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Upstream CREs Participate in the Basal Activity of Minute Virus of Mice Promoter P4 and in Its Stimulation in *ras*-Transformed Cells

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The activity of the P4 promoter of the parvovirus minute virus of mice (prototype strain MVMp) is stimulated in *ras*-transformed FREJ4 cells compared with the parental FR3T3 line. This activation may participate in the oncolytic effect of parvoviruses, given that P4 drives a transcriptional unit encoding cytotoxic nonstructural proteins. Our results suggest that the higher transcriptional activity of promoter P4 in FREJ4 cells is mediated at least in part by upstream CRE elements. Accordingly, mutations in the CRE motifs impair P4 function more strongly in the FREJ4 derivative than in its FR3T3 parent. Further evidence that these elements contribute to hyperactivity of the P4 promoter in the *ras* transformant is the fact that they form distinct complexes with proteins from FREJ4 and FR3T3 cell extracts. This difference can be abolished by treating the FREJ4 cell extracts with cyclic AMP-dependent protein kinase (PKA) or treating original cultures with a PKA activator. These findings can be linked with two previously reported features of *ras*-transformed cells: the activation of a PKA-inhibited protein kinase cascade and the reduction of PKA-induced protein phosphorylation. In keeping with these facts, P4-directed gene expression can be up- or downmodulated in vivo by exposing cells to known inhibitors or activators of PKA, respectively.

Parvoviruses are small, nonenveloped lytic viruses that can infect a variety of animals from insects to humans (40). These viruses have a single-stranded DNA genome comprising about 5×10^3 nucleotides, bracketed by terminal palindromic regions that are essential for DNA replication (2, 10). Like other parvoviruses, minute virus of mice (prototype strain MVMp) depends extensively on host cell functions under developmental and proliferative control to accomplish its infectious cycle (40). Accordingly, transcription from the viral early and late promoters (called P4 and P38 and located at map units 4 and 38, respectively) is catalyzed by the host RNA polymerase II (9) and regulated by various cellular transcription factors (1, 15, 19, 21, 34). Stable transformation of various cells correlates with enhanced parvoviral transcription (8, 39). In particular, the P4 promoter appears to be activated in *ras*-transformed derivatives (FREJ) of rat FR3T3 fibroblasts (39). Promoter P4 directs transcription of viral messengers encoding nonstructural (NS) proteins that are cytotoxic (7). Hence, characterization of the parvovirus DNA elements involved in *ras* transformation-associated stimulation of P4-directed expression may provide an insight into the mechanism of parvoviral oncolysis (36). Furthermore, besides being of interest per se, the P4 promoter may serve as an indicator of some of the cellular disturbances that link Ras to the establishment of the transformed state.

Unlike nuclear oncoproteins, which can exert their transforming properties by directly regulating gene expression, Ras is associated with the plasma membrane (29) and requires chains of intercommunicating proteins to transfer its signal to the nucleus (30). Over the past few years, considerable progress has been made in the elucidation of one of these signal transduction pathways, involving the c-*raf-1* proto-oncogene and the mitogen-activated protein (MAP) kinase cascade (14). However, Ras appears to activate more than one signal transduction pathway, depending on the biological system and experimental conditions. Some of the biological and molecular changes associated with *ras* transformation are reportedly mediated by an increase in the level of plasma membrane-bound protein kinase C (PKC) (18, 27).

These observations raise the question of which transcription factor(s) converts the signals delivered by the cytoplasmic effectors of Ras (mainly serine/threonine kinases) into changes in gene expression. Members of the Fos and Jun transcription factor family, which participate in the formation of the AP-1 complex, are prime candidates for such mediators. Indeed, these proteins can integrate signals from both PKC and MAP kinases that regulate them at transcriptional as well as post-translational levels (24). In addition, DNA motifs recognized by other proteins may be involved in Ras-induced activation of various promoters (17, 25, 28, 33, 35). Among these, CRE (cyclic AMP [cAMP]-responsive elements) and CRE-like sequences are suspected of mediating some of the transcriptional effects of Ras (17, 25, 26). CREs interact with a number of transcription factors designated by the generic name ATF/CREB. Like Jun and Fos, ATF/CREB factors belong to the group of bZIP proteins, which possess a basic DNA-binding motif (b) and a leucine zipper (ZIP), allowing their polyvalent dimerization. These factors can homo- or heterodimerize on DNA, forming ternary complexes with distinct sequence recognition specificities and *trans* activation properties (22). The

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effects of members of this family on target promoters can be modulated by various signals. In particular, phosphorylation of CREB by cAMP-dependent protein kinase (PKA) appears to enhance its transcriptional function and possibly its dimerization and/or DNA binding (24, 32, 43, 44). Interestingly, reduced PKA activity has been reported in thyroid cell nuclei when oncogenic Ras is expressed. Since Ras does not seem to regulate adenylate cyclase in higher eukaryotes, the oncoprotein is assumed to exert this effect by activating PKC, which in turn inhibits nuclear translocation of the PKA catalytic subunits (18). The cross-talk of these various pathways is worth stressing. In particular, PKA appears to inhibit the Raf-1 kinase cascade (6), implying that PKA can also affect cellular protein phosphorylation in an indirect way.

In this study, we have used the FR3T3 rat fibroblast cell line and its FREJ4 derivative, transformed by activated c-Ha-*ras* (42), to identify *ras* transformation-responsive regulatory elements in the P4 promoter. Our results suggest that the previously noted upstream CRE motifs within P4 (4, 10, 15) are involved in enhancing the rate of transcription initiation from this promoter in FREJ4 cells compared with FR3T3 cells. The factors with which these elements interact are different in normal and transformed cells, a finding in keeping with the reduced capacity of the *ras* transformant to carry out PKA-induced protein phosphorylation. Accordingly, the activity of promoter P4 can be respectively up- or downregulated by known inhibitors or activators of PKA.

MATERIALS AND METHODS

Cells and virus. The established line of Fisher rat fibroblasts FR3T3 and its c-Ha-*ras*-transformed derivative FREJ4 (37, 42) were grown in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% aseptic calf serum and 1% sodium pyruvate. MVMp was propagated in A9 cells and purified by isopycnic CsCl centrifugation as previously described (42). MVMp titers were determined by plaque assays on A9 indicator cells. Exponentially growing cells were infected at a multiplicity of 2 PFU per cell.

