JOURNAL OF VIROLOGY, Apr. 1996, p. 2369–2377 Vol. 70, No. 4
0022-538X/96/$04.00+0

Transactivation of a Cellular Promoter by the NS1 Protein of the Parvovirus Minute Virus of Mice through a Putative Hormone-Responsive Element

JEAN-MARC VANACKER,[1]* ROMUALD CORBAU,[1] GUILLAUME ADELMANT,[2] MANOUSSOS PERROS,[1]† VINCENT LAUDET,[2] AND JEAN ROMMELAERE[1]

Tumor Virology Unit, Abt 0610, Deutsches Krebsforschungszentrum, Heidelberg, Germany,[1] and Institut National de la Santé et de la Recherche Médicale U375, Centre National de la Recherche Scientifique URA 1160, Institut Pasteur de Lille, Lille, France[2]

Received 27 September 1995/Accepted 12 January 1996

The promoter of the thyroid hormone receptor α gene (c-*erbA-1*) is activated by the nonstructural protein 1 (NS1) of parvovirus minute virus of mice (prototype strain [MVMp]) in *ras*-transformed FREJ4 cells that are permissive for lytic MVMp replication. This stimulation may be related to the sensitivity of host cells to MVMp, as it does not take place in parental FR3T3 cells, which are resistant to the parvovirus killing effect. The analysis of a series of deletion and point mutants of the c-*erbA-1* promoter led to the identification of an upstream region that is necessary for NS1-driven transactivation. This sequence harbors a putative hormone-responsive element and is sufficient to render a minimal promoter NS1 inducible in FREJ4 but not in FR3T3 cells, and it is involved in distinct interactions with proteins from the respective cell lines. The NS1-responsive element of the c-*erbA-1* promoter bears no homology with sequences that were previously reported to be necessary for NS1 DNA binding and transactivation. Altogether, our data point to a novel, cell-specific mechanism of promoter activation by NS1.

Autonomous parvoviruses are small animal viruses that have oncosuppressive effects in vivo (35). Though the underlying mechanisms of oncosuppression remain largely undeciphered, the lytic action of parvoviruses on proliferating neoplastic cells constitutes a likely contributing factor (11, 35, 44). Indeed, malignantly transformed cells are choice targets for parvovirus cytotoxicity in vitro (37, 45). The parvovirus genome is an essentially single-stranded, linear DNA molecule comprising about 5,000 nucleotides (40). The left-hand part of the genome encodes nonstructural (NS) proteins that are implicated in various steps of parvovirus growth. While NS2 protein isoforms appear to be required for the full achievement of the viral life cycle in a cell-specific manner (27, 30), NS1 is necessary to parvovirus DNA replication and expression and constitutes a major viral effector of cell killing (3, 4). In the in vitro systems tested, the cytotoxic action of NS1 was found to depend on both cell proliferation (32) and transformation (29).

A variety of molecular activities have been assigned to the NS1 protein. Notably, this product modulates the expression of the two parvoviral promoters (located at 4 and 38 map units and hence called P4 and P38, respectively) (10, 17, 34). Though a transcription activation domain has been identified in the C-terminal part of NS1 (26), the precise mechanism by which this protein stimulates the P38 promoter is still an open question. The importance of the GC and TATA boxes of promoter P38 to its activation by NS1 has been recognized (1, 16). It is noteworthy that NS1 can bind to specific DNA sequences that are present in a strikingly high copy number in the parvoviral genome (9), including the P38 promoter (7). In particular, the inducing effect of NS1 can be mediated in *cis* by a P38 element named *tar* (for transactivation response) (16, 26, 34). NS1 has also been shown to interact with Sp1, suggesting that this transcription factor could mediate an indirect binding of NS1 to DNA (19). In addition to the regulation of parvovirus transcription, NS1 down-modulates gene expression driven by various heterologous promoters (13, 25, 34, 44a). The analysis of a set of deletion mutants of the NS1 gene of minute virus of mice (prototype strain [MVMp]) has failed to physically dissociate the transcriptional properties of NS1 from its cytotoxic activity (25). It is thus tempting to speculate that a cause-and-effect relationship exists between both phenomena.

In contrast to its repressive effect on the other nonparvovirus promoters tested, NS1 was found to transactivate the promoter driving the c-*erbA-1* gene (43). This gene encodes the thyroid hormone (T3) receptor (THR) α that belongs to the nuclear hormone receptor superfamily (38, 46). Members of this family act as site-specific transcriptional modulators whose activity depends on the presence of a hormonal ligand (41). Target sequences are therefore referred to as hormone responsive elements (HREs) and are derivatives of the core element A/GGGTCA (15). The physiological relevance of the NS1-induced activation of the c-*erbA-1* gene promoter is presently unclear but deserves to be considered in relation to parvovirus cytotoxicity. Indeed, MVMp infection induces the endogenous c-*erbA-1* gene and leads virus-sensitive rat fibroblasts (c-*Ha-ras*-transformed FREJ4 line) but not resistant cells (parental FR3T3 line) to accumulate THRα1 mRNAs and proteins (43). Moreover, T3 hormone (the natural ligand of THR) up-modulates the sensitivity of FREJ4 cells to the killing effect of MVMp (43). This observation thus argues for an interconnection between T3 signalling pathways and parvovirus cytotoxicity.

Altogether, these data led us to use the c-*erbA-1* promoter as a model to further analyze the mechanism of cellular transcription dysregulation by NS1. We report here that the transacti-

* Corresponding author. Mailing address: Deutsches Krebsforschungszentrum, Abt 0610, Im Neuenheimer Feld 242, P. 101949, D-69009, Heidelberg, Germany. Phone: 49 6221 424961. Fax: 49 6221 424962.

† Present address: Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520-8114.

vation of the c-*erbA-1* promoter by NS1 requires an upstream DNA motif that is unrelated to the sequences previously reported to be involved in (in)direct NS1 binding. This motif constitutes an FTZ-F1-responsive element (FRE), although FTZ-F1 (another member of the nuclear hormone receptor superfamily [12, 17, 42]) does not appear to be the FRE cognate protein in the cell type studied. While the P38 *tar* element has shown no activity on a heterologous promoter (34), the FRE is sufficient to confer NS1 responsiveness to a minimal promoter. Interestingly enough, the FRE mediates promoter transactivation in a cell-specific manner, as apparent from its responsiveness to NS1 in FREJ4 but not in FR3T3 cells under conditions in which the parvovirus P38 promoter is induced in both cell types. In this respect, the present work identifies for the first time a molecular effect of NS1 that discriminates between parvovirus-sensitive transformed cells (FREJ4) and parvovirus-resistant parental cells (FR3T3).

MATERIALS AND METHODS

Cells and transfection. The rat fibroblast line FR3T3 and its *ras*-transformed derivative FREJ4 (37) were grown in Dulbecco modified Eagle medium supplemented with 10% newborn calf serum. MVMp was produced and titers were determined according to standard procedures (45). Cell infection with MVMp was conducted as previously described (45).

Cells were transfected according to the calcium phosphate precipitation method (5), using denatured salmon sperm DNA as a carrier (up to 6 μg of total DNA per sample), and were collected 48 h after transfection. Luciferase (Luc) and chloramphenicol acetyl transferase (CAT) activities were determined and normalized to the total amount of proteins present in the cell lysates (34).

