was performed with 10 000 c.p.m. of radiolabeled DNA probes (~0.02–0.05 pmol) as previously described (5). The reaction was performed in a final volume of 20 µl buffer containing 2 µg of poly(dI-dC) (Pharmacia Biotech, Piscataway, NJ) and either the purified GST-fusion proteins or 40 µg mouse liver nuclear extract. The reactions were incubated at room temperature for 20 min and then DNase I (Boehringer Mannheim, Indianapolis, IN) was added for exactly 2 min. The digestion was stopped by adding 75 µl of 20 mM Tris–HCl (pH 7.5), 20 mM EDTA, 5 mM EGTA and 5 µg yeast tRNA, extracted with phenol/$CHCl_3$ (1:1) and ethanol precipitated. DNA resuspended in 5 µl formamide loading buffer was electrophoresed on 6% polyacrylamide/7 M urea sequencing gels (GEL-MIX 6, Life technologies, Inc., Gaithersburg, MD). The gels were dried and exposed to X-ray film (XAR5, Kodak, Rochester, NY) with a Quanta III intensifying screen (Dupont, Boston, MA), if necessary, at –70°C overnight.

Gel mobility shift experiments were performed as previously described (34). ^{32}P-labeled DNA (5000–10 000 c.p.m.; 0.01–0.05 pmol) was incubated at room temperature for 15 min in a final volume of 20 µl with various amounts of purified GST-fusion proteins, and then electrophoresed in 4% low ionic strength non-denaturing polyacrylamide gels (80:1 acrylamide/bisacrylamide)

at 80 V for 4 h. The gels were dried and exposed to X-ray film (XAR5, Kodak, Rochester, NY) at –80°C with or without intensifying screen (Dupont, Boston, MA) for 24–48 h. For quantitation, gels were exposed on Bio-Rad Imaging high sensitivity screens for 2–4 h and then analyzed on a PhosphorImager GS-250 (Bio-Rad Laboratories, Inc. Hercules, CA). Percentages of bound probe were quantitated using Molecular Analyst Version 2.1 (Bio-Rad, Richmond, CA).

Equilibrium binding affinity and dissociation rate constant determinations

The equilibrium dissociation constants (K_d) of truncated RREB1 proteins were measured by incubating serial dilutions of GST-fusion proteins with a constant concentration of each probe (0.2–0.5 nM). The original protein concentrations were 98 nM δRREB1, 25 nM GST-c4 and 50 nM GST-c0. The three probes were the GGTCCT site (HE443, 5'-GGAACTTGAGA-GAGGACCGAAGAGAAGCA, annealed with HE444, 5'-TGC-TTCTCTTCGGTCCTCTCTCAAGTTCC), the RRE site (HE445, 5'-gatcCGGTCCCCCACCATCCCCCGCCATTTCCA, annealed with HE446, 5'-gatcTGGAAATGGCGGGGGATGGTGGG-GGACCG) and a consensus binding site that was obtained (14) by the method of cyclic amplification and selection of targets (CASTing; 30) (HE447, 5'-gatcACCCCCAAACCACCC-CCCACCC, annealed with HE448, 5'-gatcGGGTGGGGGGT-GGTTTGGGGGT).

The K_d for a protein binding to a single site was calculated according to the equation (40):

$$1/K_d = C_b/C_f(C_p - C_b)$$

where C_b is the concentration of bound DNA, C_f is the concentration of free DNA and C_p is the total protein concentration. At half maximal binding ($C_b = C_f$), the equation becomes $K_d = C_p - C_b$. Because $C_p >> C_b$, the equation simplifies to $K_d = C_p$ (40). Data were analyzed using Grafit (Erithacus Software Ltd, London, UK).

Quantitative competitive gel shift assays were also used to compare the relative affinities of the proteins for the GGTCCT site (in HE443/4) and the RRE site (in HE445/6). Equal concentrations of all the proteins were added to a series of reactions with increasing amounts (0–400 pmol) of non-radioactive oligonucleotides as competitors (labeled probe concentrations were 0.20 nM HE443/4, 0.27 nM HE445/6; protein concentrations were 219 nM δRREB1, 486 nM C0 and 359 nM C4). After electrophoresis, data were collected using the PhosphorImager, as above.

The inhibition of binding by excess non-radioactive oligonucleotide is characterized by an IC_{50} value using the equation (41,42):

$$y = a/[1 + (x/IC_{50})^s] + \text{background}$$

where a is the maximum y, s is a slope factor and x is the concentration of competitors added. IC_{50} was calculated by curve fitting using four-parameter logistic/simple weighting (Grafit, Erithacus Software Ltd, London, UK). However, although we used this technique to examine relative affinities, the calculated IC_{50} values underestimate the actual affinity when the concentrations of binding site are in excess of the K_d (42,43).

