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ATF/CREB Elements in the Herpes Simplex Virus Type 1 Latency-Associated Transcript Promoter Interact with Members of the ATF/CREB and AP-1 Transcription Factor Families

Key Words
HSV-1
Latency
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Cyclic-AMP
cAMP response element

Abstract
The herpes simplex virus type 1 (HSV-1) latency-associated transcript (LAT) promoter 1 (LP1) is an inducible and cell type-specific promoter involved in regulating the production of an 8.3-kb primary LAT transcript during acute and latent infection of peripheral sensory neurons and during subsequent virus reactivation. A number of *cis*-acting regulatory elements have been identified in LP1, including two cyclic-AMP (cAMP) response element (CRE)-like sequences, designated CRE-1 and CRE-2. CRE-1 has previously been shown to confer cAMP responsiveness to LP1 and to regulate reactivation of HSV-1 from latency in vivo. A role for CRE-2 in modulating inducible activity is not yet as clear; however, it has been shown to support basal expression in neuronal cells in vitro. Electrophoretic mobility shift (EMS) analyses demonstrate that the LP1 CRE-like elements interact with distinct subsets of neuronal ATF/CREB and Jun/Fos proteins including CREB-1, CREB-2, ATF-1, and JunD. The factor-binding properties of each LP1 CRE element distinguish them from each other and from a highly related canonical CRE binding site and the TPA response element (TRE). LP1 CRE-1 shares binding characteristics of both a canonical CRE and a TRE. LP1 CRE-2 is more unusual in that it shares more features of a canonical CRE site than a TRE with two notable exceptions: it does not bind CREB-1 very well and it binds CREB-2 better than the canonical CRE. Interestingly, a substantial proportion of the C1300 neuroblastoma factors that bind to CRE-1 and CRE-2 have been shown to be immunologically related to JunD, suggesting that the AP-1 family of transcription factors may be important in regulating CRE-dependent LP1 transcriptional activity. In addition, we have demonstrated the two HSV-1 LP1 CRE sites to be unique with respect to their ability to bind neuronal AP1-related factors that are regulated by cAMP. These studies suggest that both factor binding and activation of bound factors may be involved in cAMP regulation of HSV-1 LP1 through the CRE elements, and indicate the necessity of investigating the expression and posttranslational modification of a variety of ATF/CREB and AP-1 factors during latency and reactivation.

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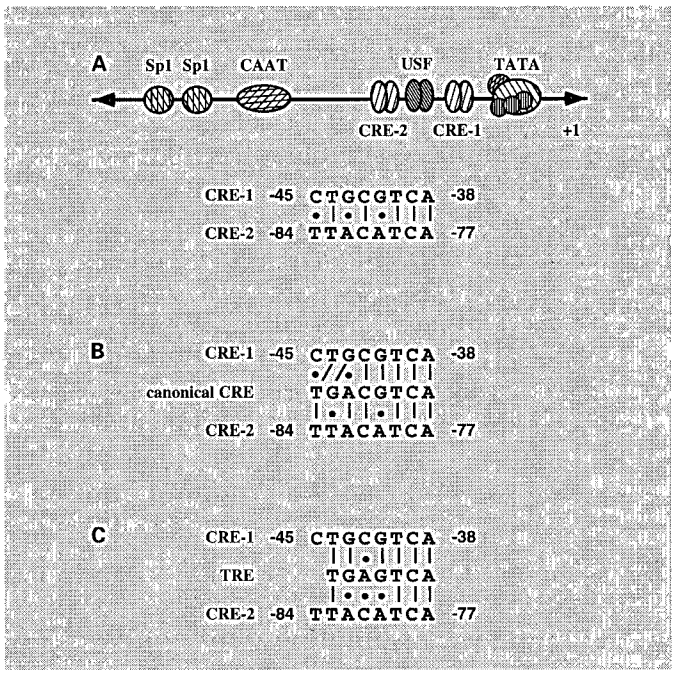

Fig. 1. Regulatory sequences of the HSV-1 LAT promoter exhibit sequence similarity to ATF/CREB and TRE consensus sequences. **A** The diagram illustrates the relative locations of several eukaryotic regulatory elements involved in the transcriptional regulation of the HSV-1 LAT promoter. USF denotes the binding site of the upstream stimulatory factor and other E-box-binding proteins [39]. CRE-1 denotes the location of the binding site of the cAMP response element identified by Leib et al. [44]. CRE-2 denotes the location of the cAMP response element-like sequence identified by Kenny et al. [38]. The nucleotide sequences of CRE-1 and CRE-2 are designated relative to the transcriptional start site. **B** A comparison of the nucleotide sequences of the HSV-1 CRE-1 and CRE-2 sites to the canonical consensus sequence for binding ATF/CREB factors. A vertical line (|) indicates matching nucleotides, while a dot (●) indicates a nucleotide mismatch. **C** A comparison of the nucleotide sequences of the HSV-1 LP1 CRE-1 and CRE-2 sites to the TRE consensus sequence. Again, a vertical line (|) indicates matching nucleotides, while a dot (●) indicates a nucleotide mismatch.

During herpes simplex virus type 1 (HSV-1) latency, infected sensory neurons express a family of colinear viral transcripts [latency-associated transcripts; LAT(s)] derived from an unstable polyadenylated 8.3-kb pre-mRNA [for reviews, see ref. 22, 68]. The major LAT species detected in neurons by northern blot hybridization analysis is a nonpolyadenylated 2.0-kb RNA. Most evidence indicates that the 2.0-kb LAT is a nonlinear, lariat-shaped molecule that is formed during a splicing reaction of the 8.3-kb transcript [20, 60, 73, 77]. Less abundant nonpolyadenylated LAT species of 1.45 and 1.5 kb in length have also been detected and are believed to be nonlinear molecules formed by splicing within the 2.0-kb LAT [67, 73]. Transcription of LAT(s) has been demonstrated to be regulated in a cell-type-specific manner by two promoters termed LAT promoter 1 (LP1) and LAT promoter 2 (LP2) [2, 12, 78]. LP1 has been shown to be the critical promoter for activating expression of LAT(s) during latency. LP2 has also been shown to possess promoter activity, and sequence within this region has been shown to augment HSV-1 LP1 activity during latency [12, 46].

A number of studies have examined virus that have deletions constructed within the LAT regulatory and coding sequence (LAT domain) to investigate the involvement of the LAT domain in the biology of latency. In summary, these studies have suggested that LAT(s) plays a role in reactivation from latency [4, 31, 42, 43, 57, 63, 71], as well as in the establishment of virus latency [47, 63, 70]. Recent reports indicate that a LAT domain-associated function may play a role in downregulating HSV-1 productive cycle gene expression during acute infection of sensory neurons [24] and possibly during latency [11]. It has been speculated that downregulation of productive infection may support the establishment of latency by reducing cytopathic effects and by evading host immune responses [24].

With the exception of the HSV-1 immediate early protein ICP4 [59], LP1 appears to be regulated primarily by cellular factors. It has, therefore, been of great interest to link LP1 activation with signaling pathways operative in neurons. LP1 contains two cAMP-response-like elements designated CRE-1 and CRE-2 which share homology to both activating transcription factor/cAMP response element-binding protein (ATF/CREB) sites and phorbol ester (TPA) response elements (TRE) (fig. 1A). In the rat pheochromocytoma cell line (PC12), CRE-1 was shown to confer cAMP inducibility to HSV-1 LP1 [1, 44]. Furthermore, a loss of function mutation constructed within CRE-1 in the context of the viral genome has been shown to reduce adrenergic reactivation of virus by epinephrine iontophoresis [6]. This evidence implies that the adrenergic/cAMP pathway within sensory neurons may function through LP1 CREs to affect viral transcription and HSV-1 reactivation. In LP1-CAT reporter expression studies involving C1300 neuroblastoma cells, both CRE-1 and CRE-2 have been shown to positively regulate LP1 basal activity in a series of experiments which utilized a panel of LP1 5′ deletion constructs [38], and in studies in which the CRE sites were mutated to block the binding of ATF/CREB factors. In addition to the cAMP second-messenger pathway, LP1 has been shown to be induced through

Ras signaling in PC12 cells by nerve growth factor and sodium butyrate [23].