Plasmid construction. Plasmid perbALuc contains the c-*erbA-1* gene promoter fragment described by Laudet et al. (23), cloned as an *Sst*I-*Hin*dIII insert upstream from the Luc reporter gene of plasmid pGL2basic (Promega). For the production of deletion mutants, the 750-bp *Alu*I fragment encompassing the c-*erbA-1* promoter was cloned in the *Hin*cII site of pUC19. The promoter sequence was recovered by *Kpn*I and *Bam*HI digestion and treated with exonuclease III (Stratagene) for various time periods at 30°C. Reaction products were blunt ended with mung bean nuclease and cloned in plasmid pUC19. The inserts were sequenced and cloned as *Sst*I-*Hin*dIII fragments into the corresponding sites of plasmid pGL2basic.

HRE-defective derivatives of the c-*erbA-1* promoter were obtained by site-directed mutagenesis. A 70-mer synthetic oligonucleotide corresponding to positions −477 to −407 (relative to the S1 major transcription start site [23]) was cloned in pAlter (Promega), and mutations were introduced according to the protocol recommended by the supplier. Wild-type and mutated sequences were recovered as *Bam*HI (filled in with Klenow polymerase)-*Kpn*I fragments and reinserted in *Sst*I (filled in with Klenow polymerase)-*Kpn*I sites in front of the deleted promoter Δ407. The resulting constructs were verified by sequencing.

To clone HRE sequences upstream from the minimal *tk* promoter, synthetic oligonucleotides C, M4, and M5 (sequences shown in Fig. 3A) flanked by *Bam*HI and *Bgl*II sites were phosphorylated and ligated. Pentamers were isolated on a 5% polyacrylamide gel and inserted into the *Bam*HI site of plasmid pBLCat5 (2).

Plasmid pRSVNS (expressing both NS1 and NS2 proteins under the control of the long terminal repeat promoter of Rous sarcoma virus) has been described elsewhere (39). To generate plasmid pRSVΔNS, pRSVNS was digested with *Bst*EII, filled in with Klenow polymerase, and religated, creating a frameshift mutation into the NS1 coding sequence. In this construct, the Rous sarcoma virus long terminal repeat directs the same NS1 mutant as in the pIPLK plasmid used in reference 43. Plasmid pULB3562 carries the P38 promoter of MVMp upstream of the CAT reporter gene (8).

Electrophoretic mobility shift essays (EMSAs). For preparation of whole-cell extracts, 1 volume of cell pellet was mixed with 1.5 volumes of buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 25% glycerol, 0.4 M NaCl, and 1 mM EDTA and allowed to lyse on ice for 20 min. After centrifugation, the total amount of protein in the supernatant was measured. Probes were labelled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Binding reactions were performed as described elsewhere (43), using 6 μg of protein extract. For supershift experiments, cell extracts were incubated with the corresponding antiserum for 30 min at room temperature prior to probe addition. Antisera xFF1rA[DEF] (directed against the *Xenopus* version of FTZ-F1) and erbA23 (directed against v-ErbA) have been described elsewhere (references 12 and 14, respectively).

UV cross-linking assays. The DNA probe was prepared by 3′-end annealing of a complementary 10-mer primer that was extended with Klenow polymerase in the presence of 5 mM 5-bromo-dUTP and 50 mCi each of [α-^{32}P]dATP, [α-^{32}P]dCTP, and [α-^{32}P]dGTP (33). The probe was incubated with 30 μg of proteins and processed for gel retardation. The gel was irradiated for 12 min at 312 nm and autoradiographed. Gel slices corresponding to retarded complexes were excised and analyzed by sodium dodecyl sulfate–8% polyacrylamide gel electrophoresis (SDS-PAGE).

RESULTS

A putative FRE in the c-*erbA-1* gene promoter is necessary for NS1-induced stimulation. We have previously shown that the promoter driving the c-*erbA-1* gene is activated in the presence of NS1 in FREJ4 cells (43). This led us to search for an NS1-responsive element(s) that mediates promoter induction in *cis*. To this end, the c-*erbA-1* promoter, cloned upstream from the Luc reporter gene (plasmid perbALuc), was serially deleted by treatment with exonuclease III. After sequencing, deletion mutants were cotransfected in FREJ4 cells with various amounts of NS1-encoding plasmid. As expected, the "full-length" promoter (wild type) was up-modulated by NS1 in a dose-dependent manner (Fig. 1). In contrast, promoter Δ407, harboring a 5′-terminal deletion of 61 bp at positions −468 to −407 relative to the S1 transcription start site (23), could no longer be transactivated by NS1. Larger deletion mutants were similarly resistant to induction by the viral product. These results indicate that the 5′ part of the c-*erbA-1* regulatory region is necessary for NS1 to stimulate the activity of the promoter.

A computer-assisted analysis of the 61-bp fragment deleted in construct Δ407 revealed the presence of a single known consensus sequence (TCAAGGTCA) between positions −443 and −435 relative to the S1 transcription start site (23). This consensus has been reported to interact with transcription factors belonging to the FTZ-F1/SF1 subgroup of nuclear hormone receptors (12, 18, 42, 47; reviewed in reference 21), hence its designation as FRE. We decided to determine whether the FRE is required for NS1 stimulation of the c-*erbA-1* promoter. To this end, a 70-bp DNA fragment corresponding to the 5′ end of the c-*erbA-1* promoter (positions −468 to −407) plus 9 nucleotides from the cloning site was subcloned, modified by site-directed mutagenesis, and inserted in front of the Δ407 deletion mutant, giving rise to the reconstituted promoters recM3 to recM5 depicted in Fig. 2A. Besides these mutants, a reconstituted wild-type promoter (recwt) was produced by supplementing Δ407 with the original 70-bp fragment. recwt served as a control, because a few extra nucleotides are present in the reconstituted promoters relative to the original sequence because of the cloning strategy used (see Materials and Methods). After sequencing, the reconstituted constructs were introduced in FREJ4 cells by cotransfection with various amounts of NS1-expressing plasmid and compared for their sensitivity to the inducing effect of NS1 (Fig. 2B). As in the case of the genuine c-*erbA-1* promoter, recwt-driven Luc expression was up-regulated by NS1. This stimulation was suppressed by the M4 and M5 mutations that are known to inactivate the FRE (12, 47). In contrast, the M3 mutation is located outside the FRE and did not prevent induction of the corresponding promoter (recM3) by NS1. Altogether, these results indicate that an intact FRE is necessary for the c-*erbA-1* promoter to be transactivated by NS1.