Plasmids and transfections. Plasmid pP4*luc* was obtained by cloning the *Sca*I-*Nco*I fragment of plasmid pP4CAT (39), containing P4 and downstream leader sequences, in front of the firefly luciferase gene (1.1-kb *Hin*dIII-*Eco*RI restriction fragment from pLucΔSS [20]) in the pBSK$^-$ vector (Stratagene). CRE-defective derivatives were obtained by site-directed mutagenesis, by substituting the *Bgl*II recognition motif (AGATCT) for a sequence of equivalent length within the CREa (nucleotides [nt] 36 to 41) and/or CREa′ (nt 68 to 81) region. The E box-defective mutant was obtained and characterized by Gu et al. (21). To clone the CRE sequence of P4 upstream from the minimal thymidine kinase (TK) promoter, a P4-derived 13-mer oligonucleotide comprising CREa (ACGT CACACGTCA) or an equivalent CRE mutant (ACGTTGCGCATCA), flanked by *Bam*HI and *Bgl*II 5′ overhangs, was phosphorylated and ligated in the presence of *Bam*HI and *Bgl*II to ensure multimerization in the head-to-tail orientation. Trimers were isolated by 5% polyacrylamide gel electrophoresis (PAGE) and inserted into the *Bam*HI site of plasmid pBLCAT5 (5).

Transfection was achieved with the LipofectAMINE reagent (Gibco BRL), as indicated by the manufacturer. Cultures (5×10^5 cells) were cotransfected with 1 μg of reporter construct and, as a standard, 0.5 μg of plasmid pTKhGH (Nichols Institute), which expresses the human growth hormone gene under control of the herpes simplex virus TK promoter. In transient-expression assays, chloramphenicol acetyltransferase (CAT) and luciferase (Luc) activities were measured at 48 h posttransfection on samples containing equal amounts of total proteins from whole-cell extracts. The results were adjusted as described previously (39), according to the transfection efficiency estimated with the help of the cotransfected plasmid standard. Stable transformants were obtained by cotransfection of 0.1 μg of plasmid pSV2neo (38) or pSV2neo EJ (37) and a 10-fold molar excess of either pP4*luc* or a derived mutant, followed by selection in medium containing G418 (500 μg/ml) for 14 days.

Nuclear protein extraction. Extractions were performed by the method of Dignam et al. (12), with the following modifications. About 5×10^8 FR3T3 or FREJ4 cells were collected in ice-cold phosphate-buffered saline and washed twice. The pellet was resuspended in buffer A (0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 0.5 mM EGTA [ethylene glycol tetraacetic acid], 2 mM EDTA, 15 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5]), and Nonidet P-40 was added to a final concentration of 0.3%. After lysis, the reaction was stopped by adding an equal volume of buffer A and centrifuging through a cushion of solution B (30% sucrose, 60 mM KCl, 15 mM NaCl, 0.5 mM EGTA, 2 mM EDTA, 15 mM HEPES [pH 7.5]). The nuclear pellet was resuspended in 12% glycerol–250 mM NaCl–1.5 mM $MgCl_2$–1 mM EDTA–0.1 mM EGTA–5 mM HEPES (pH 7.9) and incubated for 30 min at 4°C. After centrifugation (150,000 × *g*, 30 min, 4°C), the supernatant was precipitated with ammonium sulfate (0.33 g/ml), unless intended for use in in vitro kinase assays (see below), and centrifuged (85,000 × *g*, 20 min, 4°C). The pellet was resuspended in dialysis buffer (20% glycerol, 60 mM KCl, 0.25 mM EDTA, 0.125 mM EGTA, 20 mM HEPES [pH 7.9]) and dialyzed overnight. Extracts were stored at −80°C. All buffers contained a mixture of protease and phosphatase inhibitors except for extracts treated with PKA or acid phosphatase.

In vitro DNase I and in vivo UV footprinting experiments. For DNase I footprinting, the *Bam*HI-*Nco*I fragment of pMM984, an infectious full-length molecular clone of MVMp (31), was 3′ end labeled to a high specific activity on the plus strand, using the Klenow enzyme, and purified by 5% polyacrylamide native gel electrophoresis. About 10 ng of labeled probe was incubated with 100 μg of nuclear extract for 1 h on ice in 50 μl of binding buffer [6.5 mM spermidine, 0.1 mg of poly(dI-dC) per ml, 1 mM sodium phosphate (pH 7.2), 0.1 mM EDTA in 50% (vol/vol) dialysis buffer]. After incubation, 50 μl of 2 mM $CaCl_2$–10 mM $MgCl_2$ was added, and the probe was digested with various concentrations of DNase I, ranging from 0.2 to 2 μg/ml, for 30 min at room temperature. The reaction mixture was supplemented with 100 μl of 50 mM EDTA and 0.5 mg of proteinase K per ml, incubated for 30 min at 37°C and for 5 min at 90°C, phenol-chloroform extracted, ethanol precipitated, and loaded on a 7 M urea–8% polyacrylamide sequencing gel. In vivo footprinting experiments were performed as previously described (39). Briefly, total DNA from cells harvested 20 h after MVMp infection was isolated by phenol extraction, digested with RNase A, ethanol precipitated, and redissolved in 1 mM EDTA. UV irradiation (254 nm, 1,800 J/m^2) took place either immediately before cell harvest (in vivo) or after DNA purification (in vitro). Primer extension reactions were carried out with *Thermus aquaticus* DNA polymerase in order to detect UV-damaged DNA bases.

Gel retardation assays. Double-stranded oligonucleotides a′ (nt 23 to 53) and a (nt 58 to 88) were 5′ end labeled to a high specific activity by using T4 polynucleotide kinase. About 1 ng of probe (with or without unlabeled competitor at the indicated molar excesses) was incubated for 15 min on ice with 4 μg of nuclear extract in 15 μl of binding buffer (as for DNase I footprinting). In some experiments, extracts were incubated for 5 min with either sweet potato acid phosphatase (Sigma; 1 U per reaction) or the catalytic subunit of PKA (Sigma; 2 U per reaction) and 1 mM ATP prior to addition of the radiolabeled probe. Fivefold-higher amounts of acid phosphatase and PKA were used to treat extracts that had previously been phosphorylated or dephosphorylated, respectively, in vitro. Following incubation, samples were loaded onto a 6% polyacrylamide native gel.

Protein analysis by UV cross-linking. DNA probes were prepared by 3′-end annealing of a complementary 10-mer primer that was extended with Klenow enzyme in the presence of 5 mM 5-bromodeoxuridine triphosphate and 50 μCi (3,000 Ci/mmol) each of α-^{32}P-labeled dATP, dCTP, and dGTP. After purification by 20% native PAGE, probes were incubated with nuclear proteins and processed for gel retardation assays as described above, except that the reactions were scaled up fivefold. After irradiation for 10 min at 302 nm in the wet gel, retarded complexes were excised, individually eluted in 10 mM Tris–1 mM EDTA–500 mM NaCl, ethanol precipitated, and analyzed by SDS-PAGE.

