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Different binding specificities and transactivation of variant CRE's by CREB complexes

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ABSTRACT

The DNA binding specificities of CREB1 and CREB2 homodimers and the CREB2/cJun heterodimer were analyzed with a CASTing technique. All but one of the selected sequences varied from the consensus CRE (TGACGTCA) by three nucleotides or less. The profile of variations selected and the binding affinity for these sequences were unique for each CREB complex. The affinities were not effected by the palindromic nature of the sequences, but were strongly effected by flanking sequences. The strength of DNA binding *in vitro* correlated with the degree of transactivation observed in JEG-3 cells transfected with reporter plasmids harboring CRE variants, when hybrid CREB proteins fused to the VP16 activation domain were expressed. When native CREB proteins were expressed, the correlation was attenuated by the nature of the variant sequence. A CRE variant (TGACATCA) found in several natural promoters, exhibited the lowest basal transcription rate of the variants and a lower level of induction than expected when compared with the *in vitro* binding data. These results indicate that transactivation of DNA sequence elements is strongly effected by the strength of transcription factor binding, and that individual sequences can attenuate the level of induction.

INTRODUCTION

The pattern of genes expressed in a cell is altered by signal transduction pathways in response to extracellular stimuli. A stimulus begins the pathway by inducing the production of a second messenger that activates a cascade of events leading to the nucleus. Multiple homologous cellular proteins can act at identical points in a cascade. The ultimate effect on the pattern of gene expression is dependent upon the specific combination of proteins present in a particular cell. The genes to be effected are determined by specific DNA sequence elements in the promoter regions. Identical DNA regulatory elements have been found in genes activated by the same pathway.

An eight base pair DNA sequence element has been shown to mediate transcriptional effects of the second messenger cyclic AMP (cAMP) (1,2). This cAMP response element (cAMP) is identical to the ATF sites found in adenovirus (3–5). Some of the ATF sites mediate the E1A inducibility of adenovirus early genes (5). Additionally, CREs have been implicated in transactivation by HTLV1 and BLV proteins and by membrane depolarization and increased intercellular Ca^{2+} concentrations (6–10).

Multiple CRE-binding proteins have been detected in cellular extracts and a number have been purified (3,7,8,11–19). To date 10 cDNAs of CRE-binding proteins have been cloned (20–29). One of these transcription factors, CREB1, has been shown to be induced by the cAMP signal transduction pathway (30–32). Another CREB transcription factor, CREB2 (also called CRE-BP2 and ATF-2), is induced by the adenovirus E1A protein (33,34). The mechanism of action for the other CRE-binding proteins has yet to be elucidated.

All of the CRE-binding proteins contain similar functional domains, called Bzip, that consist of a basic DNA binding region followed by a leucine zipper motif. Dimerization between two of these molecules through the leucine zipper is required for DNA binding (35). All of the CRE-binding proteins form homodimers and certain members form heterodimers with other members (23,36,37). Although both CREB1 and CREB2 form heterodimers with certain other CRE-binding proteins, they do no heterodimerize with each other.

Other Bzip transcription factors include the AP1 proteins which bind to a 7 base pair DNA sequence element called an AP1 site or a TPA response element (TRE) (for a review see 38). The cJun oncogene codes for an AP1 protein that binds to an AP1 site as a homodimer and as a heterodimer with other AP1 transcription factors (for a review see 39). We and others have demonstrated that cJun also heterodimerizes with CREB2 *in vitro* and *in vivo* (26,40–43). The cJun/CREB2 heterodimer binds to a CRE with much greater affinity than to an AP1 site (40).

The availability of cDNA clones allows the manipulation and study of these transcription factors *in vitro*, however, *in vivo* studies are hindered by the number of different proteins binding

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to the same DNA sequence element. To identify DNA sequence elements which can be used as a tool to distinguish between individual CREB complexes, we used a CASTing technique (cyclic amplification and selection of targets) (44). Briefly, *in vitro* translated proteins are mixed with a pool of random oligonucleotides flanked with PCR primers. Protein–DNA complexes are immunoprecipitated, the DNA is extracted, PCR amplified and subjected to additional rounds of selection and amplification. Oligonucleotides from the final round are purified from a specific protein–DNA band on nondenaturing polyacrylamide gel, amplified, subcloned and sequenced. In this paper we compare variations of the CRE sequences selected with the CREB1 and CREB2 homodimers and the CREB2/cJun heterodimer. We identify sequences that are differentially bound by the CREB complexes and study the correlation between *in vitro* DNA binding affinity and transactivation *in vivo*.