The putative FRE forms specific complexes with cellular proteins and is sufficient for NS1-induced transactivation. The putative FRE was tested for its ability to form specific complexes with protein extracts from FREJ4 cells. A synthetic 30-bp oligonucleotide encompassing the FRE site in its c-*erbA-1* promoter context, designated C (Fig. 3A), was used as a probe in EMSAs. In order to check that the complexes formed are specific, related and unrelated oligonucleotides (see sequences in Fig. 3A) were added in excess as unlabelled competitors. As illustrated in Fig. 3B, the association of the FRE-containing oligonucleotide with proteins led to the for-

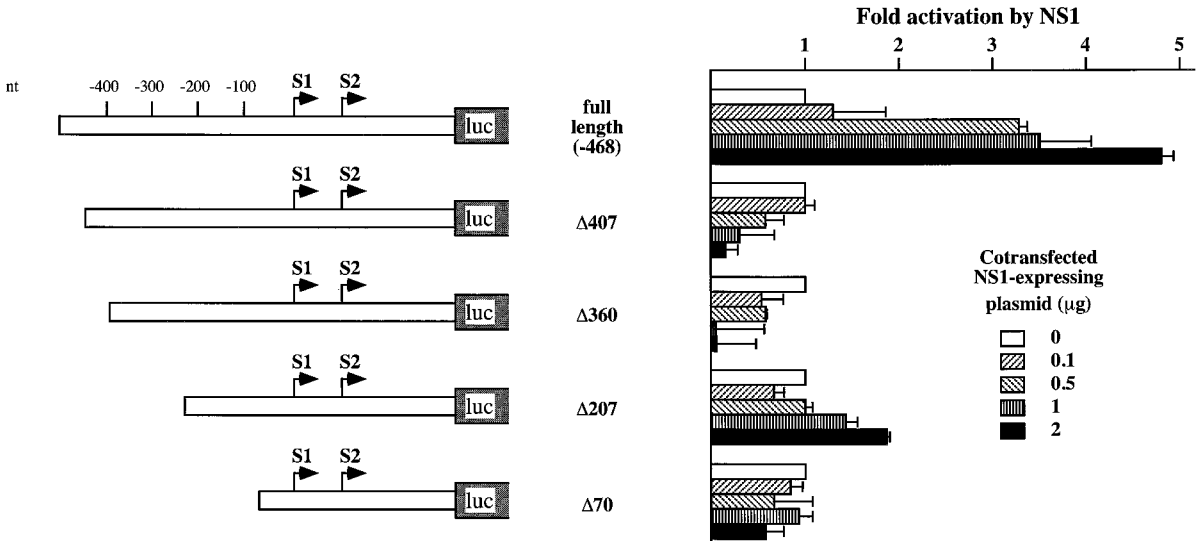

FIG. 1. Effect of NS1 protein on the activity of full-size and deleted c-*erbA-1* promoters. The full-length c-*erbA-1* promoter was sequentially deleted from the 5′ end and tested for its ability to drive expression of the Luc reporter gene in FREJ4 cells cotransfected with the indicated amounts of NS1-encoding plasmid. Positions of two major transcription start sites (S1 and S2 [23]) are indicated by arrows. Nucleotides (nt) are numbered backwards, starting from S1, in the full-length promoter. Deletion mutants (Δ) are named after the positions of their 5′-terminal nucleotides. Luc activities are expressed relative to that in NS1-free cells. Average values from three independent experiments are given with standard deviation bars.

mation of two major retarded complexes referred to as C1 and C2. The specificity of these complexes was ascertained by showing that they were suppressed by the homologous (C) but not an unrelated (G) competitor. The involvement of FRE in probe C interactions with proteins was tested with unlabelled competitors mutated in or around this element. Mutations M4, M5, M6, and M7, which all reside in the FRE, suppressed the ability of corresponding oligonucleotides to compete with the probe for complex C1 and C2 formation. In contrast, oligonucleotides mutated outside this 9-bp element were as efficient as the wild-type competitor in the suppression of C1 and C2 complexes. Therefore, the FRE proved to be important for the specific in vitro binding of a cellular protein(s) to oligonucleotides encompassing the NS1-responsive element of the c-*erbA-1* promoter. Taken with the expression data described in the previous section, these results argue for a functional role of the FRE in c-*erbA-1* promoter regulation by nuclear factors.

On the basis of these observations, some of the oligonucleotides tested in EMSAs were used to determine whether the FRE is sufficient to make a promoter NS1 inducible. Oligonucleotides encompassing the FRE were cloned as pentamers in front of the herpes simplex virus *tk* minimal promoter contained in plasmid pBL5Cat (2). After sequencing, the resulting constructs were tested by transient expression assays in the presence of various amounts of the NS1-encoding plasmid pRSVNS depicted in Fig. 4A. As shown in Fig. 4B, reporter gene expression from p5Ctk (harboring wild-type FRE) was enhanced in a dose-dependent manner by cotransfection of plasmid pRSVNS in FREJ4 cells. We have previously shown that a frameshift mutation in the NS1-coding sequence, leading to a truncation of the C-terminal part of the protein, impairs its capacity for stimulating c-*erbA-1* promoter-driven transcription (43). This NS1 mutant was placed under the control of the Rous sarcoma virus long terminal repeat, generating plasmid pRSVΔNS (Fig. 4A). Cotransfection of plasmid pRSVΔNS had no inducing effect (Fig. 4B), allowing p5Ctk transactivation to be assigned to NS1. Furthermore, expression from plasmids p5M4tk and p5M5tk (harboring the FRE mutations tested by EMSAs; see sequences in Fig. 3A) was not regulated by NS1. Altogether, these results indicate that the FRE element is sufficient to sensitize a promoter to NS1-induced transactivation.

FRE-mediated promoter transactivation by NS1 is cell specific. MVMp infection was previously shown to induce c-*erbA-1* mRNA to accumulate in *ras*-transformed FREJ4 fibroblasts but not in the nontransformed FR3T3 parental cells. This specificity is interesting since it correlates with the much greater sensitivity of the transformant to the killing effect of MVMp. It remained to be established whether this cell dependence took place at the level of transcription, in particular FRE-mediated promoter transactivation by NS1. This was first tested by comparing FREJ4 and FR3T3 cells for the effect of NS1 on expression of the p5Ctk reporter construct. As shown in Fig. 4B, NS1 failed to stimulate the 5Ctk promoter in FR3T3 cells under conditions in which the reporter was transactivated through the upstream FRE in FREJ4 cells.

Given that the FRE is involved in NS1 induction of the c-*erbA-1* promoter, it was investigated whether a similar cell specificity occurred when this promoter was the target for the viral product. To this end, the perbALuc reporter construct was introduced in FR3T3 and FREJ4 cells together with increasing amounts of NS1-encoding plasmid. As shown in Fig. 5, an NS1 dose-dependent induction of the reporter was observed in FREJ4 cells but not in FR3T3 cultures, which exhibited a repression instead. This difference was not due to a lack of NS1 production or activity in FR3T3 cells. Indeed, the viral protein was able to transactivate the MVMp P38 promoter at similar levels in both cell types, although the dose responses may not be identical (Fig. 5). It therefore appears that the extent of induction achieved by NS1 depends on both the host cell and target promoter considered. In the present rat fibroblast system, the isolation of *ras* transformants was required to reveal the FRE-mediated inducing activity of NS1.