The canonical CRE element (TGACGTCA, fig. 1B) and related elements such as the TRE (TGAGTCA, fig. 1C) are commonly found in the regulatory regions of a number of inducible cellular genes including somatostatin [52], proenkephalin [15], and *c-fos* [32], as well as some viral promoters such as those regulating the immediate early genes of HCMV [41] and the UL9 gene of HSV-1 [17]. The structure of the CRE element reflects the dimeric nature of the interacting proteins. It typically comprises two overlapping TGACG half-sites present in reverse orientation with the central CG dinucleotide comprising the overlap. However, not all CREs discovered contain the perfect 8-bp canonical sequence and certain alterations within the 8-bp element can dramatically change the affinity of the site for specific factors [3]. The TRE sequence is highly similar to the CRE with the exception that one of the two central CG nucleotides of the CRE is omitted resulting in a 7-bp element.

Transcription factors of the ATF/CREB family bind CREs as dimers to regulate transcription [for review, see ref. 9]. Several studies have shown that cAMP responsiveness of CRE-containing promoters involves the ubiquitous transcriptional activator CREB-1 whose activity is rapidly stimulated by phosphorylation on Ser 133 by cAMP-dependent protein kinase A (PKA) or calcium-regulated kinases [for review, see ref. 51]. In addition to kinase activation, CREB-1 also has been shown to exhibit constitutive transcriptional activity through the constitutive activation domain (CAD) [8, 75]. Transcriptional regulation by CREB-1 appears particularly strong when the DNA element possesses the canonical CRE motif which has strong affinity for CREB-1 homodimers. Once bound to the CRE element, CREB-1 has been demonstrated to interact directly with the basal transcriptional machinery [21, 74] or indirectly by mechanisms involving the CREB-binding protein (CBP) coactivator [13].

In addition to ATF/CREB factors, a list which includes at least CREB-1 (CREB), CREB-2 (ATF-4, TAXREB-67), ATF-1, ATF-2 (CRE-BP1, mXBP), ATFa, ATF-3, CREM, and various isoforms of these factors, CRE elements have also been demonstrated to functionally interact with a number of related 'basic-leucine zipper' (b-ZIP) transcription factors which includes members of the AP-1 (Jun/Fos) family [62]. The Jun/Fos family of b-ZIP transcription factors includes at least c-Jun, JunD, JunB, c-Fos, and Fos-related antigens Fra-1 and Fra-2. Members of the Jun family of proteins also bind to DNA as a dimer, and any combination of the three Jun proteins may dimerize or, alternatively, any of the Jun proteins may dimerize with other b-ZIP proteins such as those of the Fos family as well as ATF-2 [33], ATFa [10], and ATF-3 [14] of the ATF/CREB family. Transcription factors of the AP-1 family characteristically interact with TPA response elements (TREs) but some have been demonstrated to bind to CREs, although with significantly reduced affinity. The relevance of signaling cross-talk caused by binding of AP-1 factors to CREs in vivo is uncertain as it may be expected to cause confusion in cells responding to signals. One mechanism by which aberrant cross-talk could be controlled is through the sequence of the DNA element itself since sequence variability within the CRE or TRE can lead to increased or decreased affinity of specific b-ZIP factors [3, 61].

Another level of complexity resides in the ability of Jun factors to selectively heterodimerize with ATF/CREB proteins which alters their DNA binding specificity [10, 25, 33]. This phenomenon has led to the suggestion that ATF/CREB and AP-1 proteins should be considered as members of one superfamily. Although heterodimerization has been shown to occur both within and between the ATF/CREB and AP-1 families, there does appear to be a fair amount of selectivity in the ability of these factors to dimerize. An example of this selectivity is CREB-1 which has been shown to form heterodimers with ATF-1 but not with other members of the ATF/CREB and AP-1 families. It is known that ATF/CREB and Jun/Fos proteins can be regulated at a number of levels including expression and covalent modification; however, many details of this complex regulatory scheme remain to be elucidated.

Because the nucleotide sequence of CREs can select for interactions with different subsets of ATF/CREB or AP-1 factors, the studies presented here were conducted to compare the types of ATF/CREB and AP-1 factors that interact with LP1 CRE-1 and CRE-2 in a neuroblastoma cell line, and to determine whether the milieu of LP1 CRE-binding activity is altered by cAMP stimulation of cells. The results indicate that at least one neuronal AP-1 family member, JunD, is involved in a major fraction of the LP1 CRE binding activity. However, it is likely that LP1 CRE-1 and CRE-2 differ in their affinity towards specific JunD-containing dimers. Other factors such as CREB-1 and CREB-2 show preferential specificity towards CRE-1 and CRE-2, respectively. The studies also indicate that the LP1 CRE-1-binding activities present in nuclear extracts are modulated in C1300 neuroblastoma cells stimulated with the cAMP analog N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate (dibutyryl cAMP) and that the modulated factor(s) is most likely Jun/Fos related.

Materials and Methods

Oligonucleotides

Oligonucleotide probes containing LP1 CRE-1 and CRE-2 were synthesized by the Macromolecular Core Facility (The Pennsylvania State University College of Medicine, Hershey, Pa., USA). Blunt-ended, double-stranded oligonucleotides used in electrophoretic mobility shift (EMS) analyses were synthesized as single-stranded, complementary oligonucleotides and annealed as described [38]. The sequences of all of the HSV-1 oligonucleotides were derived directly from the published sequence of the LAT promoter 1 region of HSV-1 strain 17 [54] where nucleotide numbers are designated relative to the transcription start site. Oligonucleotides that contain mutations within the CRE elements of HSV-1 LP1 CRE-1 and CRE-2 probes are designated mCRE-1 and mCRE-2. The oligonucleotides containing the consensus DNA-binding sequences for AP-1 and Sp1 were obtained as a double-stranded oligonucleotide from Stratagene (La Jolla, Calif., USA). The oligonucleotide containing the consensus DNA-binding sequence for CRE (canonical CRE) was obtained as a double-stranded oligonucleotide from Santa Cruz (Santa Cruz, Calif., USA). Except where noted, the sequence of the oligonucleotides utilized herein are as follows, with CRE and TRE sequences underlined and mutated HSV-1 nucleotides double underlined. Canonical CRE, 5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′; TRE, 5′-CTAGTGATGAGTCAGCCGGATC-3′; HSV-1 CRE-1 oligonucleotide (−54 to −30), 5′-TGTTTTTGCTGCGTCATCTCAGCCT-3′; HSV-1 mCRE-1 oligonucleotide, 5′-TGTTTTTGCTGCGCAATCTCAGCCT-3′; HSV-1 CRE-2 oligonucleotide (−94 to −69), 5′-AAAATAAAATTACATCACCTACCCAC-3′; HSV-1 mCRE-2 oligonucleotide, 5′-AAAATAAAATTACACAACCTACCCAC-3′; Sp1, GATCGATCGGGGCGGGGCGATC. The exceptions are HSV-1 LP1 CRE-1 and CRE-2 probes utilized in figure 6A which contain nucleotides −63 to −31 (CRE-1) and −114 to −74 (CRE-2). The experimental results obtained with either set of CRE probes were nearly identical.

