In vitro binding to the ccna2 promoter region

(A) The rat ccna2 promoter region (-450+1). The transcription start site has been inferred from alignment with the human sequence. The previously described Sp1, CREB/ATF (CRE) and E2F binding sites are underlined, along with the three nonconsensus AP-1 sites (TRE-1, -2 and -3), identified by computer-aided (TRANSFAC) sequence analysis. In particular, two TRE-like heptanucleotides (CTAGTCA) were identified at positions -429 (ccna2TRE-1) and -406 (ccna2TRE-2), in addition to the variant TRE (TGAGTCC) at position -397 (ccna2TRE-3). (B) EMSA. In vitro binding of nuclear proteins from FRTL-5K-Ras cells to oligonucleotide probes containing the ccna2TREs. Nuclear extracts from FRTL-5K-Ras were incubated with the 5'-end-labeled ccna2TRE-1, ccna2TRE-2/3 and ccna2TRE-3, as described in Materials and Methods. The fos/ITRE probe was included as a positive control of AP-1 binding. DNA-protein complexes were resolved by 6% PAGE. The arrow indicates the position of specific complexes. Because of the sequence identity between TRE-1 and TRE-2, we analyzed the binding to TRE-1 compared to TRE-3. With both probes a single gel retardation product was detected, although less abundant with respect to the fos/ITRE-bound complex. In addition, the complex formed by a probe containing both the TRE-2 and TRE-3 was identical to the complex formed by the TRE-3 alone, thus excluding cooperative AP-1 binding to the two closely spaced sequences in vitro. (C) Competition analysis of the in vitro binding to the ccna2CRE, ccna2TRE-2 and fos/ITRE. Nuclear proteins were incubated with the 5'-end-labeled ccna2CRE and ccna2TRE-3 oligonucleotides. The binding specificity was determined in the presence of 20- or 100-fold excess of unlabeled oligonucleotides (wt or mutated versions: mut-ccna2CRE and mut-ccna2TRE-3), as indicated. The asterisks indicate the positions of three specific CREB/ATF complexes, while the arrow points to the AP-1 complexes. Similar results were obtained with ccna2TRE-1 (data not shown). (D) Antibody supershift analysis of the complexes binding to the ccna2CRE and ccna2TRE-2 and 3 sequences. Nuclear extracts from FRTL-5K-Ras cells were incubated with the indicated antibodies for 3 hours after binding reactions with the indicated labeled oligonucleotides, then analyzed by 8% PAGE. All the in vitro binding and supershift assays were repeated twice, with comparable results. The asterisks indicate the ATF/CREB complexes, the lower arrows the AP-1 complexes, while the upper arrows refer to the supershifted ternary complexes. At least three specific complexes are formed by the ATF probe, and supershifts are detectable with CREB (but not CREB2), ATF1 and ATF2-selective antibodies, with a major effect for the anti-ATF. In addition, specifically retarded complexes can be observed with antibodies recognizing Fra-1, or each of the Jun-family members (the lack of supershift with Sp1-specific antibodies is shown as negative control). Therefore, multiple dimers, containing ATF, Jun and Fos family members, can compete for the binding to the ccna2ATF site, in asynchronously growing FRTL-5K-Ras cells, and Fra-1 is implicated in direct binding to this sequence. (E) CRE and TRE binding activity in Fra-1 knockdown cell clones. Nuclear extracts were prepared from parental (FRTL-5K-Ras, lane 1) control (FRTL-5K-Rasneo, lane 2) and knockdown cells (shFra-1 cl2 and 7, lanes 3 and 4) were incubated with 5'-end-labeled ccna2CRE and ccna2TRE-1 and 3 oligonucleotides, followed by 6% PAGE. The Sp1 DNA-binding activity was determined, as a control between different nuclear extracts. While the interaction with the ccna2CRE site is substantially unaffected, the complexes formed by the ccna2TRE-3 and the fos/ITRE are significantly decreased in the two cell clones, with a stronger effect in the cl2, expressing the lowest level of Fra-1.