MATERIALS AND METHODS

Plasmids and vectors and oligonucleotides

In vitro transcription and translation of CREB1 and CREB2 was performed with DL1 and DL2 plasmids, respectively, which were previously described (40). Full length cJun was translated from a cDNA containing plasmid previously described (45). For transfection experiments, full length CREB1 and CREB2 cDNA's were cloned into the vector PJ6 (a gift from J.Morgenstern and H.Land) which contains the rat β actin promoter, and the SV40 intron and polyadenylation signal. Retroviruses expressing the VP16–CREB hybrids were constructed by ligating the activation domain in the SalI/RsaI fragment of the VP16 transcriptional activator gene (46) to the NcoI/HindIII fragment of CREB2 (34) or the SalI/EcoRI fragment of CREB1 (32) and inserting the resulting hybrid genes into the pBABE Neo retrovirus (47). The sequences of the designed oligonucleotides are:

D2: 5′ GGATCCAATAAATTACGTAATGGTGTGAATTC 3′
D3: 5′ GGATCCAATGCATTACGTTACAGTGTGAATTC 3′
D4: 5′ GGATCCAATTGATGATGTCATCTTGTGAATTC 3′
D5: 5′ GGATCCAATTCGGCGCGTCATCGTGTGAATTC 3′

Each designed oligonucleotide was digested with BamHI and EcoRI and ligated into the BamHI site upstream of the thymidine kinase promoter and CAT gene of the pBLCAT2 reporter plasmid (48). Individual clones were isolated and sequenced. Constructs which contained exactly two oligonucleotide inserts in identical orientations were identified and used in transfection assays.

In vitro synthesis of homodimers and heterodimer

CREB1, CREB2 and cJun proteins were translated in reticulocyte lysate (Promega) using *in vitro* capped RNA synthesized from linearized templates. For the CREB2/cJun heterodimer, the proteins were cotranslated. Gel retardation analysis using ^{32}P labeled CRE fibronectin probe was used to determine the optimal ratios of CREB2 and cJun that resulted in the visualization of only heterodimer and no homodimer band.

Gel retardation analysis

In vitro translated proteins were incubated with ^{32}P labeled probe for 20 minutes at room temperature. Reactions were electrophoresed through a 6% nondenaturing polyacrylamide gel. The gel was dried under vacuum and subjected to autoradioagraphic analysis.

Dissociation rates of CREB complexes

The dissociation rates of CREB2 homodimer and CREB2/cJun heterodimer were determined for five oligonucleotide probes, CRE fibronectin, CRE Somatostatin, ATF E4, CRE hcg and AP1 SV40, previously described (40). Briefly, CREB2 and cJun were cotranslated in reticulocyte lysate in ratios that resulted in both the CREB2 homodimer and the CREB2/cJun heterodimer bands upon gel retardation analysis with ^{32}P labeled probe. This ratio of proteins was incubated with one of the oligonucleotide probes labeled with ^{32}P for 40 minutes at 20°C to reach equilibrium. A 500 fold excess of identical unlabeled probe was added; immediately an aliquot was removed and electrophoresed into a 6% nondenaturing polyacrylamide gel. Additional aliquots were taken at 5 minute intervals and immediately electrophoresed into the same gel. The gel was dried under vacuum and subjected to autoradiography. Densitometric tracings were taken of each retarded band on the autoradiograph to determine the percent of protein–DNA complexes present at each time point relative to the initial time point. The percent of complex was plotted against time and the dissociation rate (slope of the line) was determined for both protein–DNA complexes. The dissociation of CREB2 and CREB1 off of the CRE fibronectin probe was compared similarly. All experiments were repeated with a 1000× excess of competitor and equal dissociation rates were again obtained.