Cell Lines and Nuclear Extracts

The Neuro-2a C1300 cell line (ATCC number: CCL-131) was grown in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum, 2 m*M* glutamine, 0.1% sodium bicarbonate, and 100 μg/ml each of penicillin and streptomycin. Nuclear protein extraction from Neuro-2a cells was performed essentially as described by the method of Dignam et al. [18] or Schreiber et al. [64], with nearly identical results obtained with each method. For the studies using dibutyryl-cAMP-stimulated cells, cell cultures were treated with either 1 m*M* dibutyryl cAMP (Sigma) or phosphate-buffered saline (PBS) for 24 or 8 h prior to the preparation of the extracts. The CREB-1/Sf9 extract is a CREB-1-enriched Sf9 cell nuclear extract. An *Eco*RI/*Bgl*II fragment of pRT-CR containing the human CREB cDNA [56] was inserted in the Bakpak 9 vector (Clonetech) to make the transfer vector pBac-CREB. Sf9 insect cells (ATCC number: CRL-1711) were cotransfected with the pBac-CREB transfer vector and linearized, recombination-deficient baculovirus (Baculogold, Pharmingen). Recombinant virus was recovered and used to infect 50-ml cultures of Sf9 cells. The cells were harvested 60 h postinfection and nuclear extracts were prepared by a modification of the method of Hurst et al. [35].

EMS Analyses

Oligonucleotides containing CRE-1, CRE-2, AP-1, and CREB sequences were labeled with [γ-^{32}P]-ATP by T4 polynucleotide kinase. Labeled probe (0.01–0.1 pmol; 75,000 cpm) was incubated with 2–9 μg of nuclear extract and 1 μg poly (dI-dC) for 20 min at 30°C. In antibody supershift EMS analyses, selected binding reactions may also include 100 ng (1 μl) of antibody competitor peptides. Reactions were subjected to electrophoresis at room temperature in a 5% nondenaturing polyacrylamide gel at 125 V, dried at 80°C for 45 min, and subjected to autoradiography. EMS analyses using CREB-1/Sf9 extract were performed using 2 or 4 μg of extract, 1 μg poly (dI-dC), and radiolabeled probe (75,000 cpm). Antibody supershift EMS analyses were performed by adding 0.5–1.0 μl antibody to the binding reactions 10 min prior to loading the gel. Antibodies directed against CREB-1, ATF-2, CREB-2, ATF-1, JunD, and CREM were obtained from Santa Cruz. The antibody competitor peptides used (fig. 6C) were obtained from Santa Cruz and comprised the peptide from which the antibody was raised against (200 mg/ml stock).

Results

Previous studies have clearly indicated that CRE-related elements can not only interact with ATF/CREB factors but also with members of the Jun/Fos transcription factor family [62]. The additional complexity of CRE-binding factors may provide a greater degree of flexibility in the regulation of transcription within the context of a large number of cellular and viral promoters. A sequence comparison of LP1 CRE-1 and CRE-2 with a canonical CRE and an AP-1-binding TRE is shown in figure 1. As shown, CRE-1 exhibits a high degree of sequence similarity with the TRE sequence with but just one nucleotide difference (fig. 1C). CRE-1 also shows similarity to the canonical CRE due to the shared CGTCA core element (fig. 1B). In contrast, the LP1 CRE-2 exhibits a considerably lower degree of sequence similarity when compared to the TRE sequence (only 4 out of 7 nucleotides are shared, fig. 1C). While CRE-2 exhibits a higher similarity when compared to the canonical CRE (6 out of 8 nucleotides are shared, fig. 1C), it lacks the crucial CRE half-site for recognition by CREB-1 (fig. 1B). Based on sequence analysis, one could hypothesize that HSV-1 LP1 CRE-1 would exhibit transcription factor-binding characteristics similar to both TRE and CRE elements whereas CRE-2 would exhibit binding characteristics of a CRE element.

CREB-1 Interacts with HSV-1 LP1 CRE-1

To directly address whether CREB-1 is involved in binding to LP1 CREs, a nuclear extract prepared from CREB-1-expressing Sf9 cells (CREB-1/Sf9 extract) was utilized in EMS analyses using DNA oligonucleotide

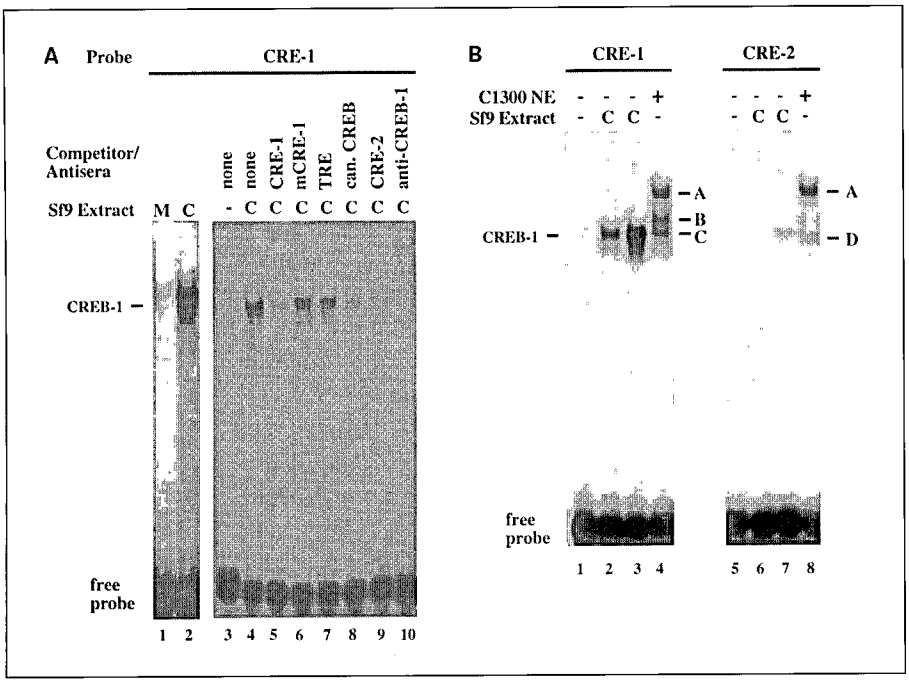

Fig. 2. A CREB-1 interacts with HSV-1 LP1 CRE-1. EMS analyses of CRE-1 probe and nuclear extracts prepared from CREB-1-expressing baculovirus-infected Sf9 cells. An aliquot (2 μg) of mock-infected Sf9 nuclear extract (M) and CREB-1-expressing baculovirus-infected cell nuclear extract (C) are shown (lanes 1 and 2). CREB-1/Sf9 nuclear extract (2 μg) was reacted with a CRE-1 probe in the absence (lane 4) or presence (lanes 5–9) of a 25-fold molar excess of competitor oligonucleotides where indicated. Antibody supershift EMS analysis was performed in lane 10 using 1 μl of anti-CREB-1 monoclonal antibody. **B** An aliquot of CREB-1/Sf9 extract (2 μg, lanes 2 and 6; 4 μg, lanes 3 and 7) was incubated with either HSV-1 LP1 CRE-1 (lanes 2 and 3), or HSV-1 LP1 CRE-2 (lanes 6 and 7) DNA probes as indicated. C1300 nuclear extract (6 μg) was reacted with each probe in lanes 4 and 8. The major DNA-protein complexes formed using C1300 nuclear extract are designated A, B, C, and D as shown.

probes encompassing the HSV-1 LP1 CRE-1 and CRE-2 sites (fig. 2). This experimental approach reduces the competitive influences of other CRE-binding proteins as well as the potential heterodimerization that can occur between various ATF/CREB and AP-1 family members. As shown (fig. 2A), a CRE-1 probe formed one major complex with factors in the CREB-1/Sf9 extract (lane 2) that did not form in studies utilizing uninfected control Sf9 cell nuclear extract (lane 1), indicating that the factor shifting the probe is the CREB-1 present in the baculovirus-infected cell nuclear extract. To confirm the identify of this factor as CREB-1, a CREB-1-specific monoclonal antibody raised against the DNA-binding and dimerization domain of CREB-1 was included in the EMS reaction (compare lane 10 to lane 4). As expected, inclusion of the antibody in the EMS reaction resulted in the abrogation and partial supershift of the complex. To test the sequence specificity of CREB-1 in the Sf9 extract, the reactions were incubated with a panel of unlabeled competitor oligonucleotides (compare lanes 5–9 to lane 4). Only the CRE-1 (lane 5), canonical CRE (lane 8), and CRE-2 (lane 9) oligonucleotides competed effectively with respect to the formation of this complex. In contrast, a mutated CRE-1 oligonucleotide (lane 6) and a TRE-containing oligonucleotide (lane 7) did not compete effectively. These observations demonstrate that CREB-1 binding is specific for ATF/CREB binding sites and that mutations placed within the CGTCA half-site of CRE-1 strongly inhibit CREB-1 binding as expected.