In vitro cAMP-dependent protein kinase assay. To measure nuclear PKA activity, nuclear extract (30 μg of protein, 20 μl) was added to 60 μl of phosphorylation solution (10 mM $MgCl_2$, 10 mM Tris [pH 7.9], 20 μM Kemptide [Sigma], 1 μM [γ-^{32}P]ATP [0.5 μCi/mol]). Reactions were carried out for 15 min at 30°C and stopped by addition of 10 μl of ice-cold 10% trichloroacetic acid. Samples were spotted on P81 paper (Whatman), dried, and washed five times with 75 mM H_3PO_4 to remove unincorporated nucleotides, and radioactivity was quantified with an Instant Imager (Hewlett Packard).

RESULTS

Upstream region of MVMp P4 promoter contains potential CRE regulatory elements. After infection of competent cells with MVMp, single-stranded viral DNA converts to duplex replicative forms believed to serve as transcription templates. Sequence analysis of the P4 promoter revealed a CRE-like motif (ACACGTCA) about 100 bp upstream from the functional TATA box. This motif is framed and designated CREa in Fig. 1A (bottom sequence). Because of its location within the terminal palindrome of the MVMp genome (Fig. 1A, hairpin configuration), CREa is duplicated as CREa′ upon conversion of the parental minus strand into a full-length replicative form (Fig. 1A, extended configuration). CREa′ is a mirror image of CREa but for a minor sequence difference (TGACGTGAT) reflecting the imperfection of the original palindrome.

In UV footprinting experiments with the permissive FREJ4

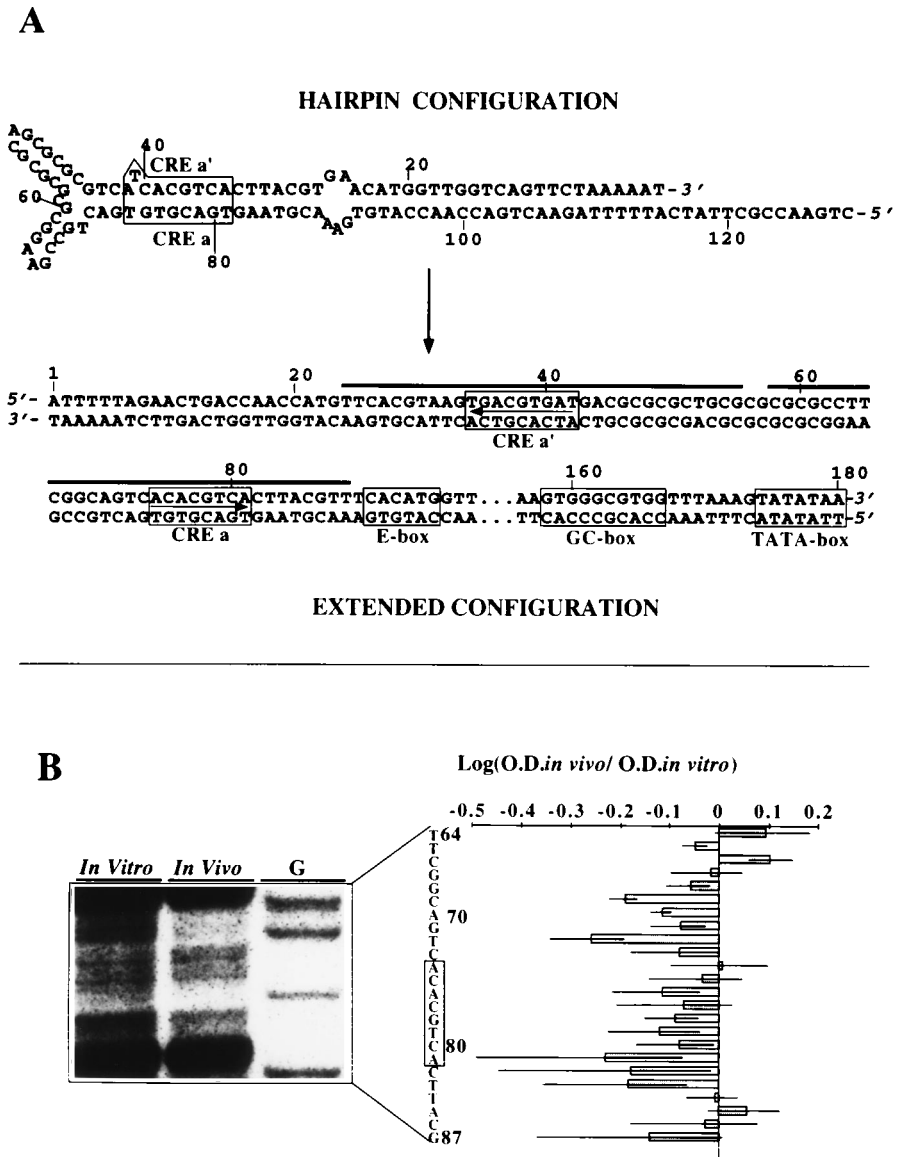

FIG. 1. Location of the CRE motifs in promoter P4. (A) Representation of the hairpin and extended configurations of the P4 promoter. Boxes indicate the CREs and the functional GC and TATA boxes. CREa and CREa′ face each other in the hairpin, and arrows show the polarity of the duplicated elements in the extended configuration. Horizontal bars delimit the oligonucleotides, a and a′, used in subsequent in vitro experiments. (B) In vivo UV footprinting analysis of the CREa region. The CRE motif is framed. In vivo and in vitro indicate that viral DNA from infected FREJ4 cells was irradiated prior to or after extraction, respectively. Lane G, Maxam and Gilbert G ladder. Bars on the right-hand side show the extent of photoprotection (log < 0) or photosensitization (log > 0) of the corresponding nucleotides. O.D., optical density. Average values from at least three experiments are given, with standard deviation bars. Nucleotides are numbered according to Astell et al. (3).

cell line, the CRE sequences of promoter P4 were tested for their ability to associate with proteins in vivo (Fig. 1B). Since a high proportion of input viral genomes (single-stranded DNA of minus polarity) are known not to be processed, we restricted our analysis to plus strands, which constitute part of the double-stranded replicative forms synthesized in infected cells. UV in vivo footprinting is based on the fact that the reactivity of DNA bases exposed to UV light depends on their mobility. DNA stretches that interact with proteins may be sterically stabilized and protected against UV-induced lesions or, on the contrary, distorted by and sensitized to them. Damaged nucleotides are revealed by their ability to arrest primer elongation by *Taq* polymerase. The region from nt 45 to 59, believed to be in a Z-DNA configuration (4), was found to block the *Taq* polymerase, precluding the analysis of nt 1 to 60 and detection of CREa′. Nt 66 to 83, encompassing the CREa consensus, were protected in vivo from UV-induced damage, suggesting their involvement in protein-DNA interactions. It is noteworthy that the interactions were measured over the whole population of viral DNA and might not necessarily concern all genomes. This could explain, at least in part, why the changes in photosensitivity were relatively small yet quite reproducible in up to four independent experiments.