CASTing technique

The random oligonucleotides and the CASTing technique described in reference 44 were used with the following alterations. Reticulocyte lysate containing equal amounts of CRE fibronectin binding activity for each CREB complex was used. Specific CREB1–DNA complexes were isolated with a polyclonal antibody raised to a carboxy-terminal 10 amino acids of CREB1. A polyclonal antibody (NJ4) raised against the carboxyl terminal 16 amino acids of CREB2 was used to immunoprecipitate CREB2 and CREB2/cJun complexes. Selected oligonucleotides from each round were amplified by polymerase chain reaction (PCR) with alpha ^{32}P dCTP and analyzed for specific binding to *in vitro* translated protein by gel retardation analysis. Equal amounts of CRE fibronectin binding activity were used in each reaction. No retarded bands were observed with selected oligonucleotides from the first CASTing round. Weak retarded bands were observed upon the second round and the intensity of the specific bands increased with each CASTing round. The retarded band from the fourth round was approximately equal in intensity for CREB1, CREB2, and CREB2/cJun and was isolated, subcloned into Bluescript (KS+) (STRATAGENE) and sequenced by the dideoxy chain termination method.

Cell culture and transfection assays

JEG-3 (human choriocarcinoma) cells were maintained Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum. Prior to transfection, 10^6 cells were seeded onto 100mm dishes and the calcium phosphate DNA coprecipitate with or without the VP16–CREB expressing retrovirus added 24 hours later. The plates were harvested 40 hours after transfection. CAT assays were performed as described (49). Each transfection included a plasmid (pJATLAC) which contains β-galactosidase-coding sequence under the control of the rat β-actin promoter. All extracts were assayed for β-galactosidase activity (50) and normalized for transfection efficiency.

RESULTS

Affinity for known CRE's

In order to separate the DNA binding activity due to *in vitro* translated CREB proteins from endogenous activity in reticulocyte lysate, truncated CREB1 and CREB2 proteins, which contain intact DNA binding and dimerization domains (40) were used. No intermediate bands corresponding to heterodimers between endogenous activity and the faster-migrating truncated proteins were observed. The DNA binding specificities of the truncated proteins were found to be identical to the full length proteins.

The dissociation rates of the CREB2 homodimer and the CREB2/cJun heterodimer from several known CRE's were compared. We have previously shown that these proteins heterodimerize in the absence of DNA and bind CRE's *in vitro* as stable dimers (40). No single occupancy intermediates were observable by gel retardation analysis. Figure 1 contains the graph derived for the dissociation rates off of the CRE fibronectin probe. Statistical analysis was performed to test the hypothesis that the dissociation rates (slopes of the lines) for CREB2 and for CREB2/cJun were equal. Hypothesis testing determined that the dissociation rates for the two different complexes were equal at the $t < 0.001$ level. The lines plotted for CREB2 homodimer and CREB2/cJun heterodimer were parallel for all five oligonucleotides tested. Because the CREB complexes had the highest affinity for the CRE fibronectin probe, this was chosen for further studies. The dissociation rates of CREB1 and CREB2 homodimers on the CRE fibronectin were determined to be equal ($t < .001$) in a separate experiment. In subsequent experiments, the amount of CRE fibronectin binding activity in the translation reactions was measured with gel retardation analysis and equal amounts were diluted into equal volumes of reticulocyte lysate before use.

Sequences of selected oligonucleotides

The CASTing technique was used to select oligonucleotides containing specific DNA binding sites for the CREB1 and CREB2 homodimers and for the CREB2/cJun heterodimer. Starting with a random pool of 5×10^9 molecules, fifty were subcloned and sequenced for each complex. Oligonucleotides which contained consensus sequences that overlapped with the primer sequence were eliminated from analysis. All but one of the oligonucleotides contained sequences that varied from the CRE consensus sequence (5′ TGACGTCA 3′) by 3 nucleotides or less. Analysis of the selected oligonucleotides revealed different profiles for each complex (Figure 2).

Less than half (12/29) of the oligonucleotides isolated with CREB1 matched the consensus CRE exactly. The nucleotide in the eighth position was most often an A (17/29), and less often a T (7/29), a G (4/29), or a C (1/29). The nucleotide in the seventh position was often an A instead of a C (12/29). Two of the oligonucleotides contained additional variations: 5′ TTACGTAA 3′ and 5′ TGACGCGC 3′.