Transient transfection assays

H4IIE-C3 rat hepatoma cells and CV-1 monkey kidney cells were plated at 0.8×10^6 or 0.4×10^6 cells per 60 mm dish, respectively. When the cells reached 25% confluence (at 18–24 h), a total of 17 μg of DNA per dish was transfected into them by calcium-phosphate coprecipitation (44). This included 5 μg of the test plasmid, 2 μg of pCMV-β-galactosidase (internal control), 5 μg of pMV7p1 (plasmid control) or pMV7p1-371 (RREB1 expression plasmid; 14) and 5 μg of pcEXV3 vector or pcEXV3-Myc-Raf-Cx (38). The DNA was allowed to remain on the cells for 4 h (CV-1 cells) or 18 h (H4IIE-C3 cells), then the medium was removed and medium containing either 20% (CV-1) or 15% (H4IIE-C3) glycerol was added for 2 min. After removal of the glycerol, fresh medium was added and incubation was continued for a total of 48 h. Cells were rinsed twice with PBS, collected and pelleted. Cells were resuspended in 100 μl lysis buffer (25 mM Tris-phosphate pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N'*,*N'*-tetraacetic acid, 10% glycerol and 1% Triton X-100; Luciferase assay system, Promega, Madison, WI) and broken by sonication. Aliquots of 30 μl of each extract were assayed for luciferase activity for 99 s using the Promega luciferase assay system in a LB9501 Luminometer (Berthold Analytical Instruments, Nashua, NH). An aliquot of 5 μl of extract was used for β-galactosidase activity measurements using the Galacto-Light chemiluminescent assay kit (Tropix, Inc., Bedford, MA). All transfections were performed in triplicate within each experiment, and experiments were repeated three times. The average non-transfected, cell-only background was subtracted from both luciferase and β-galactosidase activities. For each transfected dish, luciferase activity was normalized to β-galactosidase activity. Data from each experiment were normalized to the average value of the positive control plasmid pLJZ-1 in that experiment. Data were analyzed using Microsoft Excel and StatView (Version 4.5, Abacus Concepts, Berkeley, CA).

RESULTS

Cloning and sequencing of LZ321

In an effort to identify transcription factor(s) that bound to a *cis*-acting site present in the *ADH3* gene (10), we used a trimer of that site as probe to screen a human liver cDNA library (12,13). We isolated a partial cDNA encoding a Kruppel-type zinc-finger protein, which we named LZ321, for which there was initially no match in GenBank. This sequence encoded a protein that bound to the probe used in its isolation, but not to three other probes tested (data not shown). Re-screening the library by hybridization with our initial clone allowed isolation of overlapping cDNAs, which were sequenced (GenBank accession no. AF072825). The 5' end of the cDNA was not complete, and two of the clones retained an intron (GenBank accession no. AF072826). Recently, a Ras-responsive transcription factor called RREB1 was reported by Thiagalingam *et al.* (14); its sequence is almost identical to LZ321 in the region of overlap. RREB1 is 3577 bp and contains a 755 amino acid open reading frame, but no poly(A) site. LZ321 starts at bp 946 of RREB1 and extends 1034 bp further at the 3' end, including a poly(A) site.

RREB1/LZ321 binds to two distinct DNA sequences

DNase I footprinting demonstrated that the LZ321 protein bound to the junction region created when the oligonucleotide containing the *ADH3 cis*-acting element was trimerized, rather than to the *cis*-acting element itself (Fig. 1). Database searching revealed that this site contains a GGTCCT sequence, which is a binding site for the *Drosophila* ttk protein. This GGTCCT site is very different from the RRE (5'-CCCCACCATCCCC) in the calcitonin gene promoter with which RREB1 was isolated (14); there are only seven identities in 16 nt. The GGTCCT site differs even more from the RREB1 consensus binding site obtained using the CASTing method (30), 5'-CCCCAAACCACCCC (14) (only 4/16 identities, no different than expected by chance). δRREB1, which encodes the 705 C-terminal amino acids of RREB1 containing all four zinc-fingers (Fig. 2A), bound to the GGTCCT site in HE443/4 (Fig. 2B). This binding could be competed by a 100-fold molar excess of either self-competitor (HE443/4) or an RRE site competitor (HE445/6). Surprisingly, the RREB1 consensus site oligonucleotide HE447/8 could not compete out the binding (Fig. 2B, lane 5).

Mapping the DNA binding domain of RREB1/LZ321

Since the RREB1/LZ321 protein bound to two very dissimilar sequences, we mapped the DNA-binding domains. We tested binding of δRREB1 (containing all four zinc-fingers), C0 (a 379 amino acid peptide containing the three C-terminal zinc-fingers), C4 (a 227 amino acid peptide containing the two C-terminal zinc-fingers) and C9 (from a cDNA clone that contains an intron, and encodes a 108 amino acid peptide containing only the N-terminal zinc-finger) (Fig. 2A). All were expressed as GST-fusion proteins in *E.coli*. Gel mobility shift assays were performed using both the GGTCCT site (HE443/4) and the RRE site (HE445/6) as probes. The three peptides that contained in common the two C-terminal zinc-fingers bound to both sites (Fig. 2C). C9, containing a single N-terminal zinc-finger, did not bind to either ligand (Fig. 2C, lane 5 in both right and left figures). None of the peptides bound to the consensus RREB1 binding site HE447/8 (data not shown).