To confirm the recognition of parvoviral CREs by cellular proteins, competitive gel retardation assays were performed with radiolabeled oligonucleotides corresponding to the in vivo protected region and its upstream mirror image. Oligonucleotides a′ and a, encompassing elements CREa′ and CREa, respectively (see Fig. 1A), generated specific complexes upon incubation with nuclear extracts from FREJ4 cells (Fig. 2A).

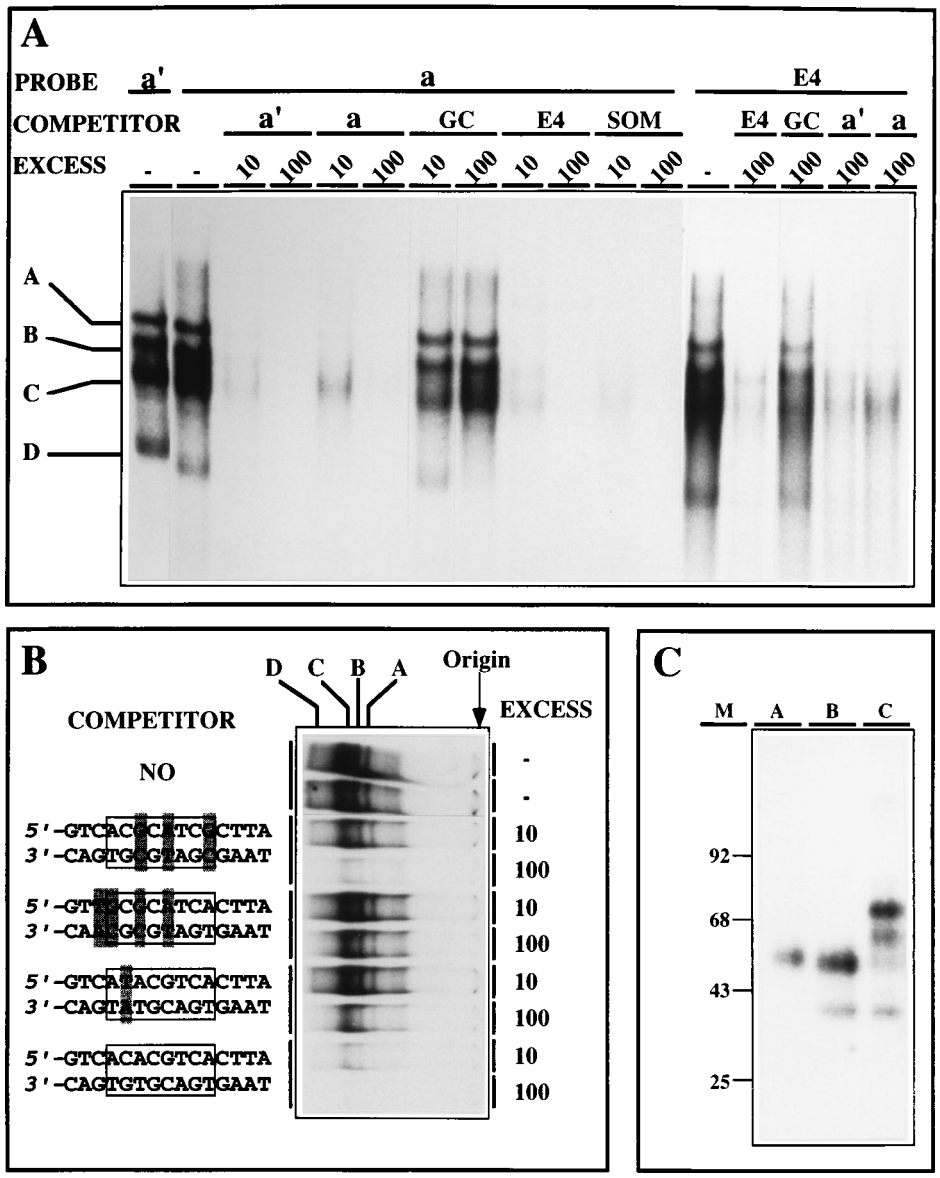

FIG. 2. Analysis of P4 CRE sequences and interacting proteins. (A and B) Competitive gel retardation assays. ^{32}P-labeled oligonucleotide probes were incubated with nuclear extracts from FREJ4 cells in the presence of the indicated excesses of unlabeled competitor and subjected to native PAGE. (A) The following oligonucleotides were used as probe and/or competitor: a and a′ (CRE regions of P4; see Fig. 1), GC (GC box of P4), and E4 and SOM (CREs from the adenovirus E4 and somatostatin promoters, respectively). (B) Oligonucleotide a was used as a probe, while competitors consisted of the same oligonucleotide (bottom two lanes) or derivatives harboring substitutions (shaded areas) in the CREa (open box) region. (C) UV cross-linking analysis. Bromodeoxyuridine-substituted oligonucleotide a, labeled on the positive strand, was incubated with FREJ4 cell extracts in the presence of a 10-fold molar excess of GC competitor. The binding reaction products were separated by native PAGE and irradiated in the gel. Retarded complexes A, B, and C (see panel A) were individually eluted and analyzed by SDS-PAGE. Lanes are labeled accordingly. M, molecular mass standards (in kilodaltons).

Despite the minor sequence difference between the two sites, due to a "mismatch" in the stem of the terminal palindrome, the retardation patterns of the two probes were indistinguishable from both qualitative and quantitative points of view. Furthermore, unlabeled a and a′ oligonucleotides added to the binding reaction mix were equally efficient in inhibiting nucleoprotein complex formation on either probe, suggesting that CREa and its mirror image CREa′ interact with identical cellular factors. As shown in Fig. 2A, three of the major complexes detected (A, B, and C) proved to be specific, since competition with a 100-fold excess of an unrelated sequence of identical length (the GC box region of promoter P4) did not affect their formation. A fourth complex (D) may be of no relevance, as its relative abundance varied from one nuclear extract batch to another and its formation was efficiently prevented by the unrelated competing sequence.

The identification of promoter P4 CREs with protein-binding motifs was confirmed in gel retardation assays with homologous oligonucleotide competitors with base substitutions at positions described as essential to complex formation on the consensus ATF/CREB motif (43). As illustrated in Fig. 2B, such mutations significantly reduced or even abolished the ability of the respective oligonucleotides to compete for protein binding.