The oligonucleotides selected with the CREB2 homodimer differed from that of CREB1 in that there was additional significant variability in the second and fifth positions. Only 5 of the sequences matched the consensus CRE exactly. The

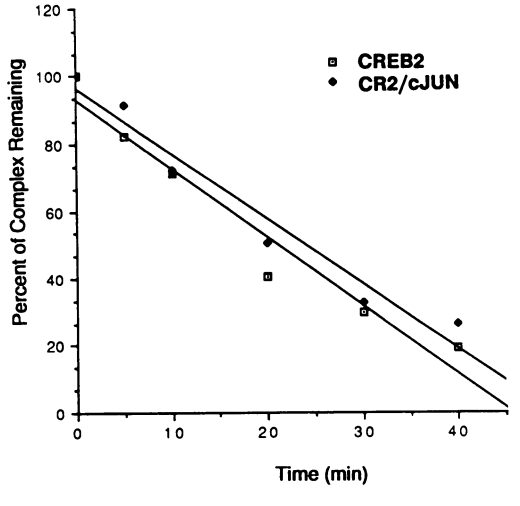

Figure 1. Plot of the dissociation rate of CREB2 homodimers and CREB2/cJun heterodimers off of the CRE fibronectin probe.

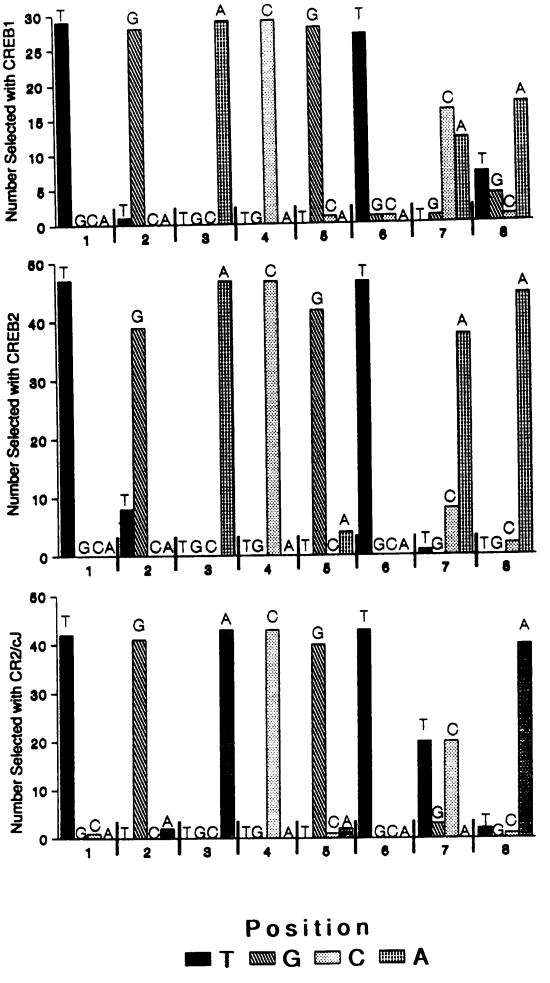

Figure 2. Frequencies of sequences selected with CREB1 homodimers (upper panel), CREB2 homodimers (middle panel) and CREB2/cJun heterodimers (lower panel). The y axis represents the number of selected oligonucleotides which contain the indicated nucleotide in the position of the CRE specified on the x axis. T, thymine; G, guanine; C, cytosine; A, adenine.

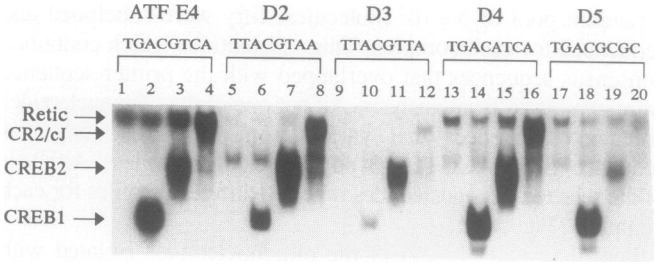

Figure 3. Affinities of CREB complexes for CRE variations. Gel retardation analysis was performed on *in vitro* translated CREB1 homodimer (lanes 2, 6, 10, 14 and 18), CREB2 homodimer (lanes 3, 7, 11, 15 and 19), CREB2/cJun heterodimer (lanes 4, 8, 12, 16 and 20), or reticulocyte lysate (lanes 1, 5, 9, 13 and 17) incubated with the ^{32}P labeled oligonucleotides indicated. CR1, CREB1; CR2, CREB2; CR2/cJ, CREB2/cJun.

