Atypical nucleoprotein complexes mediate CRE-dependent regulation of the early promoter of minute virus of mice

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The P4 promoter of the parvovirus minute virus of mice (MVMp) directs transcription of the genes encoding non-structural proteins. We have previously shown that functional upstream CRE elements contribute to both the ras oncogene-dependent activation of promoter P4 and its down-modulation by known activators of cyclic AMP-dependent protein kinase A (PKA). In the present work, the nucleoprotein complexes formed with the P4 CRE elements were characterized with regard to their polypeptide constituents and the nucleotides taking part in the interaction. Atypical interactions, both at the protein–protein and protein–DNA level, were observed, which may be a reflection of the divergence of the parvoviral CREs from the usual consensus. The CRE-mediated regulation of promoter P4 by PKA and Ras is discussed in light of these findings.

Autonomous parvoviruses are small animal viruses that exhibit oncosuppressive properties (Rommelaere & Tattersall, 1990). Although the mechanisms underlying oncosuppression are largely unknown, the preferential lytic action of parvoviruses on neoplastic cells is a likely contributing factor (Rommelaere & Cornelis, 1991). A major cytopathic effect of minute virus of mice (MVMp) and other autonomous parvoviruses was assigned to non-structural protein 1 (NS1) (Vanacker & Rommelaere, 1995). NS1 gene expression is directed by the early promoter (P4) and is induced as a result of host cell transformation, notably with the activated c-Ha-ras oncogene (Spegel et al., 1991).

Pale 4 interacts with various cellular factors, a number of which have been shown to take part in the regulation of transcription initiation (Ahn et al., 1989; Faisst et al., 1994; Fuks et al., 1996; Gu et al., 1995; Deleu et al., 1998). Since parvovirus transcription is induced in ras transformants, the role of at least some of these regulatory factors may depend on the transformation status of host cells. Accordingly, the upstream regulatory region of the MVMp P4 promoter was shown to contain duplicate sites that contribute to the differential activity of this promoter in ras-transformed vs untransformed cells (Perros et al., 1995). These sites are closely related to the consensus sequence denoted CRE (for cAMP-responsive element), hence their designation CREa and CREa’. Mutations in either of these sites significantly reduce P4-driven gene expression in FR3T3 (parental non-transformed) or FREJ4 (ras-transformed) rat fibroblasts, yet the effect is significantly more pronounced in the ras transformant, suggesting that the CREs are mediators of the ras-induced stimulation of P4. This possibility is further supported by the fact that the relative amounts of nucleoprotein complexes formed on the P4 CREs are altered by ras transformation: a fast migrating complex (denoted ‘C’) is preferentially formed with extracts from FREJ4, while two more retarded complexes (denoted ‘A’ and ‘B’) predominate in FR3T3 cell extracts. Although none of these complexes was characterized, competitive mobility shift assays suggest that their constituent proteins belong to the ATF/CREB family of transcription factors. While phosphorylation of some of these factors by cAMP-dependent protein kinase (PKA) typically leads to a CRE-mediated stimulation of transcription (Gonzalez et al., 1989; Rehfuss et al., 1991), a clear reduction of P4 activity was observed when PKA was activated by cAMP analogues in vivo (Perros et al., 1995). This is not without precedent, however, as the DNA polymerase-β gene promoter is also down-modulated under these conditions (Englander et al., 1991). In keeping with previously reported findings (Gallo et al., 1992), we found that PKA activity is reduced as a result of ras transformation, which may contribute to the observed induction of P4 promoter in cells harbouring an activated Ras oncoprotein (Perros et al., 1995).