TABLE 1. Activation of minimal TK promoter-driven gene expression by the MVMp CREa element[a]

Insert	CAT activity (% acetylation)	Activation factor[b] ± SD
DR4	1.4	1
CREa	66.1	47 ± 14
CREa$^-$	11.2	8 ± 2.5
SRE	>90[c]	>64[c]

[a] Cells were transfected with pBLCAT5 derivatives containing the indicated inserts in triplicate upstream from the minimal TK promoter (see Materials and Methods). CREa$^-$ corresponds to the second mutant from the top in Fig. 2B. CAT activities were measured 48 h after transfection and are expressed as the percentage of chloramphenicol acetylated (average values from four independent experiments).

[b] Activation factors are defined as the ratio of CAT expression from each construct relative to that from DR4-TK-CAT.

[c] CAT activities were beyond the saturation threshold of the enzyme reaction.

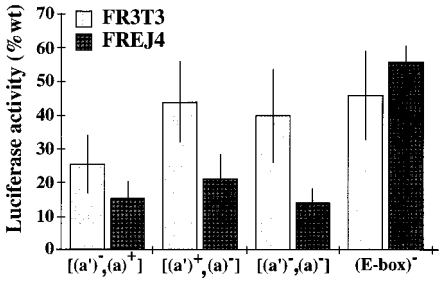

FIG. 3. Effects of mutations in the CREa and CREa′ regions on P4-driven gene expression. Either or both of the CREa [(a)$^+$] and CREa′ [(a′)$^+$] motifs were replaced by an unrelated sequence of the same size [(a)$^-$ or (a′)$^-$, respectively], and the resulting modified P4 promoters were placed upstream from the luciferase-encoding reporter gene. A construct bearing point mutations in the neighboring E box was used as a control (21). Activities were measured 48 h after transfection of FR3T3 and FREJ4 cells with the different constructs and are represented as percentages of the activity of the wild-type promoter in the respective cell line. Average values from at least eight independent experiments are shown with standard deviation bars.

The possibility that MVMp CRE sequences constitute regulatory motifs was further assessed at the functional level. To this end, three head-to-tail copies of either the wild-type or base-substituted CREa oligonucleotide were cloned upstream from a truncated heterologous TK promoter directing the expression of the *cat* reporter gene. To measure the basal activity of the minimal TK promoter, a similar construction was made with the ErbA (thyroid hormone receptor)-responsive DR4 element, which has no regulatory properties in FREJ4 cells in the absence of exogenous ErbA (41). Although it had a weaker transcriptional activator than the consensus serum-responsive element (SRE) used as a standard, the CREa-containing repeat enhanced TK promoter-driven gene expression by about 50-fold compared with expression with unstimulated DR4 (Table 1). This upmodulation was much reduced by mutations in CREa, like protein binding in competition experiments (Fig. 2B, second mutant from the top), suggesting that the P4 CRE sequences are functional under the conditions tested.

CRE elements of promoter P4 interact with ATF/CREB family transcription factors. In an attempt to identify the proteins that recognize the CREs of promoter P4, genuine CRE sequences were used in competitive gel retardation assays. As shown in Fig. 2A, a 10-fold excess of unlabeled oligonucleotide bearing the consensus CRE sequence from the somatostatin (SOM) or the adenovirus E4 promoter competed with CREa for formation of all three specific complexes. The constituent proteins of these complexes may thus belong to the ATF/CREB family of transcription factors. When used as a probe, the E4 (Fig. 2A) and SOM (data not shown) oligonucleotides formed complexes which comigrated with the retarded parvoviral CRE bands. An excess of unlabeled a or a′ oligonucleotide prevented the formation of these complexes, albeit less efficiently than the homologous competitor. Together, these observations suggest that the CREs of promoter P4 are recognized by the same proteins and with the same stoichiometry (a single protein dimer at a time) as bona fide CREs.

In order to determine whether the retarded complexes could be distinguished from each other by their protein constituents, nuclear extracts were incubated with radiolabeled oligonucleotide a or a′ in the presence of an excess of unlabeled heterologous competitor, fractionated by native PAGE prior to UV cross-linking in the gel matrix. Individual retarded complexes were eluted and analyzed by SDS-PAGE. Identical results were obtained with either strand of both probes, confirming that CREa and CREa′ form similar complexes and showing, moreover, that the constituent polypeptides are in contact with both strands of the target motif, in agreement with current models of bZIP protein interactions with DNA. The results obtained with probe a are illustrated in Fig. 2C. Complexes A, B, and C were characterized by distinct patterns of associated proteins. Although some role of protein modification cannot be ruled out, the high-molecular-weight variations detected and the capacity of ATF/CREB factors for heterodimerization argue that the CRE motif is involved in alternative complexes that differ from each other in one or more polypeptide species. The molecular mass diversity of the specifically interacting proteins (36 to 74 kDa) may be related to the known heterogeneity of the ATF/CREB family (22), although the great number of transcription factors within this size range precludes any conclusion regarding the identity of the polypeptides involved.

CREs contribute to the activation of promoter P4 in *ras*-transformed cells. Given the suspected involvement of CRE motifs in the upmodulation of some promoters by Ras (17), we investigated the possible regulatory role of these elements in the previously reported enhancement of the transcriptional activity of the P4 promoter in FREJ4 cells compared with FR3T3 cells (39). Substitution mutations were introduced into the CREa and/or the CREa′ site of a complete P4 promoter directing expression of the luciferase reporter gene. Normal FR3T3 and *ras*-transformed FREJ4 cells were transfected with the resulting constructs, together with a plasmid containing the human growth hormone gene under control of the herpesvirus TK promoter. The latter was used as a standard for evaluating the efficiency of transfection. Normalized luciferase activities of the P4 mutants are presented in Fig. 3 as percentages of the level achieved by the wild-type construct in the same cell line. Substitutions in either or both of the CRE elements suppressed P4 activity in both types of cells, suggesting that these motifs participate in promoter regulation. It is noteworthy that the inhibitory effect on P4 functioning of the substitutions in the CRE sites is stronger in *ras*-transformed than in normal cells. Since luciferase activities were at least 100-fold above the background, this cannot be a mere artifact due to the initially lower strength of the P4 promoter in FR3T3 cells. Furthermore, the neighboring E box located at nt 95 to 105 (21) contributes to a similar extent to P4 activity in FREJ4 and FR3T3 cells. Together, these observations point to specific elements, the CRE sequences, as *cis* mediators of promoter P4 stimulation in *ras*-transformed cells.