Also of interest is the fact that the CRE-2 competitor also competed more effectively for CREB-1 than the TRE or the mutated CRE-1 oligonucleotides even though this site does not contain a perfect canonical CRE half-site. This suggests that CRE sequence variation permits CREB-1 binding although as shown in figure 2B, the binding of CREB-1 to CRE-2 appears to be of lesser magnitude than to CRE-1 (compare lanes 2 and 3 to lanes 6 and 7). To determine whether CREB-1 present in C1300 neuronal cells binds to LP1 CREs, a nuclear extract prepared from these cells was also reacted with each HSV-1 LP1 CRE probe (fig. 2B, lanes 4 and 8). This cell line has been utilized as a model for studying neuronal differentiation [55, 65], and for investigating the early events of HSV-1 infection in a cell of neuronal origin [37]. To simplify the discussion of the EMS results utilizing crude nuclear extracts, the complexes that form with the probes used in these studies are designated A–D depending on relative gel mobility. Consistent with the EMS reactions utilizing CREB-1/Sf9 extract, only the CRE-1 probe forms an abundant complex (complex C) in the C1300 nuclear extracts that co-migrates with the CREB-1 complex. These observations suggest that CRE-1 has a higher affinity for CREB-1 than CRE-2, most likely as a result of the perfect CRE half-site of CRE-1.

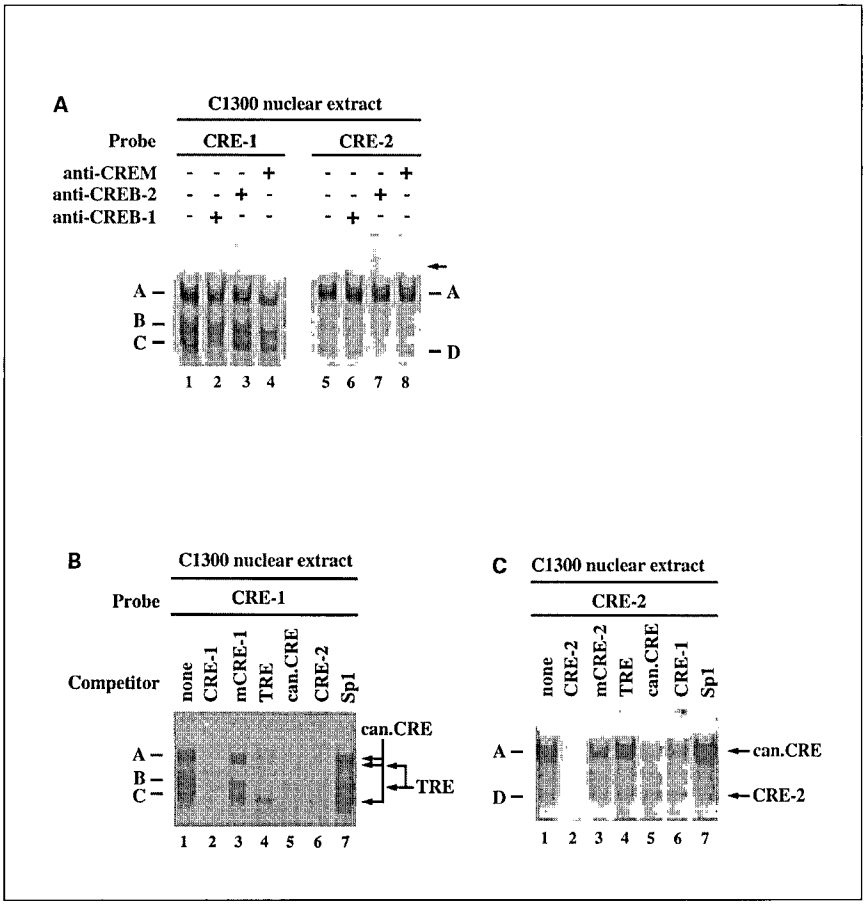

Fig. 3. A Neuronal CREB-1 and CREB-2 interact preferentially with the HSV-1 LP1 CRE-1 and CRE-2 elements, respectively. An aliquot (6 μg) of C1300 nuclear extract was incubated with the CRE-1 (lanes 1–4) or CRE-2 probe (lanes 5–8) in supershift EMS analyses. Antibodies specific for CREB-1, CREB-2, and CREM were included in the reactions as indicated. The major DNA-protein complexes formed using C1300 nuclear extract are designated A, B, C, and D as shown. The arrow indicates the position of the CREB-2 supershifted complex. **B** Factors involved in DNA-protein complexes formed using CRE-1 probe show distinct specificity for either TRE or canonical (can.) CRE sites. Competition EMS analyses were performed using C1300 nuclear extract, CRE-1 probe and a 25-fold molar excess of unlabeled CRE-1, mutated CRE-1 (mCRE-1), TRE, canonical CRE, CRE-2, and Sp1 competitor oligonucleotides as indicated. Complexes formed that show specificity for the canonical CRE or TRE are indicated by arrows on the right. **C** Factors involved in DNA-protein complexes formed using CRE-2 probe show specificity primarily toward CRE-like sites. Competition EMS analyses were performed using C1300 nuclear extract, CRE-2 probe and 25-fold molar excess of unlabeled CRE-2, mutated CRE-2 (mCRE-2), TRE, canonical CRE, CRE-1, and Sp1 competitor oligonucleotides as indicated. Complexes specific for canonical CRE or CRE-2 are indicated by arrows on the right.

To demonstrate that CRE-1 complex C contains neuronal CREB-1, this complex was abrogated using the CREB-1 monoclonal antibody (fig. 3A, compare lanes 1 and 2). In contrast, a CREB-1 supershift was not detected when the CRE-2 oligonucleotide was utilized in the supershift EMS reaction (fig. 3A, compare lanes 5 and 6). Additional supershift studies demonstrated that a CRE-2 complex (complex D) reacted with an antibody raised against CREB-2 (fig. 3A, compare lanes 5 and 7) while CREB-2 antibody had no effect on the formation of CRE-1 complexes (compare lanes 1 and 3). The CREM-reactive antibody does not significantly supershift any complexes formed with either probe. Although the CREB-2-immunoreactive complex (complex D) was formed at relatively low abundance, our studies have demonstrated that the abundance of this complex may be upregulated by the stimulation of C1300 cells with ionomycin (data not shown). CREB-2 has been implicated in transcriptional inhibition [36] as well as activation [45] through its interactions with CREs.