These observations raise the question of whether the FRE is involved in distinct associations with proteins in FREJ4 versus FR3T3 cells. This was tested in vitro by EMSAs using oligonucleotide C, previously shown to be sufficient for NS1 transactivation in FREJ4 cells. As illustrated in Fig. 6A, the specific

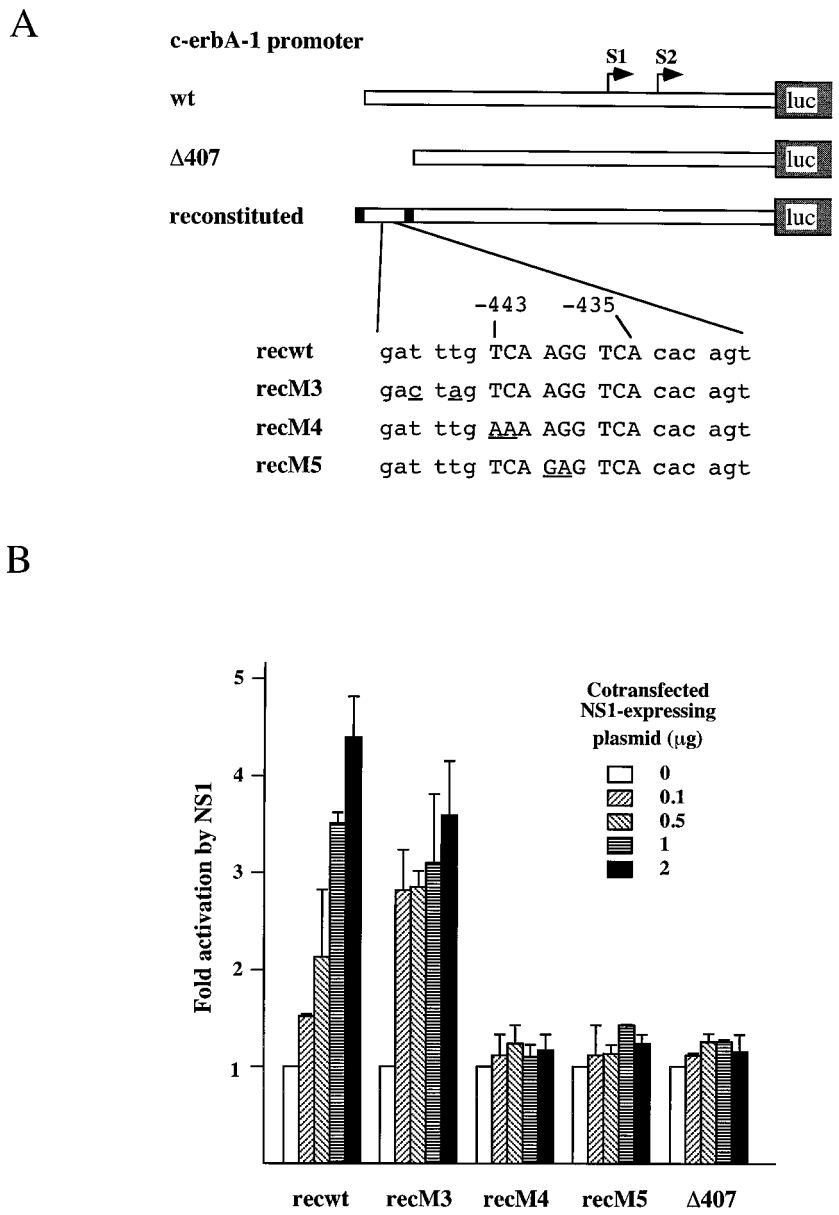

FIG. 2. Effect of point mutations in the c-*erbA-1* promoter on its responsiveness to NS1. (A) Reporter plasmids. The wild-type (wt) c-*erbA-1* promoter is shown with the S1 and S2 transcription start sites (arrows). Reconstituted constructs were obtained by inserting the upstream region that is missing from Δ407 in front of this promoter. Additional plasmid-originating nucleotides inserted as a consequence of the cloning strategy are indicated as black boxes. The upstream sequence was either wild type or mutated, giving rise to reconstituted promoters recwt and recM, respectively. The putative FRE is shown in capital letters and positioned relative to the S1 start site. Mutated nucleotides are underlined. (B) Inducing effects of NS1. FREJ4 cells were cotransfected with reconstituted reporter constructs and the indicated amounts of NS-encoding plasmid. Luc activities are given relative to that in NS1-free cells. Average values from three independent experiments are given with standard deviation bars.

complexes C1 and C2 were formed when probe C was incubated with FREJ4 cell extracts. In contrast, only C1 could be detected in the presence of proteins originating from FR3T3 cells. Although the relative abundance of C2 varied from one FREJ4 extract to the other (pointing to its dependence on the physiological state of the cells), this complex consistently distinguished the transformed line from its nontransformed parent. Cell infection with MVMp prior to protein extraction did not cause detectable changes in the retardation patterns obtained with either cell type.

The nature of the protein constituents of complexes C1 and C2 is presently unknown. Since the FRE interacts with members of the FTZ-F1/SF1 protein subfamily in other systems (12, 42, 47), the presence of this factor in complexes C1 and C2 was investigated. Prior to incubation with probe C, cell extracts were supplemented with either of the following antisera: xFF1rA[DEF] (directed against the *Xenopus* equivalent of FTZ-F1 [12]) and erbA23 (directed against v-ErbA [14] and used as a negative control). As illustrated in Fig. 6B, the retardation pattern obtained with FREJ4 cell extract was not modified by any of the antisera tested. On the contrary, the major complex formed between probe C and proteins from F9 cells (known to express the mouse version of FTZ-F1 [42]) was supershifted by xFF1A[DEF] but not by the control antiserum.

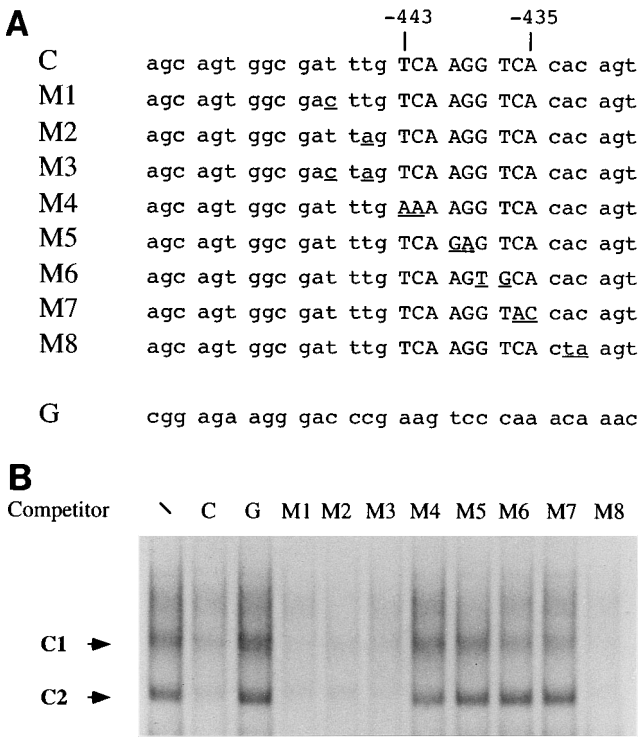

FIG. 3. Association of cellular proteins with the FRE region of the c-*erbA-1* promoter. (A) Oligonucleotides used as probes and/or competitors. Oligonucleotides C to M8 are aligned, encompass the FRE motif (capital letters; positions numbered from the S1 transcription start site), and correspond to wild-type (C) or mutated (M1 to M8, with changes underlined) c-*erbA-1* promoter sequences. Oligonucleotide G maps downstream from the c-*erbA-1* promoter and was used as an unrelated negative control. (B) Competitive gel retardation assay. ^{32}P-labelled probe C was incubated with proteins from a whole FREJ4 cell extract, in the presence or absence (−) of a 10-fold molar excess of the indicated unlabelled competitors. DNA-protein complexes were identified by EMSA. An autoradiogram of the retarded complexes is shown. Specific complexes C1 and C2 are indicated by arrows.

Therefore, the FRE of the c-*erbA-1* promoter constitutes a genuine FTZ-F1 binding site, although its cognate proteins appear to differ from this factor in the rat fibroblast system studied in the present work.