Quantitative binding studies

To test the DNA binding affinity of RREB1/LZ321 to these ligands, we performed equilibrium binding affinity studies. The δRREB1 construct was analyzed, as it was difficult to obtain sufficient quantities of the full-length RREB1/LZ321 protein. The concentration of probe was kept constant as the protein concentration was increased from 0 to 1400 nM (Fig. 3A). The binding affinity of δRREB1 to the GGTCCT site and to the RRE site was essentially the same (Fig. 3B and C; Table 1).

To confirm these results, we performed a competitive gel shift analysis (Fig. 4). Although the calculated IC_{50} values underestimate the actual affinity when the concentrations of binding site are in excess of the K_d (43,45), data confirmed that δRREB1 bound to both sites with equal affinity (Table 2).

We also carried out equilibrium binding studies on two truncated forms of LZ321, C0 and C4, to determine quantitatively how the removal of the two N-terminal zinc-fingers affected DNA binding affinity (Fig. 3B and C). Although they still bound DNA, the binding affinity was reduced. Deletion of N-terminal zinc-finger 1 (in C0) increased K_d ~3-fold over that found for δRREB1 (Table 1). Deletion of both N-terminal zinc-fingers result in ~3–8-fold increases in K_d. Similar relative reductions in affinity (increases in IC_{50}) were found in competitive gel shift analyses (Fig. 4; Table 2). Under our conditions, none of the peptides bound to the reported RREB1 consensus site.

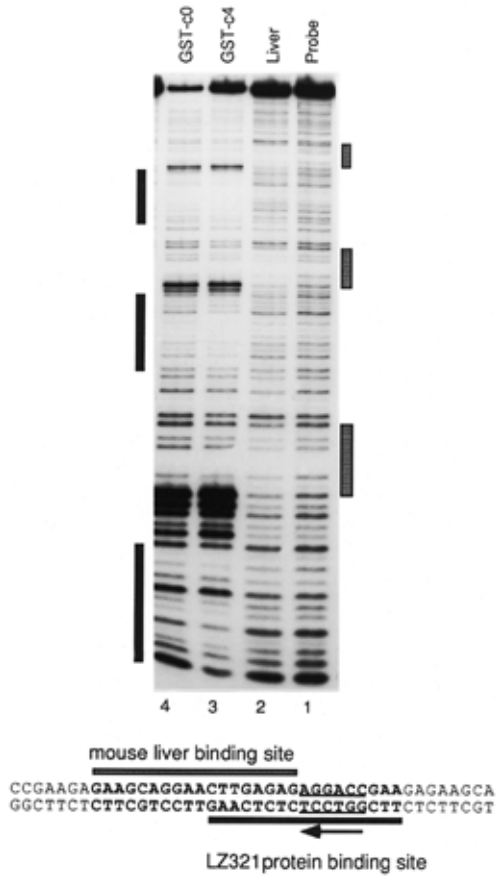

Figure 1. Footprinting of the LZ321 binding site. All reactions contain 10 000 c.p.m. probe, a trimer of HE321/2, and 2 µg poly(dI-dC). Lane 1, no protein, 0.2 U DNase I; lane 2, 40 µg liver nuclear extract, 2 U DNase I; lane 3, 75 ng GST-C4 fusion protein, 0.4 U DNase I; lane 4, 75 ng GST-C0 fusion protein, 0.4 U DNase I. The binding sites are shown below; arrow indicates the GGTCCT site.

GGTCCT site acts as an enhancer

The HE321/2 trimer, containing the GGTCCT sites, was cloned into the reporter plasmid pTATA, which has basal expression in H4IIE-C3 cells but nearly no expression in CV-1 cells (34). Constructs were made in both forward (p321F) and reversed (p321R) directions. These trimers dramatically increased transcription in transiently-transfected CV-1 cells: transcription from the p321F promoter was 126-fold that of the pTATA promoter, and transcription from p321R was 24-fold that of pTATA (Fig. 5A). In H4IIE-C3 cells, basal transcription of the pTATA plasmid was much higher than in CV-1 cells, and the increase in transcription was more modest: 7-fold for p321F and 4-fold for p321R (Fig. 5B). This *cis*-acting element stimulated transcription independent of the direction in which it was inserted, suggesting that it functioned as an enhancer.