LP1 CRE-1 Displays Characteristics of a CRE and TRE

It is apparent that other neuronal factors are involved in binding to LP1 CRE-1 and CRE-2 due to the presence of multiple complexes formed in EMS analyses using either CRE-1 or CRE-2 probes. The lowest mobility complex (complex A, fig. 2A) that forms with either CRE-1 or CRE-2 appears almost equal in abundance and mobility when the complexes formed with each probe are compared. In addition, the observation that CRE-1 forms an abundant complex (complex B) that is formed with only very low abundance with CRE-2 is of obvious interest. To investigate the sequence specificity of these complexes, we performed competition EMS analyses using a panel of unlabeled competitor oligonucleotides (fig. 3B, C) under conditions where the molar excess of homologous competitor (fig. 3B, C, lane 2) completely abrogated all DNA-protein complex formation. Interestingly, individual complexes show distinct preferences for binding either the TRE or the canonical CRE competitors.

With respect to the CRE-1 probe, the mutated CRE-1 competitor oligonucleotide, which changes the CGTCA half-site of CRE-1 to CGCAA, showed only minimal competition compared to the wild-type competitor oligonucleotide (fig. 3B, compare lane 3 with lane 1) suggesting that the CRE half-site is critical for binding all of the factors that form with the CRE- 1 probe in EMS reactions. As expected, the TRE competitor did not significantly compete for the CREB-1-containing DNA-protein complex (complex C) although it very effectively abrogated the formation of complex B and the lower portion of complex A (fig. 3B, compare lane 4 to lane 1). This suggests that complex B and at least a portion of complex A contains factors that also bind to TRE elements with high specificity. The canonical CRE competitor, in addition to showing greater overall competition, was distinct from the TRE competitor in that it showed a preference for abrogating complexes A and C compared to complex B (fig. 3B, compare lanes 5 and 4 to lane 1) indicating that factors involved in complexes A and C are most specific for binding CRE elements whereas factors involved in complex B are specific for TRE or TRE-like elements such as LP1 CRE-1. The CRE-2 competitor was similar to the canonical CRE competitor in that it also showed high overall competition compared to TRE competitor (fig. 3B, compare lane 6 to lane 1). However, CRE-2 was distinguished from the canonical CRE competitor in that it was slightly more effective with respect to abrogating complex B and slightly less effective with respect to abrogating complex C (fig. 3B, compare lane 6 to lane 1). As a negative control, the Sp1 site competitor showed no detectable abrogation of any complexes under these conditions (fig. 3B, compare lane 7 to lane 1). The complexes that form most specifically with either the canonical CRE or TRE sequences are indicated by arrows to the right of figure 3B. These observations as a whole suggest that LP1 CRE-1 can potentially function as either a CRE or TRE site since it binds factors that are highly specific for both of these elements, although a more detailed functional analysis of the promoter is necessary to test this hypothesis.

LP1 CRE-2 Displays Some Characteristics of a Canonical CRE

Similar to the competition EMS analyses performed utilizing CRE-1 as probe, studies were also performed with a CRE-2 probe (fig. 3C). In summary, the most abundant complex (complex A) formed with CRE-2 is abrogated entirely by self-competition (compare lane 2 to lane 1), and to a high degree by the canonical CRE (compare lane 5 to lane 1), and CRE-1 (compare lane 5 to lane 1) competitors, whereas complex A is abrogated only marginally by the TRE competitor (compare lane 4 to lane 1) and not at all by the Sp1 competitor (compare lane 7 to lane 1). A mutation created within the CRE-2 site (TTACATCA changed to TTACACAA) was shown to significantly block the ability of the CRE-2 competitor oligonucleotide to compete for complexes A and D (fig. 3C, compare lanes 3 and 1 to lanes 2 and 1) indicating that the CGTCA-like half-site of CRE-2 (CATCA) is required for efficient binding of C1300 factors to CRE-2. These results are consistent with the results of figure 3B and overall suggest that the factors that comprise complex A with CRE-2 are similar to those that form complex A with CRE-1. It is not likely that they are exactly the same, however, since the TRE competitor was less efficient at abrogating complex A formed with the CRE-2 probe (fig. 3C, compare lanes 4 and 1) than complex A formed with the CRE-1 probe (fig. 3B, compare lanes 4 and 1).

The factors within complex A that exhibit a TRE specificity appear to be involved in forming the lower half of this complex in EMS analyses using CRE-1 and CRE-2 probes, which suggests that complex A is heterogeneous and that only a fraction of the factors that make up this complex show a high level of specificity for discriminating a canonical TRE from a CRE. CRE-2 also shows specificity unique among all of these probes in that the formation of complex D (fig. 3C, lane 1) is only competed significantly by a CRE-2 competitor (fig. 3C, compare lane 2 to lane 1) and not by any of the other competitor oligonucleotides (fig. 3C, lanes 4–7). Previously (fig. 3A), we have demonstrated that complex D is supershifted by anti-CREB-2 antibody and therefore these data suggest that CREB-2/ATF-4 may have sequence specificity that is different than CREB-1, involving a consensus sequence more similar to LP-1 CRE-2 (TTACATCA) than the canonical CRE, the TRE or LP1 CRE-1.

JunD Interacts with LP1 CREs

Since a substantial proportion of the DNA-protein complexes formed with the HSV-1 LP1 CRE elements were not reactive to antibody specific for CREB-1 or CREB-2, we proceeded to examine the factors involved in forming the additional complexes. Kenny et al. [38] demonstrated that the slowest migrating complexes that form when C1300 nuclear extract was reacted with a CRE-2 probe were related to the ATF family of transcription factors by antibody supershift EMS analyses. To examine the identity of these factors, antibody raised against ATF-1 or ATF-2 was reacted with the CRE-1 and CRE-2 DNA probes (fig. 4). The addition of ATF-1 antibody into the

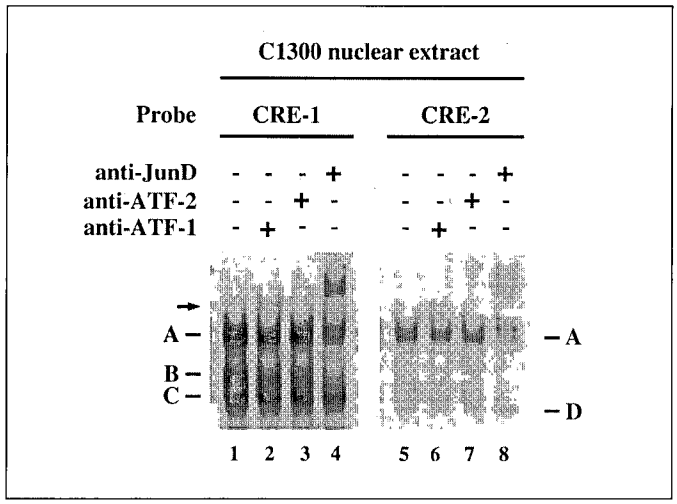

Fig. 4. JunD is major component of HSV-1 LP1 CRE-1 and CRE-2 binding activity in C1300 nuclear extracts. An aliquot (6 μg) of C1300 nuclear extract was incubated with the CRE-1 (lanes 1–4) or CRE-2 probe (lanes 5–8) in supershift EMS analyses. Antibodies specific for ATF-1, ATF-2, and JunD were included in the binding reactions as indicated. The major DNA-protein complexes formed using C1300 nuclear extract are designated A, B, C, and D as shown. The arrow indicates the position of the ATF-1 supershifted complex.