As a preliminary step toward the understanding of the cell dependence of protein interactions with the c-*erbA-1* FRE, the FREJ4-specific C2 complex was compared with C1 with respect to the apparent molecular masses of involved polypeptides. To this end, we performed UV cross-linking experiments consisting of scaled-up preparative EMSAs, in-gel irradiation, excision of individual retarded complexes, and SDS-PAGE analysis. As shown in Fig. 6C, complexes C1 and C2 were each characterized by a distinct polypeptide of around 65 and 80 kDa, respectively. Although some role of protein modification cannot be formally ruled out, the large variation observed in the apparent molecular masses argues that FRE interacts with different polypeptides, giving rise to the alternative C1 and C2 complexes. In the pair of cell lines studied, binding of the 80-kDa species appeared to correlate with promoter sensitivity to NS1 induction.

DISCUSSION

The NS1 protein is known to be able to *trans* regulate parvovirus as well as various heterologous promoters (10, 13, 16, 25, 34). The present work led to the identification of a novel NS1 regulatory pathway which can be distinguished from previously described ones by the following features: (i) the NS1-responsive *cis*-acting promoter determinant constitutes a nuclear receptor-binding site (designated FRE), (ii) this motif can mediate an inducing effect of NS1 when placed out of its promoter context, (iii) a cellular promoter (the c-*erbA-1* regulatory region) can be the target for an activating effect of NS1, and (iv) the FRE-mediated inducing action of NS1 exhibits a striking cell specificity.

The c-*erbA-1* promoter contains an NS1-responsive FRE element. Results presented in this article show that the TCAAGGTCA sequence from the c-*erbA-1* upstream regulatory region is necessary for NS1 to stimulate gene expression driven by the c-*erbA-1* promoter. A/GGGTCA represents a consensus core element (designated HRE) for the binding of members of the nuclear receptor superfamily (15, 41). These proteins act as site-specific transcriptional modulators whose binding and/or activity depends on the presence of a hormonal ligand. Exceptions to this rule are the so-called orphan receptors that still await the identification of a natural ligand and may act as constitutive modulators (22; reviewed in reference 21). Nuclear receptors exhibit a complex pattern of DNA interaction. Some receptors are able to homo- or heterodimerize in the presence of direct, inverted, or everted HRE repeats (15). On the other hand, motifs containing a single HRE define monomeric protein binding sites (46). In this case, the sequence upstream from HRE determines the specificity of nuclear receptor recognition. In particular, the TCAAGGTCA sequence represents the consensus element for DNA interaction with the FTZ-F1/SF1 subgroup of nuclear receptors (12, 18, 42, 47; reviewed in reference 21) and is therefore referred to as FRE. It is worth noting that the FRE has no homology with the DNA sequences to which NS1 was recently reported to bind (7, 9), including the *tar* element which participates in the transactivation of the parvovirus P38 promoter by NS1 (16, 34). Furthermore, the FRE is unrelated to the GC box that also contributes to P38 activity and interacts with an NS1-binding transcription factor (16, 19).

In agreement with its designation, the c-*erbA-1* promoter FRE proved able to mediate the DNA binding (Fig. 6B) and promoter inducing (data not shown) activities of the FTZ-F1 protein. It should be stated, however, that FTZ-F1 does not appear to be the FRE cognate protein in the rat fibroblast system analyzed in this work. Indeed, no change in the FRE retardation pattern was observed in EMSAs, when FREJ4 cell extracts were preincubated with antiserum directed against FTZ-F1 and able to supershift the FRE complex formed with proteins from F9 cells (known to express FTZ-F1 [42]). Another nuclear receptor (germ cell nuclear factor) has recently been reported to bind to the FRE (6). This factor is also unlikely to be the protein partner of FRE in FREJ4 cells, since UV cross-linking experiments performed with FREJ4 cell extracts led to the detection of two FRE-associated polypeptide species with apparent molecular masses (65 and 80 kDa) different from that of germ cell nuclear factor (56 kDa). Therefore, the FRE-binding proteins of FREJ4 cells remain to be identified and are presently designated X65 and X80 after their respective molecular masses. From their capacity for specific recognition of the FRE motif, X65 and X80 can in all likelihood be assigned to the superfamily of nuclear hormone receptors. Both proteins were indeed present in the nucleus, as apparent from the similar FRE retardation patterns obtained with whole-cell (Fig. 3 and 6) and nuclear (data not shown) extracts. X65 and X80 are each characteristic of a distinct retarded complex, suggesting that they bind to FRE in an independent and mutually exclusive way. This view is consistent with the fact that the consensus core element AGGTCA is