As a first step towards unravelling the molecular mechanism of this modulation, the DNA sequences interacting with
Fig. 1. Mapping of nucleotides participating in the interaction of the CREa region with proteins. (A) Cu-OP footprinting analysis of the complementary (+) and viral (−) strands in the CREa region. Oligonucleotide a was labelled on either strand, incubated with extracts from FREJ4 cells and fractionated by native PAGE. Retarded complexes [lanes A, B and C marked according to Perros et al. (1995); see also Fig. 2 A] and free probe (lanes F) were digested in the gel with Cu-OP prior to elution and sequencing gel electrophoresis. Maxam and Gilbert ‘G’ sequence ladders were used to locate the protected regions that are indicated by open boxes on the right-hand side of the autoradiograms. (B) DEPC and DMS interference experiments performed with oligonucleotide a. DEPC- or DMS-modified probes labelled on the (+) or (−) strands, respectively, were incubated with nuclear extracts from FREJ4 cells and subjected to native PAGE. Free probe (lanes F) and specific complexes (lanes A–C) were eluted and individually analysed by sequencing gel electrophoresis. Filled (●) and open (○) circle symbols on the left-hand side of the autoradiograms indicate strongly and weakly interfering modifications, respectively. (C) Representation of the results of experiments illustrated in panels (A) and (B), in relation to the sequence of oligonucleotide a. Brackets delimit the regions protected from Cu-OP digestion. Filled (●) and open (○) circle symbols indicate strong and weak interference of the corresponding modified bases, respectively.

proteins in each of the three specific complexes formed with the P4 CREs (designated A, B and C; see Perros et al., 1995) were mapped in situ by copper–o-phenanthroline (Cu-OP) footprinting analysis. Nuclear extracts from FREJ4 cells were prepared as described previously (Perros et al., 1995). For DNA probes encompassing each of the P4 CREs we used the double-stranded oligonucleotides a′ and a (nt 23–53 and nt 58–88 from the MVMp genome, respectively), end-labelled on either strand with Klenow DNA polymerase. Gel retardation assays were performed as described by Perros et al. (1995), except that the wet gel was treated with Cu-OP according to Kuwabara & Sigman (1987). Treated retarded complexes were individually
eluted from the gel in 10 mM Tris (pH 8), 1 mM EDTA, 500 mM NaCl. After ethanol precipitation, samples were loaded onto a 20% polyacrylamide sequencing gel. Similar results were obtained with oligonucleotide probes encompassing CREa and CREa’, and are illustrated for the former in Fig. 1(A). Clear-cut protection was observed over a single DNA stretch centred around the CRE-like sequence on each strand (brackets in Fig. 1C), suggesting that this is the recognition motif for the proteins involved in all three complexes. Interestingly, the protected zone extends beyond the stretch homologous to the CRE consensus, and is wider than protected regions described for CREs from other promoters (Weih et al., 1990). This localization was corroborated by methylation and carboxyethylolation interference experiments. Probes labelled with the Klenow enzyme were modified by dimethyl sulfate (DMS) or diethyl pyrocarbonate (DEPC) according to Sturm et al. (1987), prior to mobility shift assays. Individual retarded complexes were eluted, hydrolysed as described and analysed by 20% polyacrylamide sequencing gel electrophoresis. The results for oligonucleotide ‘a’ are illustrated in Fig. 1(B). As depicted in Fig. 1(C), the formation of all three complexes was suppressed by modification of the same nucleotides, which were all located within the Cu-OP protected regions and were scattered over the CRE motif. Binding interference was observed following modification of bases that lay beyond the DNA motif homologous to the CRE consensus, in agreement with above-mentioned Cu-OP data. Moreover, the interfering nucleotides differ from those described for other systems (Weih et al., 1990; von der Ahe et al., 1990). In conclusion, although chemical nuclease mapping experiments corroborate the involvement of the motifs inferred from competitive gel retardation assays (Perros et al., 1995) in the nucleoprotein complexes, they reveal that parvoviral CREs are different from bona fide CREs in terms of their mode of interaction with nuclear proteins.