To test this view, we examined whether *ras* transformation is

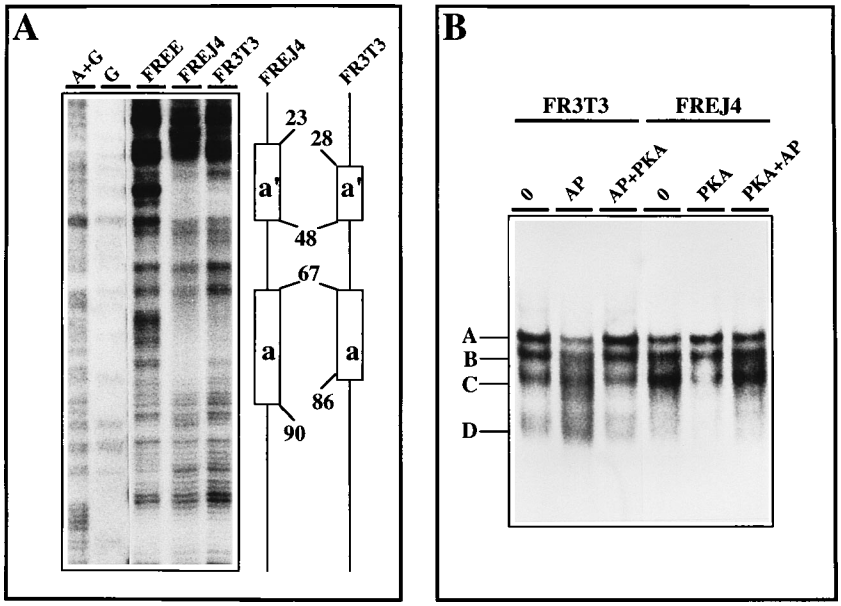

FIG. 4. Comparison of FREJ4 and FR3T3 cells for protein interactions with the CREs. (A) DNase I footprinting analysis of the upstream region of promoter P4. The fragment of MVMp duplex DNA carrying nt 1 to 260 was ^{32}P labeled at the 3′ end of the positive strand and incubated or not with extracts from FREJ4 or FR3T3 cells. Unreacted (lane Free) and reacted (lanes FREJ4 and FR3T3) probes were partially digested with DNase I and loaded onto a sequencing gel. Maxam and Gilbert G and A+G ladders were used to locate the protected zones, which are indicated by boxes on the right. Nucleotide numbers are according to Astell et al. (3). (B) Gel retardation assay of oligonucleotide a probe incubated with extracts from normal (FR3T3) or transformed (FREJ4) cells. Binding reaction mixes were fractionated by native PAGE either directly (lanes 0) or after treatment with acid phosphatase (AP) and/or PKA. Retarded complexes A to D are marked.

associated with changes in the interactions of P4 CRE elements with cellular factors. Nuclear extracts from FR3T3 and FREJ4 cells were first compared for their ability to protect the P4 promoter region encompassing the CREa and CREa′ repeats against DNase I digestion. As shown in Fig. 4A, both extracts protected two main segments, referred to as a and a′ and corresponding to the in vivo photoprotected zone CREa and its mirror image CREa′, respectively. However, the footprint sizes and digestion patterns obtained with the two cell types were found to differ. In particular, the FREJ4 a and a′ footprints were symmetrically extended towards the 5′ and 3′ ends, respectively compared with the FR3T3 footprints. It therefore appeared that *ras* transformation might be associated with a change in the nature of the protein(s) interacting with the CREs of promoter P4. This was confirmed by comparing FREJ4 and FR3T3 extracts for their ability to alter the electrophoretic mobility of oligonucleotides a (Fig. 4B) and a′ (data not shown). Although the mobility shifts induced by FR3T3 proteins were qualitatively indistinguishable from those described above for FREJ4 cells, *ras* transformation was found to correlate with a reproducible difference in the relative abundance of the retarded complexes: while complex C predominated over A and B in the presence of FREJ4 extracts, the contrary was true in the FR3T3 system.

CRE motifs of promoter P4 respond to PKA. It has previously been shown that Ras reduces the intranuclear level of cAMP-dependent protein kinase (PKA), a major regulator of ATF/CREB family transcription factors (18). Accordingly, in vitro treatment of FREJ4 nuclear extracts with the purified catalytic subunit of PKA prior to incubation with the radiolabeled probe was found to stimulate the formation of complexes A and B over that of C, reproducing the retardation pattern obtained with FR3T3 cell extracts (Fig. 4B). The effect was reversed by supplementing the in vitro-phosphorylated extracts with acid phosphatase. Conversely, in vitro treatment of FR3T3 nuclear extracts with acid phosphatase increased the relative abundance of complex C up to the level seen with FREJ4 cell extracts, while subsequent incubation with PKA restored the initial retardation pattern. These results suggest that Ras favors C-type occupancy of the CRE motif by limiting PKA-induced phosphorylation of proteins belonging to this complex and/or by stimulating a PKA-sensitive process promoting its formation. This hypothesis was tested in vivo by treating FREJ4 cells with dibutyryl cAMP (dbcAMP), a membrane-soluble activator of PKA, prior to nuclear extract preparation. In agreement with the in vitro data, the cAMP analog reduced the proportion of C versus A and B complexes to the value observed with FR3T3 cell extracts (Fig. 5A). This change paralleled the capacity of dbcAMP to raise the intranuclear PKA activity of FREJ4 cells to the FR3T3 level, as determined by measuring the ability of the same nuclear extracts to phosphorylate in vitro a synthetic peptide (Kemptide) containing a consensus target sequence for the PKA catalytic subunit (Fig. 5A).

The increased P4 promoter activity in FREJ4 cells compared with FR3T3 cells and its greater dependence on CREs suggest that the Ras-associated shift in the occupancy of these motifs (favoring complex C over A and B) may enhance P4 strength. Since the formation of complex C is antagonized by addition (in vitro) or activation (in vivo) of PKA, we decided to test this hypothesis by determining (i) whether PKA modulators regulate P4 activity and (ii) whether this effect is mediated in *cis* by the CRE motifs. To this end, cells were stably transfected with the luciferase reporter gene under the control of either the wild-type P4 promoter or mutated derivatives carrying substitutions within the CRE sequences. In agreement with our working hypothesis, treatment of stable transfectants with the PKA activator dbcAMP was found to depress P4-driven gene expression (Fig. 5B and C). Mutations in either or both of the CREa and CREa′ sites decreased P4 responsiveness to the