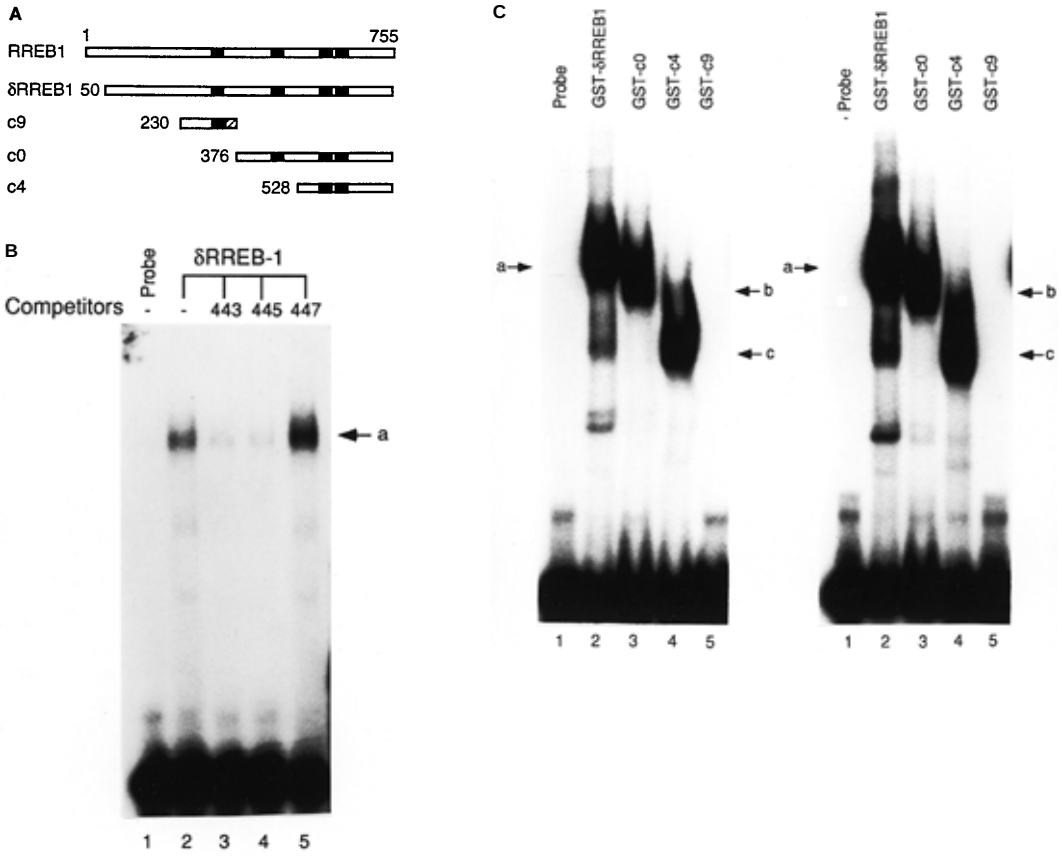

Figure 2. Gel retardation analyses of protein–DNA binding. (**A**) Schematic diagram of truncated protein constructs used in mapping DNA binding domain. Solid boxes show positions of zinc-fingers. Constructs c9, c0 and c4 are named after the lambda clone from which they were excised. All were produced as GST-fusion proteins as described in Materials and Methods; dRREB1, c0 and c4 all extend from the amino acid noted at the left of the line to the end of the polypeptide; numbering follows RREB1 (14). The intron in c9 leads to synthesis of the protein to amino acid 338 followed by 35 amino acids derived from the intron sequence (hatched). (**B**) Competition for δRREB1 binding. 5000 c.p.m. ^{32}P-labeled GGTCCT site HE443/4 oligonucleotide, 500 ng of poly(dI-dC) and 0.4 μg of GST-δRREB1 protein were used in each reaction. Lane 1, control with no protein; lane 2, 0.4 μg of GST-δRREB1 without any competitor; lanes 3–5: 0.4 μg GST-δRREB1 protein with 100-fold molar excess of cold oligonucleotides (HE443/4, HE445/6 and HE447/8) as competitors. Arrow labeled 'a' points to the shifted band. (**C**) Binding of truncated proteins. Each reaction contained 5000 c.p.m. ^{32}P-labeled probe (left = GGTCCT site in HE443/4; right = RRE in HE445/6), 500 ng of poly(dI-dC) and 1 μg of GST-fusion protein. For both: lane 1, free probe; lane 2, 1 μg GST-δRREB1, lane 3, 1 μg GST-C0, lane 4, 1 μg GST-C4; lane 5, 1 μg GST-C9. Arrows a, b and c show three shifted bands; a is the same as in (B).

To test the transactivation activity of RREB1/LZ321, an RREB1 mammalian expression vector was cotransfected with the GGTCCT-site constructs p321F and p321R. RREB1 alone did not increase the transactivation activity in either cell line under the experimental conditions used (Fig. 6). Since RREB1 is a putative Ras downstream effector (14), we tested cotransfections with an activated Raf construct, Raf-Cx (38), overexpression of which can produce constitutively activated Raf-1 protein kinase activity in a Ras-independent manner. In the H4IIE-C3 cells, transcription from the p321F promoter was stimulated 4-fold by the activated Raf-1; there was essentially no stimulation of p321R (Fig. 6A). In CV-1 cells, activated Raf-1 did not stimulate the reporter constructs (Fig. 6B).