The P4 CRE–protein complexes were further characterized by antibody supershift analysis and immunoprecipitation of UV-crosslinked complexes, followed by SDS–PAGE. For the supershift experiments, gel retardation assays were performed as described by Perros et al. (1995), except that nuclear extracts in the gel retardation buffer were supplemented with 1 μg of antibodies prior to addition of the radiolabelled probe. UV-crosslinking experiments were carried out as previously described (Perros et al., 1995), except that the reaction mixture was irradiated in solution and immunoprecipitated with specific antibodies prior to fractionation by SDS–PAGE.

(i) Complex A. In the gel retardation assays (Fig. 2A), complex A was efficiently supershifted by two distinct antibodies that recognize both CREB1 and its ΔCREB isofrom (lanes 3 and 4), while an antibody specific solely for the full-length CREB1 protein had no effect (lane 2). This result is consistent with the UV-crosslinking experiments, showing that a single protein of about the size of (Δ)CREB1, namely 45 kDa (Montminy & Bilezikjian, 1987), was immuno-precipitated in the form of a complex with CREa by the anti-CREB1/ΔCREB antibody X-12 (Fig. 2B, lane 1). These data argue for the ΔCREB homodimer being the protein constituent of complex A, although the participation of full-length CREB1 cannot be ruled out.

(ii) Complex B. In the supershift assays, complex B behaved like complex A regarding sensitivity to antibodies produced by Hurst et al. (1990) against CREB1/ΔCREB (Fig. 2A, lane 4). Yet, anti-CREB1/ΔCREB antibody X-12, which could efficiently supershift complex A, had no detectable effect on the mobility of complex B (Fig. 2A, lane 3). This suggests that complexes A and B both contain (Δ)CREB1, but differ with respect to their other constituent(s). Indeed, an antibody directed against ATF1 could efficiently supershift complex B but not A (Fig. 2A, lane 6). This observation is in keeping with immunoprecipitation data showing that a set of polypeptide species of about 43 kDa, corresponding to the size range of the different phosphorylated forms of ATF1 (Hurst et al., 1990), was immunoprecipitated in association with CREa by the anti-ATF1 antibody (Fig. 2B, lane 3). Altogether, these data suggest that complex B consists of a CREB1–ATF1 heterodimer.

(iii) Complex C. Similarly to complex B, complex C was supershifted by the anti-ATF1 antibody (Fig. 2A, lane 6). This is consistent with our previous in situ UV-crosslinking analysis which revealed the presence of an approximately 43 kDa protein species in complex C (Perros et al., 1995). In addition, this complex was found to contain a polypeptide constituent of about 74 kDa, which is too large to be assigned to ATF1. The mobility of complex C was not affected by other antibodies directed against CREB2 (Fig. 2A, lane 5), ATF2, Jun, Fos or CREM (data not shown). Yet, two different anti-ATF2 antibodies led to the immunoprecipitation of polypeptides crosslinked to P4 CREs (Fig. 2B, lanes 4 and 5) and showing a size pattern similar to that of the non-ATF1 component detected in complex C by in situ crosslinking (Perros et al., 1995). This raises the possibility that complex C may be an ATF1–ATF2 heterodimer. However, this assignment remains questionable since the formation of such heterodimers is thought to be unlikely under physiological conditions (Hai et al., 1989) and could not be confirmed in our system by supershift experiments or attempts at complex reconstitution with baculovirus-produced proteins (data not shown). In any case, results from the supershift and UV-crosslinking studies suggest that the partner of ATF1 in complex C does not belong to the family of CREB–CREM proteins, with which this factor has been shown to interact (Hurst, 1995). Indeed, members of this family have molecular masses in the range 40–50 kDa, whereas the apparent molecular mass of the unknown component of complex C is 74 kDa. Moreover, antibodies against CREB and CREM failed to supershift the complex in gel retardation assays, further substantiating our hypothesis that complex C contains an atypical associate of ATF1. The recently identified parvovirus initiation factor PIF
Fig. 2. Characterization of the polypeptides that interact with P4 CREs. (A) Gel retardation assays were performed with FREJ4 nuclear extracts as previously described (Perros et al., 1995), except that binding reactions were supplemented with specific antibodies prior to electrophoretic separation: pre-immune serum (lane 1); anti-CREB1 240 (lane 2), anti-CREB1/ΔCREB (X-12, lane 3; and from Hurst et al. (1990), lane 4); anti-CREB2 (C-20, lane 5); anti-ATF1 (C41–5.1, lane 6). (A)–(C), specific retarded complexes (arrows). Similar results were obtained with probes harbouring either CREa (illustrated) or CREa (data not shown). (B) BrdU-substituted oligonucleotide α, labelled on the (−) strand, was incubated with FREJ4 cell extracts, UV-irradiated and immunoprecipitated with specific antibodies prior to fractionation by SDS–PAGE. Antibodies were directed against CREB1/ΔCREB (lane 1), CREB2 (lane 2), ATF1 (lane 3) or ATF2 (F2BR1, lane 4 and C-19, lane 5). M, Molecular mass standards (in kDa); arrows and bracket, positions of the protein components of complexes A, B and C, as described in Perros et al. (1995). Polyclonal antibodies against ATF1 were a generous gift of H. Hurst (ICRF, London). Antibodies directed against CREB1 (240), CREB1/ΔCREB (X-12), ATF1 (C41–5.1), CREB2 (C-20) and ATF2 (F2BR1 and C-19) were purchased from Santa Cruz Biotechnology.