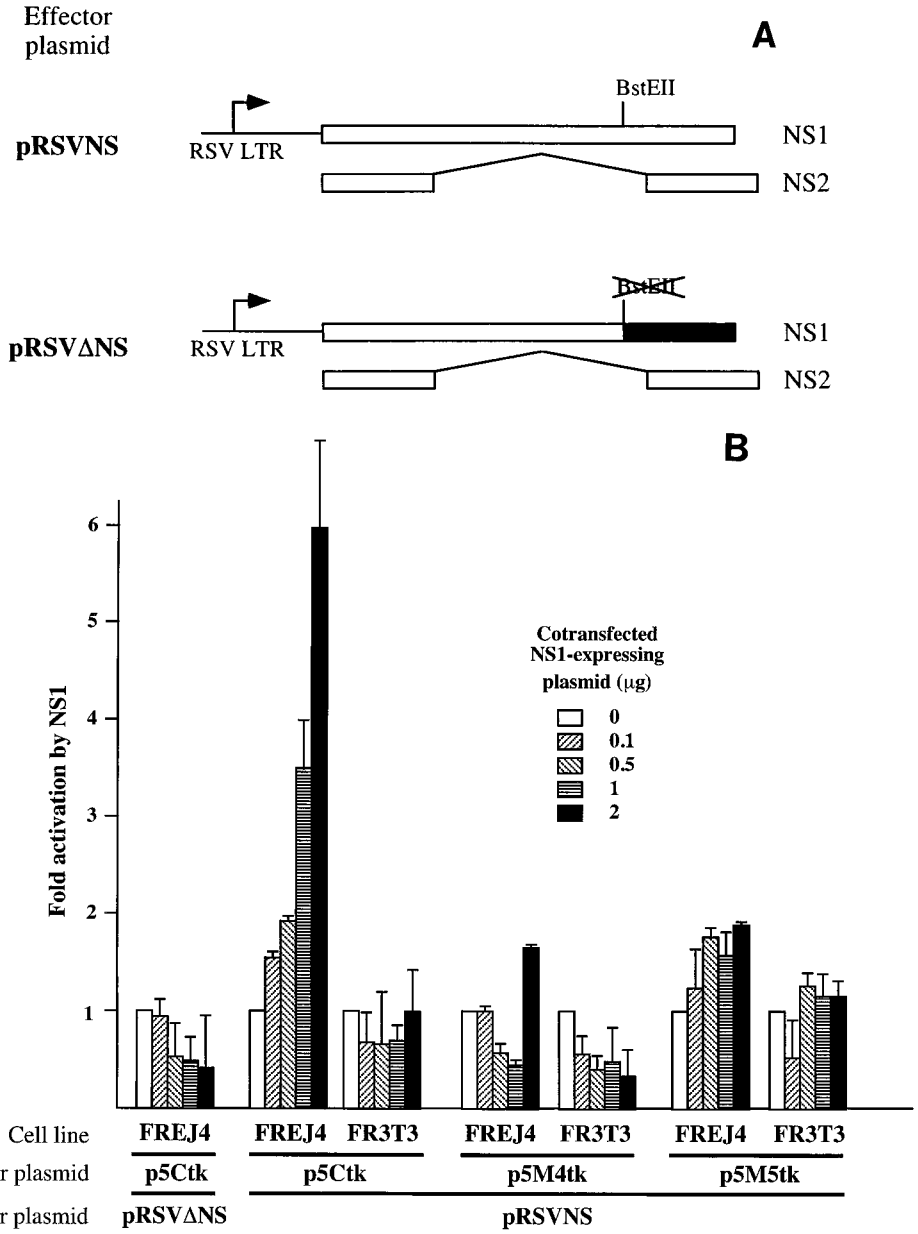

FIG. 4. Effect of NS1 on a minimal promoter supplemented with FRE. (A) Effector plasmids. pRSVNS encodes wild-type NS1 and NS2 species (boxes) under the control of Rous sarcoma virus long terminal repeat (RSV LTR) (arrow). The *Bst*EII restriction site present in this construct is disrupted in pRSVΔNS, resulting in an NS1-specific frameshift mutation. The truncated part of the protein is indicated as a black box. (B) Inducing effect of NS1. FREJ4 and FR3T3 cells were cotransfected with reporter constructs harboring the CAT gene under the control of a minimal *tk* promoter supplemented with five copies of oligonucleotide C (p5Ctk), M4 (p5M4tk), or M5 (p5M5tk), together with the indicated amounts of pRSVNS or pRSVΔNS plasmid. CAT activities were determined 48 h after transfection and are plotted relative to the values measured in NS1-free cells. Average values from three independent experiments are given with standard deviation bars.

present as a single copy in the FRE, while this sequence has to be repeated to allow dimeric protein interaction.

FRE confers NS1 responsiveness to a heterologous promoter. Sensitivity to the inducing effect of NS1 could be transferred to a heterologous promoter (here the minimal *tk* promoter) by supplementing it with the FRE. This is in contrast to the *tar* element of the parvovirus P38 promoter, which constitutes a *cis* determinant for NS1 transactivation but could not be shown to function out of its original promoter context (34). The level of stimulation achieved by NS1 through the FRE is low (four- to sixfold), whether this motif is present as a single copy in the c-*erbA-1* promoter or is cloned as a pentamer in front of the minimal *tk* regulatory region. There is precedent for the fact that a multimeric insertion does not necessarily result in a cooperative activation. In particular, tandem repeats of FRE were reported to mediate pleiotropic effects ranging from inhibition (42) to synergistic stimulation (12, 28) through low-level activation (18), depending on the regulatory protein and host cell involved. A rate-limiting step may consist of the recruitment of a factor(s) necessary for transactivation. It is also worth noting that the minimal *tk* promoter is inhibited in cells induced to express NS1 (data not shown), which could mask in part the FRE-mediated transactivation of plasmid p5Ctk by the parvovirus product.

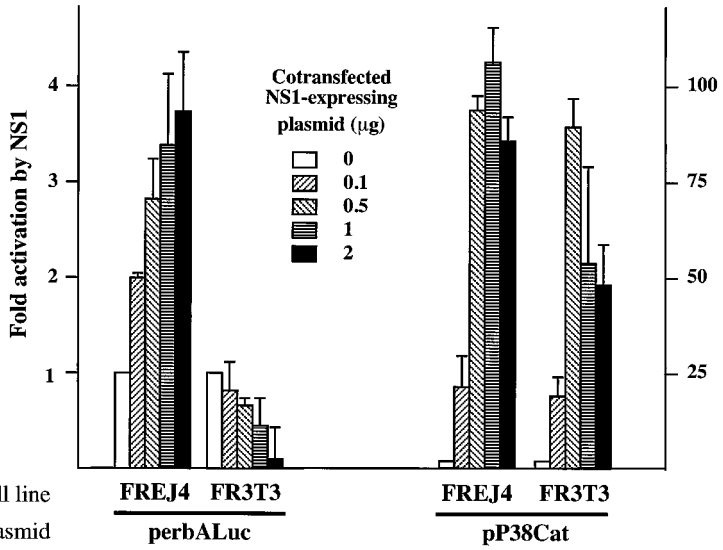

FIG. 5. Cell specificity of promoter transactivation by NS1. FREJ4 and FR3T3 cells were cotransfected with reporter constructs driven by the cellular c-*erbA-1* promoter (perbALuc; 2 μg) or the parvoviral P38 promoter (pP38Cat [pULB3562]; 0.5 μg), together with the indicated amounts of pRSVNS plasmid. Luc and CAT activities were determined 48 h after transfection and are plotted relative to the values measured in NS1-free cells. Average values from three independent experiments are given with standard deviation bars.

A cellular promoter can be transactivated by NS1. The cellular promoters that were reported until now to be regulated by NS1 are actually repressed by this protein (13, 25, 34, 42a). The present work shows that not only parvovirus (16) but also cellular promoters can be targets for NS1 inducing activity. The mechanism by which the NS1 protein controls transcription is still unclear. By means of NS1 fusion proteins comprising the LexA DNA binding domain, it was shown that the C terminal of NS1 can transactivate a promoter to which it binds through the LexA operator (26). Interaction of the genuine NS1 protein with DNA has recently been reported to occur either directly through sequences containing repeats of the ACCA motif (9) or indirectly through association with transcription factor Sp1, which binds to GC-rich elements (19). Computer-assisted analysis did not reveal ACCA-like repeats in the c-*erbA-1* promoter. On the other hand, the c-*erbA-1* regulatory region lacks a TATA box but contains a number of GC-rich elements, like promoters driving nuclear hormone receptor genes (reference 41 and references therein). Yet these GC boxes proved insufficient for the c-*erbA-1* promoter to respond to NS1, which required the unrelated FRE. There is, however, a possibility that Sp1 or similar proteins still contribute to the NS1-induced stimulation of the full c-*erbA-1* promoter by cooperating with FRE-binding polypeptides. Such