Table 1. DNA binding affinities of CREB complexes for CRE variations

OLIGO	SEQUENCE	DNA BINDING AFFINITES		
		CR1	CR2	CR2/cJ
fn	CCG TGACGTCA CCC	++++	++++	++++
215	GCA TTACGTAA CCA	+	++++	++++
216	AAA TTACGTAA TGG	+	++++++	++++
226	AAA TTACGTAA TAG	++	+++++	++++
245	TTA TTACGTAA TGT	++	+++++	++
252	TAA TTACGTAA TGC	-/+	++++	++
24	GCG TGACGTAT ATT	+	++	++
229	AAA TGACGTAT TGC	+++	++++	++
210	CAC TGACGTAA AGA	++++	++++	++
322	TCA TCACGTAA TCT	++	++++	++
32	CGC TGACCTCA TCA	++++	++++	++++
317	AGA TGACATCA TCA	++++	++++	++++
26	TAA TGACATAA TCC	-/+	+++	-/+
27	ATA TGACCTAA CCA	++	+++	++
29	TAA TGACATCA CTA	+	++++	++
164	CGA TGACGCGC CGA	++	++	-/+
260	GCA TTACGTTA CAG	-/+	+++	-/+
330	TGG CAACGTGC CAA	++++	+++	?

Nucleotides which differ from the consensus CRE are underlined. The number of + symbols indicates the degree of binding; −/+, barely detectable binding; ?, high background interference; CR1, CREB1; CR2, CREB2; CR2/cJ, CREB2/cJun; fn, fibronectin.

majority (37/47) varied at the seventh position and 5 varied at both the second and seventh positions. The last nucleotide was most often an A (45/47) and was a C in two instances. Four of the oligonucleotides contained an A instead of a G at the fifth position. One unique oligonucleotide contained a T in both the second and seventh positions: 5′ TTACGTTA 3′.

Less than half of the oligonucleotides isolated with the CREB2/cJun heterodimer matched the CRE consensus seuence exactly (18/43) and approximately half contained an A in the seventh position (21/43). The nucleotide in the last position was most often an A (41/43) and was a T in the other two cases. Three of the oligonucleotides varied at the fifth position. One that did not match either consensus varied at four positions: 5′ CAACGTGC 3′.

A minority of the consensus sequences were perfect palindromes for CREB1 (12/29 or 41%), CREB2 (10/47 or 21%) and CREB2/cJun (18/43 or 42%). The majority of oligonucleotides contained a purine 5′ and a pyrimidine 3′ to the

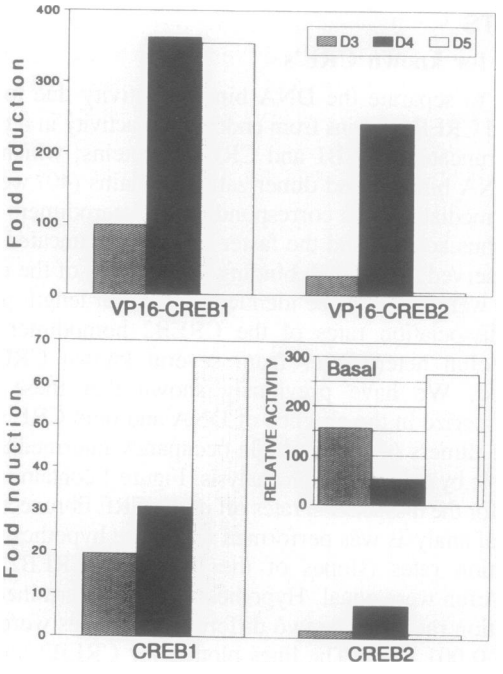

Figure 4. Transcriptional activation of CRE variations. Human choriocarcinoma JEG-3 cells were transfected with the pBLCAT2 reporter containing two copies of the indicated CRE variation. Fold induction was determined by dividing the CAT activity (corrected for transfection efficiency) determined in the presence of hybrid or native CREB proteins by that determined in its absence. The insert in the lower panel shows the relative activity of the reporter plasmids in the absence of exogenous CREB proteins.

consensus sequence for CREB1 (17/29 or 59%), CREB2 (35/47 or 75%) and for CREB2/cJun (38/43 or 88%).