DISCUSSION

RREB1/LZ321 binds to two distinct DNA binding sites

Sequence-specific *cis*-element recognition by transcription factors is central to the control of gene expression. Some transcription factors, such as Oct-1, have broad binding specificity, which often corresponds to broad functional activity (46,47). Here we report that RREB1/LZ321 binds to two distinct DNA sequences, an RRE site and a *ttk*-related site. The RRE in the calcitonin gene mediates stimulation of transcription in response to Ras activation in TT human medullary thyroid cancer cells (14). RREB1 was cloned from these TT cells by DNA affinity screening using catenated RRE sites as probe (14). We cloned the same transcription factor, LZ321, from a human liver cDNA library using a probe that contained the ttk consensus sequence GGTCCT. Although the sequences of these two sites differ greatly (only 7/16 identity), RREB1/LZ321 can bind to both sequences with equal affinity, as demonstrated by gel shift experiments (Fig. 2), equilibrium binding affinity studies (Fig. 3, Table 1) and competitive gel shift analyses (Fig. 4, Table 2). Given the major difference in sequence between these two sites, it is likely that RREB1/LZ321 could bind to many other sequences that share parts of the binding site. RREB1/LZ321 could thus act on a wider range of genes than originally suspected. Surprisingly, under our conditions we could not demonstrate binding to a consensus sequence (14) that was determined using the CASTing method (30).

Table 1. Dissociation constant of RREB1/LZ321 polypeptides

	Dissociation constant (K_d) (nM)		
	GGTCCT site	RRE site	RREB1 consensus
GST-δRREB1	49 ± 10[a]	60 ± 16[a]	NB[b]
GST-C0	152 ± 38	155 ± 64	NB
GST-C4	399 ± 113	167 ± 17	NB

Dissociation constants of RREB1/LZ321 fusion proteins GST-δRREB1, GST-C0 and GST-C4 (Fig. 2A) were determined by equilibrium binding analyses using electrophoretic mobility shift assays with increasing amounts of each polypeptide. Three different sequences were used: the GGTCCT site contained in HE443/4, the RRE site contained in HE445/6 and the RREB1 consensus site contained in HE447/8.

[a]Average of two experiments.

[b]NB, no binding.

±, standard errors derived from curve fitting.

Table 2. IC_{50} determined by competitive gel mobility shift assays

	IC_{50} values (nM)			Corrected IC_{50} values (nM)	
	GGTCCT site	RRE site	P_t (nM)	GGTCCT site	RRE site
GST-δRREB1	400 ± 17	399 ± 31	219	181	180
GST-C0	1305 ± 40	1164 ± 175	486	819	678
GST-C4	1844 ± 520	779 ± 83	359	1485	420

Competitive gel mobility shift experiments were used to find the IC_{50} values of the fusion proteins with two different sequences: the GGTCCT site contained in HE443/4 and the RRE site contained in HE445/6. These were converted to apparent dissociation constants, and corrected for the concentration of binding sites by subtracting P_t from the K_d(app).

P_t, the protein concentration in each study.

±, standard errors derived from curve fitting.

The C-terminal two zinc-fingers of RREB1/LZ321 are sufficient for specific DNA ligand binding

RREB1/LZ321 contains four zinc-fingers, two of which are widely spaced: the N-terminal zinc-finger is 125 amino acids away from finger 2, and finger 2 is 98 amino acids away from finger 3. Zinc-fingers 3 and 4 are separated by the 7 amino acid Kruppel H/C link TGERPYX. Since certain zinc-finger proteins, such as Evi1 (48) and MZF-1 (49), have two independent domains that bind to two distinct DNA sequences, we tested whether binding of LZ321/RREB1 to distinct sequences was due to different binding domains. The truncation studies reported here showed that the C-terminal two zinc-fingers of RREB1/LZ321 are sufficient to determine the binding specificity to both ligands (Figs 3 and 4; Tables 1 and 2). The other two fingers in RREB1/LZ321 and/or their adjacent amino acid sequences increased the binding affinity to both sequences. This is consistent with data from other zinc-finger proteins, in which two zinc-fingers are often enough for sequence-specific binding (4). NGFI-A family members, such as NGFI-C, Egr3 and Krox20, share very similar C_2H_2 zinc-finger DNA binding domains, but their binding affinities to consensus binding sites differ as a result of the different protein contexts of the DNA-binding domains (42).

GGTCCT site is an enhancer

We demonstrated that the GGTCCT site could act as an enhancer in both H4IIE-C3 and CV-1 cells, greatly increasing transcription from a weak, TATA-containing promoter even in the absence of specific Ras stimulation (Fig. 5). In the CV-1 cells, where the background activity of the pTATA plasmid was low, stimulation was very dramatic: 24–126-fold. This demonstrates that RREB1/LZ321 can stimulate transcription of genes containing either a GGTCCT-site or an RRE (14). Promoters containing these elements (and probably related sequences) would be expected to respond to the Ras–Raf pathway. Cotransfection with RREB1 alone did not further activate the promoters in either cell line. Overexpression of an activated Raf construct in the same cells did stimulate promoter activity in H4IIE-C3 cells, but only of one construct; there was no effect in CV-1 cells. While this is surprising, it might be because the levels of Raf signaling in these cells even in the absence of the cotransfected plasmid suffice.