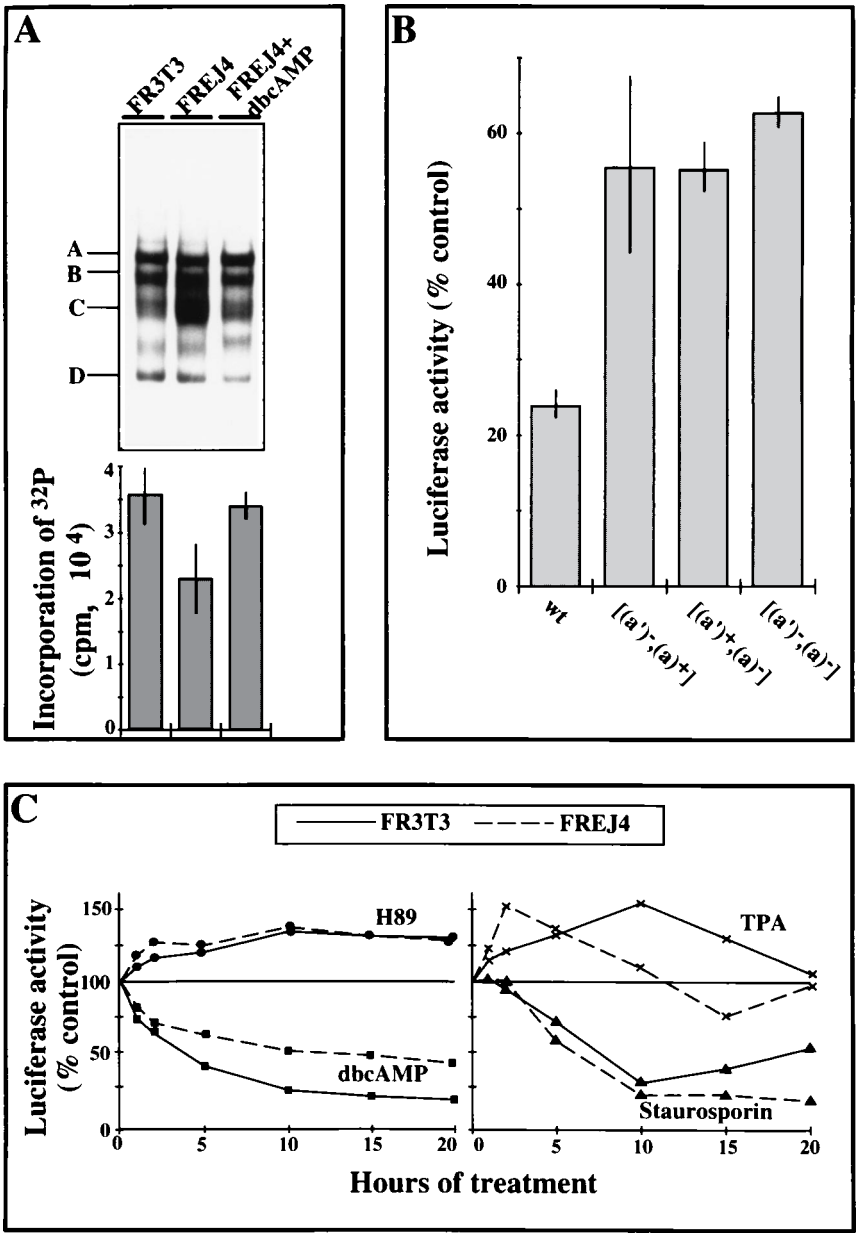

FIG. 5. Influence of modulators of PKA and PKC activities on protein-DNA interactions and functioning of promoter P4. (A) FREJ4 cultures were incubated for 24 h with dbcAMP and compared with untreated FREJ4 and FR3T3 cells for the ability of nuclear extracts to retard the migration of radiolabeled oligonucleotide a. PKA activities of the same extracts were measured by in vitro phosphorylation of a synthetic substrate in the presence of [γ-^{32}P] ATP and are expressed as incorporated radioactivities in the bottom histogram (average values from 10 experiments with standard deviation bars). (B) FR3T3 clones were selected for integration of the luciferase reporter gene driven either by the wild-type P4 promoter (wt) or by substitution mutants altered in the outer [$(a')^-$, $(a)^+$], inner [$(a')^+$, $(a)^-$], or both [$(a')^-$, $(a)^-$] CRE motifs. Luciferase activities were measured after a 12-h incubation in the presence of dbcAMP and are represented as percentages of the values obtained from the same clone in the absence of the drug. (C) Stably transformed clones of FR3T3 (– – – –) and FREJ4 (——) cells expressing the luciferase gene under the control of the wild-type P4 promoter were treated with PKA (left-hand graph) or PKC (right-hand graph) inducers and inhibitors. Luciferase activities were measured at various incubation times and are expressed as percentages of the values obtained for the same but untreated clone. ●, H89 (5 μM); ■, dbcAMP (1 mM); ×, tetradecanoyl phorbol acetate (100 ng/ml); ▲, staurosporin (1 μM).

inhibitory effect of dbcAMP (Fig. 5B), suggesting that these elements cooperate in PKA-sensitive activation of the promoter. It should be mentioned that the double CRE mutant of P4 still exhibited residual suppression in the presence of the cAMP analog. This might be due to additional responsive elements in the promoter or to the indirect effects of the treatment. Furthermore, P4 activity was reduced by forskolin, another PKA activator (data not shown), and stimulated by H-89, a specific PKA inhibitor (Fig. 5C). Interestingly, the inhibitory effect of dbcAMP on P4-driven gene expression was less pronounced in FREJ4 than in FR3T3 cells, suggesting that the lower nuclear PKA activity of the *ras* transformant is not due to a limitation of the cAMP supply. As reported for other systems (18), Ras is more likely to inhibit the nuclear translocation of the PKA catalytic subunit due to the activation of PKC. Accordingly, we observed a reduction of P4 activity after treatment with the specific PKC inhibitors staurosporin (Fig. 5C) and H-7 (data not shown) and stimulation of this activity

by tetradecanoyl phorbol acetate (TPA), a PKC activator (Fig. 5C). The TPA effect, one should note, was transient and faded earlier in FREJ4 than FR3T3 cells, a finding attributable to the known feedback regulation of the enzyme and to PKC activation in *ras*-transformed cells.

It should also be stated that the effects of PKA and the various PK modulators, reported above for the FREJ4 line, could be fully reproduced with an uncloned population of *ras*-transformed cells (data not shown). This multiclonal culture was selected for neomycin resistance after transfection of FR3T3 cells with pSV2neoEJ and exhibited both phenotypic transformation and EJ *ras* expression. Therefore, the distinct responses of the P4 CRE motifs, as seen in FREJ4 cells, do not appear to be specific for this clone but are rather general features of *ras*-transformed FR3T3 cells.

DISCUSSION

Interaction of members of the ATF/CREB family of transcription factors with parvoviral CREs. Ahn et al. (1) have previously reported that the activity of promoter P4 depends to a large extent on proximal GC and TATA boxes but is also modulated by upstream regulatory sequences. A recent report by Gu and collaborators (21) demonstrates the importance of the upstream Y box for the full activity of P4. In the present work, two CRE motifs, CREa and CREa′, were identified in the upstream region of P4. CREa and CREa′ are located within a terminal palindromic sequence and, but for a minor difference corresponding to mismatches in this structure, are inverted complementary copies of each other.