Affinities of CREB complexes for CRE variations

Oligonucleotides containing sequences which varied significantly from the original CRE consensus 5′ TGACGTCA 3′ were chosen for further analysis. An equal amount of fibronectin CRE binding activity for each dimer was incubated with the selected oligonucleotides and subjected to gel retardation analysis (results summarized in Table 1). Sequences flanking the consensus had a strong effect on the strength of binding. Five sequences containing both second and seventh position variations (5′ TTACGTAA 3′) were bound poorly by CREB1 and strongly by CREB2. The affinity of CREB2/cJun for these oligonucleotides varied from intermediate to strong. Sequences containing only the position seven variation also gave variable results with no logical pattern.

The ability of CREB1 to bind to CRE's containing the fifth position variation was also tested. CREB1 was able to bind to sequences containing this variation only when the second and seventh nucleotides were not varied, G and C, respectively (oligos 32 and 317). If either or both of the nucleotides in these positions 2 and 7 were T or A, respectively, CREB1 as well as CREB2/cJun binding was drastically reduced (oligos 26, 27 and 29). CREB2 bound well to all of the minority sequences regardless of the nucleotides present in positions 2 and 7.

Unique oligonucleotides which varied from the consensus's were also tested. The unique CREB1 oligonucleotide (164) was bound at a low level by both CREB1 and CREB2 and very poorly

by CREB2/cJun. The unique CREB2 oligonucleotide (260) was bound well by CREB2 and very poorly by CREB1 and CREB2/cJun. Unfortunately, the unique CREB2/cJun oligonucleotide (330) was bound so well by endogenous activity in the reticulocyte lysate that CREB2/cJun binding could not be detected above this high background. CREB1 and CREB2 bound very well to this sequence.

Sequences that exhibited bias between the different dimers were selected for further studies. To eliminate any effects of the random sequences in the oligonucleotides, only the consensus and three flanking nucleotides were used in the design of new probes. These designed probes all contained identical sequences 5′ and 3′ of the DNA binding sites. DNA sequences from oligonucleotides 216, 260, 317 and 164 were used to construct oligonucleotides D2, D3, D4 and D5, respectively. The affinity of each dimer for the designed oligonucleotide was tested with gel retardation analysis (Figure 3). D2 was bound well by CREB2 homodimers and CREB2/cJun dimers, however CREB1 homodimer binding was slightly reduced. D3 was bound very well by CREB2 and very poorly by the other dimers, D4 was bound equally well by all three dimers, and D5 was bound preferentially by CREB1.

Transactivation of CRE variations by CREB homodimers

The relationship of *in vitro* DNA binding to transactivation *in vivo* was studied with transient transfection assays. Transcriptional induction by CREB1 and CREB2 proteins *in vivo* occurs through two very different mechanisms. To overcome these differences, one of the strongest activators of transcription known (45), the VP16 acidic activation domain, was fused to each of the CREB proteins. The potency of VP16 transactivation should mask any differences between the strengths of CREB1 and CREB2 transactivation. Therefore, if the degree of transactivation by a transcription factor is directly dependant on the strength of DNA binding, then transactivation of variant CRE's by the VP16−CREB hybrids should correlate with the affinity of CREB1 and CREB2 for these sequences.

Two copies of each designed oligonucleotide were ligated into a reporter plasmid containing the thymidine kinase TATA box and the chloramphenicol acetyltransferase (CAT) gene. These plasmids were transfected into JEG-3 cells in the presence and absence of vectors expressing the VP16−CREB hybrids. Transactivation of reporter plasmids by VP16−CREB1 and VP16−CREB2 was assessed with transient transfection assays of CAT activity (Figure 4, upper panel). The ability of VP16−CREB hybrids to transactivate the reporter plasmid directly correlated with the *in vitro* DNA binding affinities (compare Figures 3 and 4). VP16−CREB1 transactivated the reporter plasmid containing low affinity oligonucleotide (D2) 3.5 fold less well than those containing higher affinity oligonucleotides (D4 and D5). VP16−CREB2 transactivated the reporter plasmid containing the high affinity oligonucleotide (D4) 5-fold more than the reporter containing the lower affinity oligonucleotide (D3). The reporter plasmid containing the oligonucleotide bound poorly by CREB2 *in vitro* (D5), was poorly induced by VP16−CREB2. Transactivation by VP16−CREB2 was consistently lower than transactivation by VP16−CREB1. This difference was also observed for the intact CREB proteins (Figure 4, lower panel), however the degree of difference in activation ability was not quantitatively similar between the hybrid and intact CREB proteins. The difference in transactivation for the hybrid proteins was 11.4, 4.0, and 60.0 for D3, D4, and D5, respectively, while the difference for the intact CREB proteins was 3.6, 1.5, and 88.5, for D3, D4, and D5, respectively.