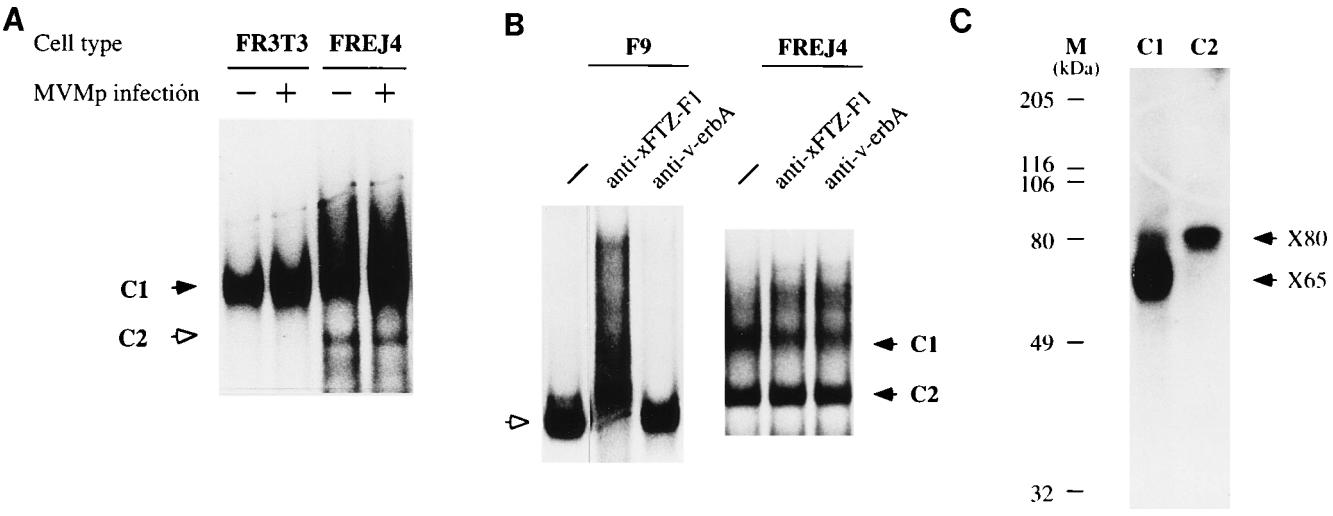

FIG. 6. Characterization of FRE-protein complexes. (A) EMSA of the association of oligonucleotide C probe (Fig. 3A) with proteins from MVMp (+)- or mock (−)-infected FR3T3 and FREJ4 cells. Specific complexes C1 and C2 (Fig. 3B) are indicated by arrows. (B) Effect of antibodies directed against FTZ-F1 or v-ErbA on the formation and electrophoretic mobility of probe C complexes with proteins from FREJ4 or F9 cells. Specific complexes are indicated by closed (FREJ4) and open (F9) arrows. (C) UV cross-linking analysis of polypeptide constituents of complexes C1 and C2. ^{32}P-labelled, bromodeoxyuridine-substituted oligonucleotide C was incubated with whole extracts from FREJ4 cells. Specific complexes C1 and C2 were resolved by EMSA and individually analyzed by SDS-PAGE after UV cross-linking. Polypeptide species are named after their apparent molecular masses and are indicated by arrows. M, standard molecular mass markers (in kDa).

a cooperation between Sp1 and nuclear receptors was recently exemplified with regard to the regulation of the acylcoenzyme A oxidase promoter (20).

The parvovirus P38 promoter harbors no FRE. There is now good evidence that NS1 binds to a site within the *tar* element of P38 to up-regulate this promoter (7). Therefore, FRE-mediated induction constitutes one of several pathways by which the activity of a promoter can be stimulated by NS1. It is presently unclear how NS1 exerts its influence through the FRE element. EMSAs performed with in vitro-synthesized NS1 protein (data not shown) or infected cell extracts (Fig. 6A) gave no indication of NS1 interaction with FRE. However, this evidence cannot be taken as definitively negative, since the recently described binding of NS1 to ACCA-containing DNA fragments proved to be ATP dependent (7) and could not be demonstrated by the EMSA technique used in the present work (9). Interestingly, nucleoside triphosphate (NTP)-binding-site mutant NS1 molecules (K405R and K405M, both defective in P38 transactivation [31]) were found to be unable to stimulate the c-*erbA-1* promoter (data not shown). Therefore, NS1 binding to DNA, transactivation of P38, and up-regulation of the c-*erbA-1* promoter share the same dependence upon an intact NTP-binding site in NS1. On the other hand, a qualitative difference in FRE-bound polypeptides was detected between cells that are respectively sensitive and resistant to NS1-induced activation of the c-*erbA-1* promoter. It follows that cellular proteins may intermediate between NS1 and FRE to achieve promoter activation, though direct binding of NS1 to the FRE cannot be ruled out. Prior infection of cells with MVMp did not alter the EMSA patterns obtained with the FRE probe, making it unlikely that NS1 modulates expression of the X65 and X80 polypeptide constituents of retarded FRE complexes. Alternatively, NS1 may act by binding to the FRE cognate proteins, modifying them at the posttranslational level or regulating their activity, which could pass undetected in EMSAs and be achieved either directly or through other proteins.

Cell specificity of FRE-mediated promoter activation by NS1. Whether harbored by the c-*erbA-1* region or placed in front of the minimal *tk* promoter, the FRE allowed NS1 to stimulate gene expression in *ras*-transformed FREJ4 cells but not in the progenitor FR3T3 cell line. This variation contrasts with the capacity of NS1 for FRE-independent induction of the parvoviral promoter P38 in both cell types. In this respect, FRE-mediated transactivation constitutes a first NS1 molecular action that exhibits cell specificity. It is noteworthy that a differential effect of NS1 on c-*erbA-1* promoter-driven gene expression was also detected between a *src* transformant and the parental nontransformed cell line (data not shown) and is therefore not unique to the clones analyzed in this study. Additional work is required, however, to assess the extent of this differential effect as a function of the oncogene and cell type involved.

The underlying mechanism of the dependence of NS1 transactivation on *ras* transformation is presently elusive but may be tentatively related to the differential association of FRE with proteins from FREJ4 versus FR3T3 cells. One FRE-protein complex was shared by both cell types and involved a 65-kDa polypeptide component, as determined by UV cross-linking experiments using FREJ4 (Fig. 6C) and FR3T3 (data not shown) cell extracts. In contrast, a second complex, consisting of FRE and an 80-kDa polypeptide from FREJ4 cells, could not be detected with FR3T3 extracts and may be envisioned as participating in one way or another in promoter transactivation by NS1.

The c-*erbA-1* target promoter analyzed in this study originates from human cells (23, 43) and was introduced by transfection in rat fibroblasts. It is noteworthy that the promoter of the rat c-*erbA-1* gene was recently cloned (24) and also contains the 5′-TCAAGGTCA-3′ FRE at a similar position relative to the transcription start site. The perfect conservation of this motif in an otherwise highly divergent environment argues for the importance of FRE in the regulation of the THRα-encoding gene. Furthermore, the FRE can be expected to make the endogenous rat c-*erbA-1* gene NS1 inducible in a cell-specific fashion. This is in agreement with our previous work showing that MVMp infection leads FREJ4 but not FR3T3 cells to accumulate c-*erbA-1* mRNAs and corresponding THRα proteins (43). Furthermore, parvovirus infection does not stimulate expression of the THRβ-encoding gene (43), the promoter of which does not comprise a FRE (36).

A connection may be made between the *trans*-regulatory and cytotoxic functions of NS1. (i) The specificity of NS1-induced activation of FRE-containing promoters for FREJ4 (*ras*-transformed) versus FR3T3 (parental untransformed) cells is reminiscent of the recently reported requirement of oncogenic transformation for the sensitization of the latter cells to the killing effect of NS1 (29). (ii) The transcriptional and cytotoxic activities of NS1 could not be dissociated so far by introducing mutations in the NS1 gene (25). (iii) FREJ4 cell killing by MVMp is aggravated in the presence of T3 hormone (the natural ligand of NS1-induced THRα) (43). Altogether, these results argue for dysregulation of cellular gene expression being a marker or even one of the causes of NS1-induced cell death.

ACKNOWLEDGMENTS

We are grateful to C. Dreyer (Tübingen, Germany) and J. Ghysdael (Orsay, France) for the generous gift of antisera. We are indebted to P. Tattersall and J. Nüesch for providing NS1 mutants. K. Sucker is thanked for technical assistance.

This work was supported by the Commission of the European Communities (Biomedicine and Health Research Programme). R.C. is a fellow of the Ministere de l'Education Nationale (France).