recognizes the CRE region of MVM and comprises a p70–85 subunit (Christensen et al., 1997a, b), which has an observed molecular mass similar to that of the unknown constituent of complex C. However, PIF binds coordinately to two copies of the ACGT motif (Christensen et al., 1997b) and can be distinguished in this respect from the protein constituents of complex C regarding DNA binding specificity (see Fig. 1). Moreover, the nonspecific competitor poly(dI–dC), which was included in all our gel retardation assays, was reported to inhibit PIF binding to DNA (Christensen et al., 1997a). Therefore, the partner of ATF1 in complex C is unlikely to be a PIF component.

Analysis of the proteins interacting with P4 CREs led to the partial characterization of the three specific complexes involving these elements. Complex A appears to be a CREB1 homodimer that may consist of ΔCREB, while complex B is an ATF1–CREB1 heterodimer. Similar complexes regulate a variety of cellular and viral promoters, mediating their activation but in none of the reported cases their downmodulation by PKA (Ellis et al., 1995; Rehfuss et al., 1991). Complex C contains ATF1 in addition to (an)other unidentified protein(s). The apparent molecular mass (74 kDa) of the latter protein together with antibody analyses suggest that this polypeptide may not be one of the frequently described partners of ATF1 (Hurst, 1995; Montminy, 1997). This unprecedented association is likely to account for the peculiarity of P4, vs consensus CRE, regarding their interaction with proteins. Activation of PKA results in the preferential occupancy of P4 CREs by complexes A and B, while ras transformation (with concomitant decrease in PKA activity and P4 activation), favours the formation of complex C. The composition of complex C is likely to hold the key to the
unusual CRE-mediated down-modulation of P4 in response to PKA activation. Indeed, it is unlikely that complexes CREB1–CREB1 and CREB1–ATF1 exert a repressive effect on the parvoviral promoter. The PKA-dependent modulation of P4 more probably reflects the relative capacities of complexes A, B and C to activate P4-driven transcription. Should complexes A and B contain the ΔCREB isof orm of CREB1, which lacks a portion of the transregulati ng domain (Ruppert et al., 1992), an overall reduction of P4 functioning may take place under conditions (e.g. PKA activation) allowing these complexes to supplant a more potent complex C. The known reduction of PKA activity following Ras expression (Gallo et al., 1992) may favour the formation of the more potent activator complex C, thus contributing to activation of P4. The proof of this working hypothesis must await a more detailed functional analysis of P4 CREs after site-directed mutagenesis, and the characterization of the unknown cellular protein(s) that interact(s) with these motifs in addition to those identified in the present work.

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