Potential targets for RREB1/LZ321

We used the GGTCCT core binding sequence (5'-TGAGA-GAGGACCGAA-3') to search the NCBI non-redundant database for other potential targets for RREB1/LZ321. Several selected mammalian genes that have a GGTCCT site in their promoter or 5' untranslated regions are shown in Table 3. These are potential targets of RREB1/LZ321. It should be noted, however, that the ability of RREB1/LZ321 to bind to divergent sequences suggests that far more genes could be subject to regulation by this transcription factor, and thereby to Ras/Raf regulation.

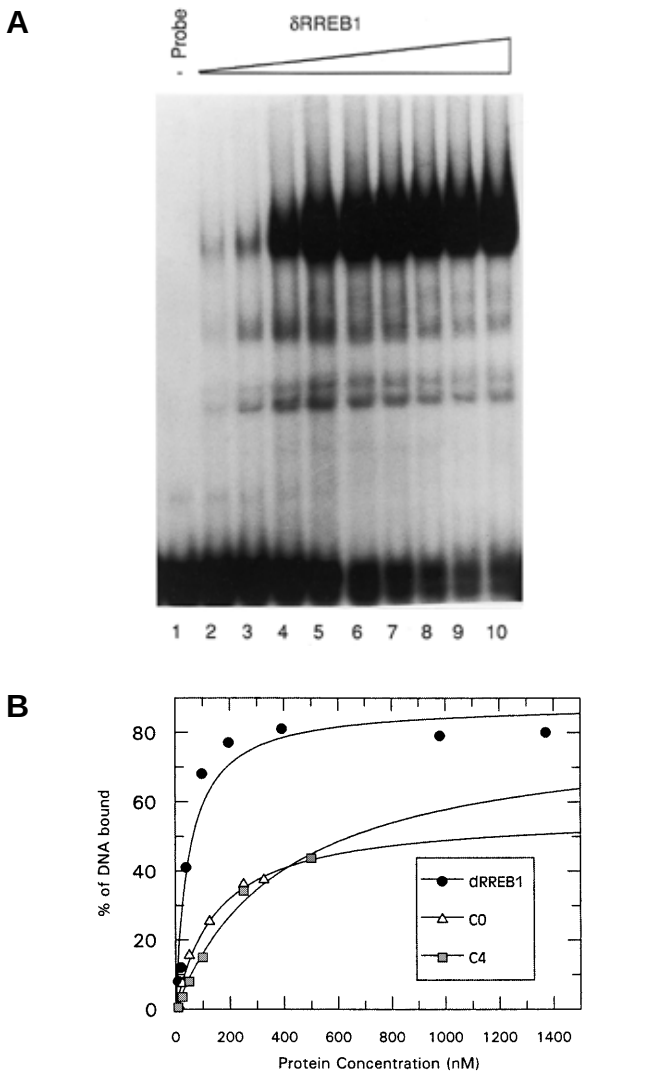

Figure 3. Equilibrium binding analysis of δRREB1 with two distinct binding sites. (**A**) Electrophoretic mobility shift assay with increased amount of δRREB1 and ^{32}P-labeled GGTCCT site (HE443/4 at 0.29 nM). Protein concentration for GST-δRREB1 from lane 2 to lane 10 were 9.8, 19.6, 49, 98, 196, 490, 686, 980 and 1960 nM, respectively. (**B** and **C**) Saturation binding curves of GST-δRREB1 (dRREB1), GST-C0 (c0) and GST-C4 (c4) to the GGTCCT site [HE443/4; (B)] and to the RRE site [HE445/6; (C)]. Data were plotted as percentage of DNA bound versus concentration of protein (nM).