Transactivation by the full length wild type CREB proteins correlated with the *in vitro* DNA binding affinities, except that transactivation of D4 was lower than expected when compared with the *in vitro* binding specificities (Figure 4, lower panel). The basal transcription rate of D4 was lower than that of D3 and D5 as well (Figure 4, lower panel insert).

DISCUSSION

This study demonstrates that CREB complexes consisting of CREB1 and CREB2 homodimers and CREB2/cJun heterodimers have similar but unique DNA binding specificities. Using a CASTing technique, all but one of the sequences selected varied from the consensus CRE by no more than three nucleotides. Each complex, however, selected a unique profile of variations at individual nucleotide positions (Figure 2). CREB1 exhibited the most stringent profile with variations occurring in the seventh and eighth position and only once in the second position. The CREB2/cJun heterodimer selected sequences with variations in the fifth as well as the seventh and eighth positions. CREB2 homodimers exhibited the least stringent profile with variations occurring in the second, fifth, seventh and eighth positions.

Although only of the sequences selected with CREB1 contained the fifth position variation, this sequence was bound strongly by CREB1 (Table 1). Combining the second or seventh variation with the fifth, however, significantly reduced CREB1 as well as CREB2/cJun binding. Given that the fifth position variation was observed in less than one percent of CREB2 selected sequences, the lack of this variation in CREB1 selected sequences is most likely due to the number of analyzable sequences obtained. CRE's containing a T in the fifth position have been identified in the mouse tissue plasminogen activator gene (t-PA), the p450scc gene, the rat and bovine glycoprotein hormone alpha genes, and the retinoic acid receptor β gene (51−55). CREB1 has been identified as one of the proteins bound by this sequence (54).

The minority of sequences selected with all three complexes were perfect palindromes, and CREB2 appeared to select against them (79 % asymmetry). No correlation was observed between the symmetrical nature of the CRE sequence and *in vitro* DNA binding specificities. Although the consensus sequence consists of eight nucleotides, flanking sequences clearly effect the binding affinities of all three complexes (Table 1). Nucleotides immediately flanking the 5′ and 3′ sides of the consensus consisted of a purine and a pyrimidine, respectively, in the vast majority of cases.

In previous studies, we observed identical affinities of CREB2 homodimers and CREB2/cJun heterodimers for the fibronectin CRE oligonucleotide (40). We confirmed this with off rate analysis and used this probe to determine optimal ratios of CREB2 and cJun for heterodimer formation. In order to obtain exclusively heterodimers, an excess of cJun protein was needed. This indicates that the CREB2 homodimer is more stable than the heterodimer. The excess of cJun and/or the stability of the heterodimer, may explain why the CREB2 homodimer did not materialize when the heterodimer was incubated with a probe that is bound preferentially by the homodimer (Figure 3, lane 12). Others have demonstrated that the CREB2/cJun heterodimer can stably coexist in solution with the cFos/cJun heterodimer (26). In this study, we identify sequences which are bound differentially

by the CREB2 homodimer and the CREB2/cJun heterodimer (Table 1 and Figure 3). The *in vivo* DNA binding specificity of CREB2/cJun could not be evaluated because the equilibrium of homo- and hetero-dimers can not be controlled *in vivo*.