CREa and CREa′ could not be distinguished from consensus CREs in competitive mobility shift assays, suggesting that they may recognize similar proteins, with a stoichiometry of one polypeptide dimer per motif. Indeed, protein binding to CREa or CREa′ could be impaired by mutating these sequences at sites shown to be essential to consensus CRE recognition, as shown by gel retardation assays. Furthermore, preliminary experiments with in vitro-translated factors (11) or specific antibodies (33b) support the involvement of members of the ATF/CREB family—in particular, ATF1, ATF2, and CREB1—in complex formation with the CRE motif of promoter P4. The CREa and CREa′ motifs took part in three specific protein complexes distinguishable by their electrophoretic mobility and their polypeptide constituents. Yet the great diversity, modifications, and cross-reactivity of the proteins binding to CREs have precluded us so far from establishing the definite identity of these constituents.

Contribution of CREs to activation of promoter P4 in *ras*-transformed cells. The activity of the parvoviral P4 promoter is stimulated upon *ras* transformation of the host cell (39). P4 directs the expression of the parvoviral transcription unit encoding the cytotoxic NS proteins (7). Since transformation of many cells exacerbates the parvoviral cytopathic effect (36), knowledge of P4 regulation is interesting not only as regards *ras* transformation but also as a potential clue to one of the mechanisms by which parvoviruses distinguish transformed from normal cells. Various factors seem to mediate the effect of Ras on transcription in different systems (17, 35). Among the possible candidates, CRE-recognizing proteins were found to participate in the Ras-induced activation of some promoters. For instance, dedifferentiation of thyroid cells in the presence of Ras is associated with a reduced affinity of nuclear proteins for CRE motifs, and the stimulatory effect of Ras on the β-polymerase promoter or on the long terminal repeat of intracisternal A particles requires an intact CRE (17, 18, 25). However, the factors that mediate this regulation in *trans* have not yet been characterized.

The data presented in this report suggest that the parvoviral CRE sites are involved in the activity of promoter P4. Substitution of an unrelated sequence for either of these sites significantly reduced P4-driven gene expression in both normal FR3T3 and *ras*-transformed FREJ4 cells. It is worth noting that specific CRE recognition is also required for the activation of the MVMp left-hand origin of replication by the parvovirus NS-1 product (11). The present results indicate that the CRE motifs contribute to P4 activity in the absence of NS-1 and are associated with proteins irrespective of whether cells are infected (in vivo footprints) or not (in vitro footprints). Nevertheless, the putative cross-talk of CRE-binding and NS-1 factors, as inferred from above-mentioned replication initiation data, deserves to be considered within the context of the previously reported modulation of promoter P4 by NS-1 (13, 23).

Variations inherent in the functional assays (efficiency of transfection, modulation of the TK promoter used as a standard, stability of the reporter gene products) preclude any direct comparison of P4 activities between transformed and parental cell lines, yet the effect of the CREa and CREa′ mutations was up to 2.5 times more severe in the Ras transformant, suggesting that the CRE motifs contribute to stimulating P4 in this cell line. Ras-responsive elements are often reported to be bipartite, consisting of an AP-1 or CRE motif and a nearby Ets-binding site (35). It is worth noting in this respect that evidence of the involvement of Ets in P4 promoter regulation has recently been obtained (16a). Furthermore, the CRE motif of P4 is present in two copies, which both appear to contribute to the Ras responsiveness and dbcAMP sensitivity of the parvoviral promoter.

That the CRE sequences contribute to the responsiveness of promoter P4 to Ras in our model system is supported by the fact that *ras* transformation was accompanied by an increased propensity of these sequences to associate in vitro with proteins in the form of complex C rather than A or B. Treatment of FREJ4 cultures with dbcAMP, furthermore, both reversed this effect and caused CRE-dependent inhibition of P4 promoter activity. Together, these observations raise the intriguing possibility that the shift of the CRE motif occupancy towards complex C may be relevant to P4 activation in FREJ4 cells.

PKA sensitivity of P4 promoter activation mediated by CREs. By means of PKA catalytic subunits (in vitro) or PKA activators (in vivo), we were able to reverse two effects of *ras* transformation on promoter P4: the proportion of DNA-protein complexes A and B versus C was increased, and the promoter strength was reduced. Both of these changes were mediated by the CRE motifs. A straightforward interpretation of these observations would be that PKA modifies a factor(s) interacting with P4 CRE elements and is inhibited by Ras. This scenario is supported by the ability of PKA to phosphorylate CREB (32) and by the reduction of PKA activity in the presence of Ras (18). Yet the known cross-talk of PKA with other Ras signalling pathways converging on transcription factors (6) raises the possibility that other effectors besides PKA may control the Ras-induced change in protein association with the CREa/a′ motifs. The significant yet moderate decrease in PKA activity detected in FREJ4 versus FR3T3 cells may thus not be the only clue to the preferential formation of complex C in the *ras* transformant. P4 activity was reduced in normal FR3T3 cells by site-directed mutagenesis of the CRE motifs, arguing for the occupancy of these sites by an activating complex. This is consistent with the fact that FR3T3 cell extracts fulfilled high PKA conditions, and PKA was reported to activate the tran-

scriptional function of CREB (18). It should be stated, however, that normal cell extracts gave rise to two major complexes (A and B) with P4 CRE sequences. Therefore, the overall positive transcriptional effect mediated by CREa/a′ does not rule out that a repressor (such as CREM [16]) takes part in one of the complexes. By favoring the formation of complex C over A and B, Ras may thus stimulate promoter P4 as a result of the substitution of an activating factor for a less efficient or even inhibitory one.

The reduced nuclear PKA activity in FREJ4 cells may be due to stimulation of PKC. Previous reports indicate that PKC is both activated by Ras transformation (29) and responsible for the inhibition of the nuclear translocation of PKA catalytic subunits (18). Furthermore, the inhibitory effect of the cAMP analog dbcAMP on the P4 promoter was stronger in FR3T3 than FREJ4 cells, suggesting that downregulation of PKA in the transformed line is not due to limitation of cAMP. Thus, PKC is a likely intermediate between Ras and PKA on the pathway leading to P4 promoter modulation. In keeping with this view is the ability of activators or inhibitors of PKC to stimulate or repress P4-driven gene expression, respectively. Furthermore, the level of diacylglycerol, a potent activator of PKC, is about two times higher in *ras*-transformed than in parental cells (33a).

In conclusion, we have revealed duplicated CRE DNA motifs that contribute to the basal activity of promoter P4 and to its stimulation in *ras*-transformed cells. These elements are recognized by proteins which belong to the ATF/CREB family of transcription factors and which bind to the CREs in the form of distinct complexes. The relative abundance of these complexes appears to be controlled by protein kinases, in particular PKA, and varies concomitantly with the activation of the promoter in *ras*-transformed cells. Since cells transformed by a variety of agents are sensitized to parvoviral infection (36), it would be interesting to determine whether the regulatory pathway identified in this work is Ras specific or whether it can be triggered by other oncoproteins.

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