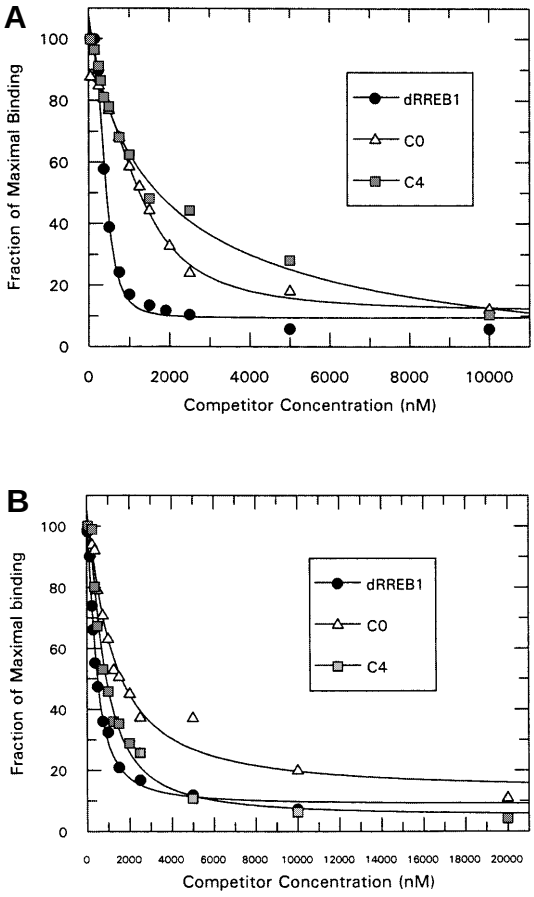

Figure 4. Relative equilibrium binding constants of δRREB1, C0 and C4 proteins (IC_{50}s determination) by quantitative competitive gel shift assays. Data are plotted as fraction of maximal binding versus competitor concentration (nM), at a fixed concentration of labeled probe. (**A**) GGTCCT site (HE443/4; 0.20 nM). (**B**) RRE site (HE445/6; 0.27 nM).

Related proteins

There are some interesting parallels between RREB1/LZ321 and the *Drosophila* developmental protein ttk. The GGTCCT sequence is a ttk (p69 isoform) binding site (27,28). ttk, like RREB1/LZ321, is a C_2H_2 zinc-finger protein of the Kruppel family. Thus ttk and RREB1/LZ321 share a similar binding site and belong to the same protein family, although the two proteins have no sequence homology outside of their zinc-finger regions. Both proteins are downstream targets of Ras/Raf activation (14,23–25). These similarities suggest that RREB1/LZ321, like ttk, may play an important role in cellular differentiation.

A human protein named Finb (finger protein in nuclear bodies; GenBank accession no. D49835), recently cloned from human breast cancer cells (50), is apparently a much longer version of LZ321/RREB1. Finb has 15 zinc-fingers (50). RREB1, LZ321 and the C-terminal region of Finb are nearly identical in nucleotide sequence, with the minor differences possibly due to polymorphisms and/or sequencing errors. The region originally reported as 5' untranslated sequence in RREB1 continues the alignment with the coding region of Finb, with some minor sequence differences. RREB1 was reported to be in a 9 kb

Table 3. Putative RREB1/LZ321 target genes with the GGTCCT binding site

Gene	Sequence	Position	Accession no.
GGTCCT site	TTC**GGTCCT**CTCTCA		
Human β-interferon promoter	gat**GGTCCT**CTCTCt	–52/–46	M11286
Human inducible nitric oxide synthase (NOS2A) promoter	ccC**GGTCCT**CTCTCA	–3323/–3359	X97821
Mus musculus serotonin transporter (SET) promoter region	cTg**GGTCCT**CTCTtA	–494/–508	U26452
Mouse thioredoxin	caC**GGTCCT**CTCTCA	–371/–384	D21855
Porcine thimet oligopeptidase	aag**GGTCCT**tcacat cTC**GGTCCT**CTCTgc	–436/–450 +37/51	AB000426
Porcine ApoA1	GTC**GGTCCT**CTCaCc	–479/–465	Z14124
Mouse bone morphogenic protein 2	cct**GGTCCT**CTCTCt	–821/–838	AF074942
Mineralocorticoid receptor promoter, human Tupaia belangeri (shrew)	aTC**GGTCCT**CTCTgt	+100/+115	M80582 M16801 Z75077
Homo sapiens chloride channel ABP mRNA, 5' UTR	cTC**GGTCCT**CTCcCg	+67/52	AF034607
Rabbit caldesmon	ATC**GGTCCT**CTCTtt	+49/63	L37147
Porcine D-amino acid oxidase	cTg**GGTCCT**CTCTCc	+37/54	M16972

Shown are the names of selected mammalian genes that contain, within their promoter or 5' untranslated sequences, good matches to the GGTCCT sequence reported here. Upper case letters represent matches to the GGTCCT site studied here, lower case letters represent mismatched nucleotides. Note that given the wide binding specificity of RREB1/LZ321, many other sequences and therefore many other genes should be targets of regulation by this factor.

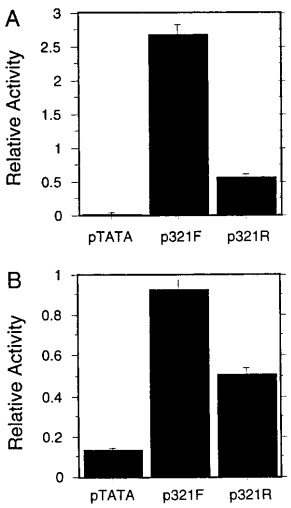

Figure 5. Transient transfections with the GGTCCT site. A trimer of the HE321/322 oligonucleotide was inserted into pTATA in both directions (p321F, direction as in *ADH3* promoter; p321R, reversed) as described; this trimer contains three GGTCCT sites. After transient transfection assays, transcriptional activities of these plasmids were determined relative to pLJZ-1, an *ADH3* promoter plasmid, in CV-1 cells (**A**) and in H4IIE-C3 cells (**B**). Bars show mean; lines indicate standard error.