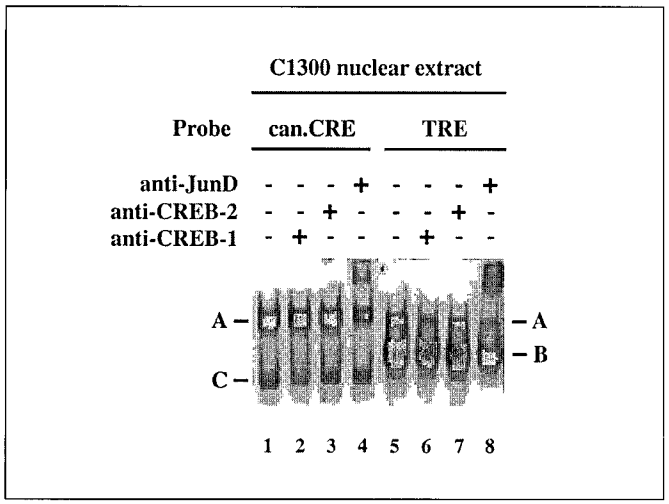

Fig. 5. Interaction of CREB-1, CREB-2, and JunD with the canonical (can.) CRE and TRE. An aliquot of C1300 nuclear extract (6 μg) was incubated with the canonical CRE (lanes 1–4), or TRE probe (lanes 5–8) in supershift EMS analyses. Antibodies directed against CREB-1, CREB-2, and JunD were included in the reactions as indicated to determine the immunoreactivity of the complexes.

EMS reactions resulted in a small abrogation of complex A and a small amount of supershifted complex indicated by an arrow (fig. 4, lane 2). This was observed in studies involving CRE-1 (fig. 4, compare lanes 1 and 2) and to an even lesser extent with the CRE-2 probe (fig. 4, compare lanes 5 and 6). Little if any immunoreactive material was detected using antibody raised against ATF-2. These observations suggest that ATF-1 and ATF-2 are unlikely to play prominent roles in regulating LP1 activity in C1300 cells under the conditions examined.

Since AP-1-related factors have also been implicated in binding to CREs [62] and because the TRE competition EMS analyses suggest that some of the LP1 CRE binding activity appears to require a binding sequence similar to a TRE, supershift EMS analyses were conducted using a number of antibodies that react to members of the Jun and Fos transcription factor families. While some of these antibodies reacted weakly (data not shown), the JunD antibody reacted very strongly with CRE-1- (fig. 4; compare lanes 1 and 4) and CRE-2- (fig. 4; compare lanes 5 and 8) containing DNA-protein complexes. More specifically, the JunD antibody supershifted a portion of complex A in studies utilizing either CRE-1 or CRE-2 probes, as well as complex B that forms with the CRE-1 probe. The fact that JunD antibody only partially supershifted complexes A and B indicates that both of these complexes are heterogeneous and therefore include other factors not yet identified. These results are not surprising due to the high number of b-ZIP proteins that can putatively bind to these sequences. The result that JunD was found as a primary constituent of complexes A and B is consistent with the competition studies (fig. 3B, C) in which it was demonstrated that both of these complexes display specificity toward the AP-1-binding TRE competitor oligonucleotide. It is possible that AP-1 factors such as c-Jun, JunB, c-Fos, and FosB may interact with LP1 CREs but only a very small amount of supershifted material was observed when antibodies raised against these factors were used in EMS analyses (data not shown). These results indicate that JunD is one of the primary Jun family members that binds to LP1 CREs and affects transcriptional regulation in a C1300 neuronal cell line.

Supershift EMS analyses were also conducted using C1300 nuclear extracts and the canonical CRE and TRE probes (fig. 5). As shown, a CREB-1-containing complex (complex C) is readily identified using the canonical CRE probe and CREB-1 monoclonal antibody (fig. 5, compare lanes 1 and 2). The addition of CREB-1 antibody into the EMS reaction containing the canonical CRE probe (fig. 5, lane 2) resulted in the partial abrogation of a complex that

comigrates with CREB-1 complexes observed in EMS reactions containing the CREB-1/Sf9 extract (fig. 2, lanes 2–4). Antibody directed against CREB-2 did not react significantly with any of the canonical CRE-containing complexes (fig. 5, compare lanes 1 and 3). As shown in figure 5 (lanes 5–8), complexes formed using the TRE probe did not react significantly with either the CREB-1 or CREB-2 antibodies.

Similar to LP1 CRE probes, a significant supershift was observed when antibody specific for JunD was utilized in EMS reactions (fig. 5, compare lanes 1 and 4 or lanes 5 and 8). As previously shown with LP1 CRE-1 and CRE-2 probes, JunD immunoreactivity was also observed with both canonical CRE and TRE probes in the comigrating low mobility complexes (complex A). However, the major TRE-associated complex (complex B) is not formed with the canonical CRE probe and this complex also contains JunD immunoreactive material based on the partial abrogation and supershift of this complex in supershift EMS analyses utilizing the TRE probe (fig. 5, compare lanes 5 and 8). The nonsupershifted material in the major TRE-containing complex (fig. 5, lane 8) is likely to be comprised of other Jun/Fos-related factors. Since the heterodimeric partner of Jun proteins can affect the DNA-binding sequence specificity of the complex, it is likely that JunD is present in C1300 cells as a dimer with at least two different proteins, and this may result in two JunD-containing complexes which have different sequence specificity. If this is the case, then the results suggest that one of the JunD dimers (involved in forming complex B), binds TREs in a highly selective manner, whereas another dimer (involved in forming complex A) is less sequence selective and binds both CRE- and TRE-like elements.

In summary, when one compares the DNA-protein complexes that form with the HSV-1 LP1 CRE-1 and CRE-2 probes with the canonical CRE and TRE probes, it can be concluded that all four oligonucleotide probes share the feature of interacting with JunD-related factors. However, the composition of the JunD complexes is not equivalent because JunD is involved in two different complexes with very different mobility. The low mobility JunD complex (complex A) involves proteins which interact with ATF/CREB elements (CRE-1, CRE-2, and canonical CRE) while proteins comprising the higher mobility JunD complex (complex B) preferentially interact with the TRE and CRE-1 elements. These results suggest that LP1 CRE-1 represents a hybrid binding site with characteristics of an ATF/CREB element and a TRE. CRE-2 on the other hand appears more like an ATF/CREB binding site than a TRE with the notable exception that this element selects against interactions with CREB-1, likely due to the absence of an intact CGTCA half-site, and it has the unique characteristic among all sites examined in that it binds CREB-2.

Dibutyryl cAMP Treatment Alters LP1 CRE-Binding Activities

Since CRE-1 has been documented to facilitate cAMP-induced LP1 *trans*-activation [44], we were interested in determining whether treatment of cells with a cAMP analog would alter LP1 CRE-binding activities. To examine this possibility, C1300 cells were treated with 1 m*M* dibutyryl cAMP for 24 h and nuclear extracts prepared from these cells were compared in EMS analyses with extract prepared from PBS-treated control cultures. The resultant nuclear extracts were compared in EMS analyses using DNA probes containing the TRE, canonical CRE, CRE-1, or CRE-2 binding sites (fig. 6). A loss of the bottom half of the slowest migrating pair of complexes (complex A) was observed when either CRE-1 (fig. 6A, compare lanes 1 and 2) or CRE-2 (fig. 6A, compare lanes 3 and 4) probe was utilized in EMS analyses. In contrast, as indicated by the arrow, a relative increase in binding was observed in the faster migrating complex (complex B) detected with the CRE-1 (fig. 6A, compare lanes 1 and 2) and TRE (fig. 6, compare lanes 7 and 8) probes. Four different nuclear extracts prepared from dibutyryl cAMP-stimulated C1300 cells as well as two experiments using forskolin stimulation have yielded the same basic results. This supports the possibility that regulation may be mediated by the redistribution of a preexisting factor such as JunD from one complex to another as well as upregulation and downregulation of specific factors.

The sequence specificity of the complexes formed using cAMP-stimulated cells was examined by competition EMS analyses (fig. 6B). The results demonstrate that the most abundant complex formed using this extract (complex B) is preferentially competed by the TRE competitor rather than the canonical CRE competitor, indicating that the factors that comprise this complex are likely to be in the AP-1 family of transcription factors. All of the complexes that formed in the competition EMS analyses were sequence specific as demonstrated by the absence of competition when mutated CRE-1 or Sp1 competitors were used. Overall, the competition results using the cAMP-stimulated extracts (fig. 6B) are similar to those performed using unstimulated extracts (fig. 3B) with the only difference being the intensification of complex B relative to complexes A and C in the stimulated cell extracts.