It was hypothesized that fusing the potent VP16 acidic activation domain to the CREB proteins would overcome or mask their differences in transactivation potential of the native CREB proteins. The degree of transactivation would therefore correlate with the strength of DNA binding observed *in vitro*. Although a correlation between binding affinity and transactivation was observed, our hypothesis was not true, because the CREB1 hybrid consistently transactivated more strongly than the CREB2 hybrid. One explanation for this difference, is that the VP16 activation domain is not maximal in this system and that the CREB1 portion of the VP16−CREB1 hybrid contributes to the level of transactivation observed. Although the full length CREB1 cDNA was used in construction of the VP16−CREB1 hybrid, the VP16−CREB2 hybrid consisted of only the 253 carboxyl terminal amino acids of CREB2. Therefore, the CREB2 portion of the VP16−CREB2 could not contribute to the level of transactivation because it does not contain the putative metal binding finger that has been demonstrated to be required for transactivation by CREB2 (56). Another explanation for the difference in the degree of transactivation between the VP16CREBs, is that CREB2 is negatively regulated in JEG-3 cells and that this regulation also effects the hybrid protein.

The native CREB1 also transactivated the variant CRE's more strongly than native CREB2, however the degree of difference in activation ability was not quantitatively similar between the hybrid and wild type proteins. The reason for the difference in transactivation between the native CREBs may be that CREB1 is a better transactivator in JEG-3 cells in the absence of E1A. Another explanation could be that CREB2 is negatively regulated. This difference may be caused by cellular proteins interacting with the CREBs to effect their binding, dimerization and/or transactivation potential. Given the differences in the CREB1 and CREB2 protein sequences outside of their DNA binding and dimerization domains, and their different inducers, cAMP, and E1A, respectively, it is very likely that they are regulated by different cellular proteins.

Although transactivation by the hybrid proteins strongly correlated with the degree of *in vitro* binding affinities, transactivation of the full length proteins appeared to be influenced by the basal strength of the reporter plasmid. The reporter plasmid bearing the D4 oligonucleotide which contains the fifth position T substitution found in the natural promoters discussed above had the lowest basal activity. To date, there are no known natural CRE's corresponding to the D3 and D5 variants. Others have also observed a lower basal activity for this variant in comparison to other CRE's in JEG-3 cells (54). In our studies, the potent VP16 activation domain was uneffected by the basal strength of the promoter, however with the intact CREB proteins, the degree of transactivation of the D4 sequence was lower than expected in relation to the other variants. The ability of DNA sequence elements to differentially effect transactivation by the same bound transcription factor in an identical promoter context has also been observed by others (57−59). It has been postulated that the transcription factor proteins take on an inactive conformation when bound to negative promoter elements and a functionally active conformation when bound to positive elements. Although the effect we observed is only an attenuation and not an inhibition, conformational differences in the CREB proteins when bound to individual sequences may be responsible. The increase in CREB1's transactivation potential by phosphorylation has been postulated to be caused by a conformational change leading to exposure of glutamine-rich activation domains (60). Conformational changes could effect the ability of these transcription factors to interact with coactivators. This indicates, that the D4 variation functions to attenuate the degree of transactivation by CREB complexes observed on other CRE's.

CREB2 is ubiquitously expressed in tissues, however the ratio of full length and alternatively spliced forms varies (61). CREB2 has been implicated in signal transduction in the brain, because high levels of expression have been observed in the pyramidal cells of the hippocampus (61), and deletion of the chromosome 2q31−33 which involves the CREB2 gene has been associated with multiple anomalies and mental retardation (61). Expression of CREB2 has been shown to be increased in regenerating liver and in some tumors implicating it in cellular proliferation (61). cJun is an immediate early gene whose expression is increased in response to growth factors and mitogens and has no known alternatively spliced forms (62−67). The cJun proto-oncogene has been implicated in cell cycle progression (68). CREB1 is also ubiquitously expressed, however its alternatively spliced forms are expressed in equal ratios (29,70).

Although these transcription factors co-exist in a variety of tissues and bind to identical DNA sequence elements, they are not redundant. Our results demonstrate that CREB1, CREB2 and CREB2/cJun exhibit different binding affinities for variant CRE's and that CREB1 and CREB2 transactivate these variants to different degrees. Numerous other studies have demonstrated that these transcription factors are regulated by the level of expression of their full length and alternatively spliced mRNAs, by their specificities of heterodimerization, by the affinities of the heterodimers for specific DNA sequences, by phosphorylation, and by interaction with other cellular factors.

In conclusion, we have identified CRE variations which are bound with different affinities by CREB complexes. The degree of transactivation of these sequences by CREB proteins correlated with the strength of binding observed *in vitro*, but other factors including the nature of the DNA sequence attenuated the response.

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