transcript in all tissues tested except adult brain (14), and there was evidence for smaller transcripts in TT cells. We found that in addition to a major LZ321 transcript of ~9.7 kb, there were multiple smaller transcripts (7.0, 4.6, 1.2 and 1.0 kb) in heart, pancreas and muscle tissues (51). Multiple transcripts (9.5, 6.0, 4.4, 2.3 and 2.0 kb) were also found for Finb in embryo, placenta and various human cell lines (50). The composite RREB1/LZ321 cDNA is 4.4 kb, which correlates well with the minor transcripts of 4.4–4.6 kb. RREB1 was shown to be a single-copy gene that mapped to chromosome 6p25 (52). We found evidence for alternative splicing in LZ321, with several cDNA clones containing an unspliced intron (above). There is also evidence of alternative splicing in Finb (50). These data suggest that Finb and LZ321/RREB1 are products of the same gene, and that there is alternative splicing. It is also possible that the shorter LZ321/RREB1 are truncations of the larger protein, or are synthesized from an alternative, downstream promoter.

A chicken homolog of the human RREB1 gene has also recently been cloned (53) (GenBank accession no. AF013754). It, too, encodes a much longer protein described as having 16 zinc-fingers, the C-terminal portion of which is homologous to RREB1/LZ321. Sequence alignment demonstrates that the homology to Finb extends to the N-terminus of both proteins, and encompasses the key Cys and His residues of all 15 zinc-fingers in Finb. Changing the reported sequence of Finb to the consensus among the three human cDNAs at several positions increases the homology. The reported extra zinc finger of the chicken gene lies between fingers 11 and 12 of Finb, lacks one of the conserved His residues, and does not show substantial homology to Finb. On the basis of this alignment, we suggest it is not functional. The chicken gene is also expressed in all tissues except brain. The strong conservation of zinc-fingers and the similar pattern of expression suggest that the homology reflects conserved function. All three genes contain consensus MAPK phosphorylation sites.

We have identified, by database searching, a homologous protein from *Drosophila*, HINDSIGHT (GenBank accession no. DMU86010). HINDSIGHT is a developmentally important gene involved in germ-band retraction (54). HINDSIGHT has 14 C_2H_2 zinc-fingers, of which numbers 1, 2, 6, 7, 8, 10, 13 and 14 align with the zinc-fingers of Finb and the chicken homolog, and 9 aligns with the probably non-functional extra zinc-finger of the chicken gene.

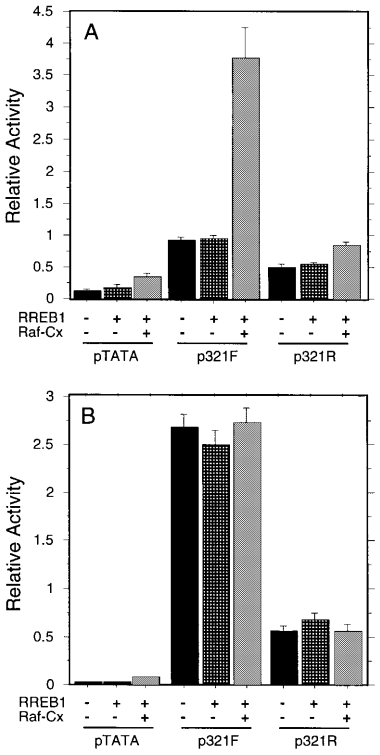

Figure 6. Effect of RREB1 and activated Raf on transcriptional activity of the GGTCCT site in H4IIE-C3 (**A**) and CV-1 (**B**) cells. The plasmids used in Figure 5 were cotransfected into cells along with expression vectors for RREB1 (pMV7p1-371) or RREB1 plus an activated Raf construct Raf-Cx (pcEXV-myc-Raf-Cx), as noted below. –, no cotransfected DNA; +, cotransfection with the indicated plasmid. Bars depict relative promoter activity; lines indicate standard error.

The proteins from these three widely diverged species are most similar in the region encompassing the last two zinc-fingers with their H/C link. These are the two zinc-fingers that we have shown convey binding specificity in LZ321/RREB1. The human Finb/LZ321/RREB1 and the chicken RREB1 homolog share 47/49 identities in the region from the first C of Finb finger 14 to the last H of finger 15, with the two differences being conservative substitutions of Ser for Thr. The identity extends further to both sides of these fingers as well. HIND-SIGHT has 28/49 identities and 10 conservative substitutions in this region. This striking conservation argues for the importance of this gene to the organisms, and suggests conserved DNA binding sites.

Summary

cDNAs encoding a transcription factor in the Ras–Raf pathway have been cloned using two very different *cis*-acting sequences as probes. We have demonstrated that the protein encoded by these cDNAs, RREB1/LZ321, binds both sequences with the same affinity. This binding specificity is due to the C-terminal pair of zinc-fingers, but the affinity is affected several-fold by the sequences toward the N-terminus. We have also demonstrated that a GGTCCT-containing element acts as an enhancer, which can be further stimulated by activated Raf. This transcription factor belongs to a conserved family of factors that share these specificity-determining zinc-fingers, and might be important in the regulation of a wide array of genes.

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