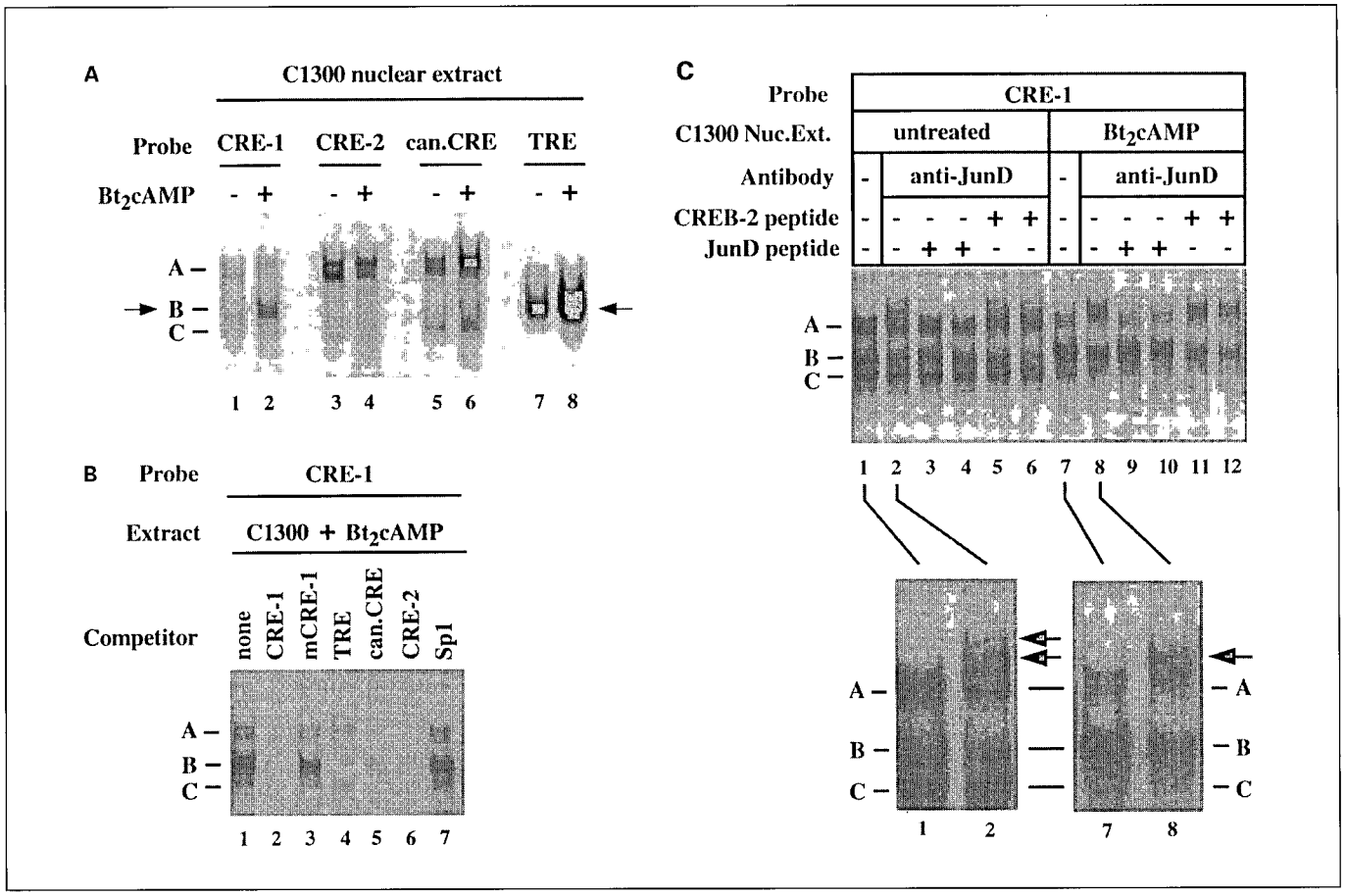

Fig. 6. Treatment of C1300 cells with dibutyryl cAMP (Bt_2cAMP) alters DNA-protein complex formation with HSV-1 LP1 CREs. **A** Neuronal C1300 cells were treated with 1 m*M* dibutyryl cAMP for 24 h prior to the preparation of nuclear extracts. An equivalent volume of each extract was tested for complex formation with a CRE-1 (lanes 1 and 2), CRE-2 (lanes 3 and 4), canonical (can.) CRE probe (lanes 5 and 6) or TRE probe (lanes 7 and 8). The major DNA-protein complexes formed using C1300 nuclear extract are designated A, B, and C as shown. **B** Competition EMS analyses using CRE-1 probe, Bt_2cAMP-stimulated (8 h) nuclear extracts (3 μg), and 25-fold molar excess of unlabeled CRE-1, mutated CRE-1 (mCRE-1), TRE, canonical (can.) CRE, CRE-2 and Sp1 competitor oligonucleotides. **C** Anti-JunD supershift of CRE-1 complexes formed using unstimulated and Bt_2cAMP-stimulated (8 h) C1300 nuclear extracts (3 μg). Reactions containing 0.5 μl anti-JunD antibody and 1 μl competitor peptides are indicated above. At the bottom is a close-up of lanes 1, 2, 7, and 8 to point out changes in the mobility of supershifted bands marked by arrows.

Since previously obtained evidence indicates that JunD is a major AP-1 family member that binds to CRE-1 (fig. 4), we tested the possibility that JunD may be involved in the changes in complex formation observed (fig. 6A, B). Toward this end, we included anti-JunD antibody into the EMS reactions as indicated in figure 6C. The supershifts obtained utilizing the CRE-1 probe and JunD antibody are shown with EMS reactions containing unstimulated (fig. 6C, compare lane 1 to lane 2) and stimulated (fig. 6C, compare lane 7 to lane 8) cell extracts, respectively. Antibody specificity was confirmed by blocking the supershifts using the anti-JunD epitope peptide in EMS reactions containing unstimulated cell extracts (fig. 6C, compare lane 2 to lanes 3 and 4) and stimulated cell extracts (fig. 6C, compare lane 8 to lanes 9 and 10), whereas an unrelated peptide did not block the supershifts (fig. 6C, compare lane 2 to lanes 5 and 6, and compare lane 8 to lanes 11 and 12). These results demonstrate that JunD is a significant component of CRE-1-binding activity in both extracts. A very close examination of the autoradiograph suggests that JunD may be involved in the cAMP-induced changes in DNA-binding activity as assessed by a small change in the mobility of supershifted complexes when lanes 2 and 8 (bottom of fig. 6C) are

compared. This change appears to involve a switch from two supershifted bands in unstimulated cell extracts to one supershifted band in stimulated cell extracts. It is hypothesized that this may be the result of altered JunD dimer formation induced by the treatment of C1300 cells by dibutyryl cAMP.

Many possibilities exist to explain the observed results, including increased or decreased expression of factors at the RNA and/or protein level, as well as covalent modification of the existing proteins. Either of these general mechanisms could result in the alteration of dimer formation between b-ZIP proteins or the relocalization of factors within subcellular compartments that in turn could modulate the nuclear concentration, affinity, or sequence specificity of nuclear CRE-binding activities. JunD has been observed to be a member of an AP-1 complex induced by treatment of myeloid cell lines with a cAMP analog [58]. These analyses suggest the possibility that the cAMP second messenger system may regulate HSV-1 LP1 activity by two different mechanisms. The first mechanism may involve the phosphorylation of Ser 133 on CREB-1 bound to LP1 CRE-1. The second mechanism, which is likely to occur with slower and more sustained kinetics, may be mediated through alterations in binding of other nuclear transcription factors that bind to LP1 CREs or other LAT promoter regulatory elements and may include JunD or other members of the AP-1 family.

Discussion

Regulation of Factors That Bind to CRE Elements

The function of ATF/CREB family members and the signals that they respond to appear to be different between family members [for review see ref. 9, 51]. CREB-1 can stimulate basal and cAMP-induced transcriptional activity. ATF-1-mediated *trans*-activation has been shown to be stimulated by cAMP, however, the level of *trans*-activation with ATF-1 does not appear to be as high as with CREB-1, and it may actually antagonize CREB-1-dependent activation by competing for CREs, depending on the cellular level of CREB-1 homodimer [19]. CREB-2 and certain CRE modulator (CREM)-related factors have also been shown to inhibit CRE-dependent *trans*-activation by competing with more active factors for binding [36, 50]. Our DNA-protein-binding analyses indicate that between HSV-1 LP1 CRE-1 and CRE-2, CRE-1 is the primary target for CREB-1. This evidence makes it important to consider CREB-1 as a functional LP1 *trans*-activator. LP1 CRE-1 interacts with additional factors to form DNA-protein complexes that were equal in abundance compared to the CREB-1 DNA-protein complex, indicating that factors other than CREB-1 may be of significant functional importance. In contrast to these observations, when EMS analyses were performed with LP1 CRE-2, CREB-1 was not strongly detected in DNA-protein complexes, raising the possibility that CRE-2 does not respond to cAMP stimulation, at least via interactions with CREB-1.

Our studies indicate that at least one member of the Jun family (JunD) is involved in a substantial percentage of the DNA-protein complexes that form between both LP1 CREs and factors present in C1300 nuclear extracts. The identification of the Jun family of cellular proteins as possibly participating in the transcriptional regulation of the HSV-1 LP1 is of great interest. LP1 CRE-1 differs from the consensus TRE by just one nucleotide. Our analyses demonstrate that a CRE-1 probe forms at least two JunD-immunoreactive complexes when reacted with C1300 nuclear extracts. These complexes have mobilities that match complexes formed with the TRE probe and are abrogated by inclusion of a TRE competitor oligonucleotide into the binding reactions. LP1 CRE-2 does not have a high degree of sequence similarity to the TRE consensus sequence and consistent with this observation, CRE-2 complexes are not effectively competed by a TRE competitor oligonucleotide. However, Jun factors may function through the CRE-2 element via Jun heterodimers that bind to non-TRE elements with high affinity. The heterodimeric partner of Jun may be of the ATF family or it could possibly involve other related b-ZIP proteins.

The regulation of c-Jun, JunD, and JunB is not identical, and has the potential to differ between cell types and between the types of cellular signals utilized. In the nervous system, JunD is typically expressed at high constitutive levels in neurons of the brain [28] and in dorsal root ganglia (DRG) [16] whereas c-Jun is expressed at low constitutive levels or in highly cell type-specific patterns. Not all of the Jun-containing dimers contribute equally to the process of transcriptional regulation. For example, JunD has been shown to activate proenkephalin transcription in a manner dependent on the cAMP second messenger pathway while c-Jun functioned constitutively and JunB had no effect alone but blocked activation by JunD [40]. In a similar study using human neuroblastoma SK-N-MC cells, ATF-3 was shown to bind the proenkephalin CRE2 element (Enk-CRE2) as a JunD heterodimer and to induce proenkephalin gene expression in a cAMP-responsive manner [14]. It is interesting that Enk-CRE2 and LP1 CRE-1 show 100% sequence similarity within the seven core nucleotides which implies that these two elements

may share very similar affinity for the same transcription factors. Since cAMP activation of the proenkephalin promoter in neuronal cells has been shown to involve JunD and ATF-3 interactions with Enk-CRE2, it is interesting to speculate that Jun D and ATF-3 may play an important role in regulating cAMP-induced LP1 activity via the CRE elements due to the shared sequence similarity between Enk-CRE2 and LP1 CRE-1. Our studies support the possible involvement of JunD in LP1 function, although, we only detected low levels of ATF-3 in our EMS analyses (data not shown) and ATF-2 antibody is without substantial effect on DNA-protein complex formation (fig. 4).

Other investigators have reported increased levels of expression of c-Jun and c-Fos in trigeminal ganglia during acute HSV-1 ocular infection and during virus reactivation [72]. It is possible that c-Jun and c-Fos also interact with LP1 CREs and that they were not readily detected in our studies due to low basal expression and/or the absence of relevant signal transduction pathway stimulation. Alternatively, c-Jun may not bind to LP1 CREs with high affinity relative to JunD. The regulation of other b-ZIP proteins implicated in our studies have not been reported in investigations of cellular gene expression during the establishment, maintenance, or reactivation of latent HSV-1 infection of the peripheral nervous system. However, they have been shown to be differentially regulated in neurons following the stress of axotomy [27]. Due to the potential significance of the Jun/Fos family in regulating HSV-1 LP1 activity, future studies should address these factors as potential transcriptional effector molecules in a regulatory pathway that involves LP1 and HSV-1 latency and reactivation.

Function of CRE Elements in HSV-1 Latency

CRE elements are known to play important roles in regulating the inducible expression of a number of cellular genes through the cAMP and calcium second messenger systems. The receptors that transduce these signals are typically coupled to G-proteins and include the adrenergic receptor family, as well as receptors for neuromodulatory peptides, and prostaglandins. Recent studies indicate that regions of LP1 and LP2 including the CRE-1 element play a significant role in epinephrine-induced HSV-1 reactivation [4–6, 29, 30]. It has been suggested that cAMP-inducible transcription factor binding elements such as CREs and AP-2 sites as well as DNA methylation within the regions necessary for efficient induced reactivation facilitate reactivation by altering LAT promoter structure and transcriptional activity. Also, recent evidence suggests that histone acetylase activity is associated both directly with CBP [53] and indirectly with the CBP binding protein P/CAF [76]. Because CBP has been shown to interact directly with phosphorylated CREB-1 and CREB-2/ATF-4 [45], both LP1 CRE sites may play a role in recruiting this histone acetylase to the LAT promoter in order to relieve transcriptional inhibition caused by chromatin structure.

Recent studies have been conducted using LAT-negative HSV-1 in a hyperthermia-induced HSV-1 reactivation model. However, the reactivation defect associated with the LAT-negative HSV-1 strains was shown to be related to the establishment of latency in fewer neurons [70]. The reaction of sensory ganglia to HSV infection is consistent with the neurobiological responses observed during nerve regeneration of peripheral processes following injury, and includes alterations of neuropeptide production [26], neurite sprouting [49, 66], and upregulation of AP-1 factors [72]. It is possible that these regenerative responses are linked to signaling pathways that regulate LAT promoter activity. In support of this hypothesis, studies have shown LP1 activity to be the highest in trigeminal neurons during the acute phase of ganglionic infection, after which LAT transcription declines to lower basal levels [48]. A number of reports have shown that the Neuro 2a clone of C1300 cells follows a pattern of neuronal differentiation following treatment with agents that induce an elevation of intracellular cAMP levels [7, 55, 65]. It is possible that the biochemical events involving alterations in CRE-binding activity that we observe in the C1300 cell line using a cAMP analog mimic some of the stress-induced regenerative events that occur in sensory neurons during HSV infection. In support of this hypothesis is the result which shows that a peptide hormone (alpha-melanocyte-stimulating hormone) released from the pituitary gland and keratinocytes following injury and inflammation can elevate intracellular cAMP levels in dorsal root ganglion cells, stimulate neurite outgrowth, and facilitate regeneration of peripheral nerves [34, 69]. Further elucidation of the cellular events associated with HSV infection are required to determine the validity of this model.

Acknowledgments

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