REFERENCES

1. **Ahn, J. K., Z. W. Pitluk, and D. C. Ward.** 1992. The GC box and TATA transcription control elements in the P38 promoter in the minute virus of mice are necessary and sufficient for transactivation by the nonstructural protein NS1. J. Virol. **66:**3776–3783.
2. **Boshart, M., M. Klüppel, A. Schmidt, G. Shutz, and B. Luckow.** 1992. Reporter constructs with low background activity utilizing the cat gene. Gene **110:**123–130.
3. **Brandenburger, A., D. Legendre, B. Avalosse, and J. Rommelaere.** 1990. NS1 and NS2 proteins may act synergistically in the cytopathogenicity of parvovirus MVMp. Virology **174:**576–584.
4. **Caillet-Fauquet, P., M. Perros, A. Brandenburger, P. Spegelaere, and J. Rommelaere.** 1990. Programmed killing of human cells by means of an inducible clone of parvoviral genes encoding non-structural proteins. EMBO J. **9:**2989–2995.
5. **Chen, C., and H. Okoyama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
6. **Chen, F., A. J. Cooney, Y. Wang, S. W. Law, and B. W. O'Malley.** 1994. Cloning of a novel orphan receptor (GCNF) expressed during germ cell development. Mol. Endocrinol. **8:**1434–1444.
7. **Christensen, J., S. F. Cotmore, and P. Tattersall.** 1995. Minute virus of mice transcriptional activator protein NS1 binds directly to the transactivation region of the viral P38 promoter in a strictly ATP-dependent manner. J. Virol. **69:**5422–5430.
8. **Cornelis, J. J., N. Spruyt, P. Spegelaere, E. Guetta, T. Darawshi, S. F. Cotmore, P. Tattersall, J. Tal, and J. Rommelaere.** 1988. Sensitization of transformed rat fibroblasts to killing by parvovirus minute virus of mice correlates with an increase in viral gene expression. J. Virol. **62:**3438–3444.
9. **Cotmore, S. F., J. Christensen, J. P. F. Nüesch, and P. Tattersall.** 1995. The NS1 polypeptide of the murine parvovirus minute virus of mice binds to DNA sequences containing the motif $[ACCA]_{2-3}$. J. Virol. **69:**1652–1660.
10. **Doerig, C., B. Hirt, J.-P. Antonietti, and P. Beard.** 1990. Nonstructural protein of parvoviruses B19 and minute virus of mice controls transcription. J. Virol. **64:**387–396.
11. **Dupressoir, T., J.-M. Vanacker, J. J. Cornelis, N. Duponchel, and J. Rom-**

melaere. 1989. Inhibition by parvovirus H-1 of the formation of tumors in nude mice and colonies in vitro by transformed human mammary epithelial cells. Cancer Res. **49:**3203–3208.

12. **Ellinger-Ziegelbauer, H., A. K. Hihi, V. Laudet, H. Keller, W. Wahli, and C. Dreyer.** 1994. FTZ-F1-related orphan receptors in *Xenopus laevis*: transcriptional regulators differentially expressed during early embryogenesis. Mol. Cell. Biol. **14:**2786–2797.
13. **Faisst, S. R., S. Faisst, C. Grangette, J. R. Schlehofer, and J. Rommelaere.** 1993. NFκB upstream regulatory sequences of the HIV-1 LTR are involved in the inhibition of HIV-1 promoter activity by the NS1 proteins of autonomous parvoviruses H-1 and MVMp. Virology **197:**770–773.
14. **Freake, H. C., A. Santos, Y. Goldberg, J. Ghysdael, and J. H. Oppenheimer.** 1988. Differences in antibody recognition of the triiodothyronine nuclear receptor and c-erbA products. Mol. Endocrinol. **2:**986–991.
15. **Glass, C. K.** 1994. Differential recognition of target genes by nuclear receptor monomers, dimers and heterodimers. Endocr. Rev. **15:**391–407.
16. **Gu, M.-L., F. X. Chen, and S. L. Rhode.** 1992. Parvovirus H-1 P38 promoter requires the transactivation region (*tar*), an Sp1 site and a TATA box for full activity. Virology **187:**10–17.
17. **Hanson, N. D., and S. L. Rhode.** 1991. Parvovirus NS1 stimulates P4 expression by interaction with the terminal repeats and through DNA amplification. J. Virol. **65:**4325–4333.
18. **Honda, S., K. Morohashi, M. Nomura, H. Takeya, M. Kitajima, and T. Omura.** 1993. Ad4BP regulating steroidogenic P-450 gene is a member of steroid hormone receptor superfamily. J. Biol. Chem. **268:**7494–7502.
19. **Krady, J. K., and D. C. Ward.** 1995. Transcriptional activation by the parvoviral nonstructural protein NS1 is mediated via a direct interaction with Sp1. Mol. Cell. Biol. **15:**524–533.
20. **Krey, G., A. Mahfoudi, and W. Wahli.** 1995. Functional interactions of peroxisome proliferator-activated receptor, retinoid-X receptor, and Sp1 in the transcriptional regulation of the acyl-coenzyme-A oxidase promoter. Mol. Endocrinol. **9:**219–231.
21. **Laudet, V., and G. Adelmant.** 1995. Lonesome orphans. Curr. Biol. **5:**124–127.
22. **Laudet, V., C. Hänni, J. Coll, F. Catzeflis, and D. Stéhelin.** 1992. Evolution of the nuclear receptor gene superfamily. EMBO J. **11:**1003–1013.
23. **Laudet, V., J.-M. Vanacker, G. Adelmant, A. Bègue, and D. Stéhelin.** 1993. Characterization of a functional promoter for the human thyroid hormone receptor alpha (c-*erbA-1*) gene. Oncogene **8:**975–982.
24. **Lazar, J., B. Desvergne, E. C. Zimmerman, D. B. Zimmer, M. A. Magnuson, and V. M. Nikodem.** 1994. Role for the intronic sequences on expression of thyroid hormone receptor α gene. J. Biol. Chem. **269:**20352–20359.
25. **Legendre, D., and J. Rommelaere.** 1992. Terminal regions of the NS-1 protein of the parvovirus minute virus of mice are involved in cytotoxicity and promoter *trans* inhibition. J. Virol. **66:**5705–5713.
26. **Legendre, D., and J. Rommelaere.** 1994. Targeting of promoters for transactivation by a carboxy-terminal domain of the NS1 protein of parvovirus minute virus of mice. J. Virol. **68:**7974–7985.
27. **Li, X., and S. L. Rhode.** 1991. Nonstructural protein NS1 of parvovirus H-1 is required for efficient viral protein synthesis and virus production in rat cells *in vivo* and *in vitro*. Virology **184:**117–130.
28. **Lynch, J. P., D. S. Lala, J. J. Peluso, W. Luo, K. L. Parker, and B. A. White.** 1993. Steroidogenic factor 1, an orphan receptor, regulates the expression of the rat aromatase gene in gonadal tissues. Mol. Endocrinol. **7:**776–786.
29. **Mousset, M., Y. Ouadrhiri, P. Caillet-Fauquet, and J. Rommelaere.** 1994. The cytotoxicity of the autonomous parvovirus minute virus of mice nonstructural protein in FR3T3 rat cells depends on oncogene expression. J. Virol. **68:**6446–6453.
30. **Naeger, L., J. Cater, and D. Pintel.** 1990. The small nonstructural protein (NS2) of the parvovirus minute virus of mice is required for efficient DNA replication and infectious virus production in a cell-type-specific manner. J. Virol. **64:**6166–6175.
31. **Nüesch, J. P. F., S. F. Cotmore, and P. Tattersall.** 1992. Expression of functional parvoviral NS1 from recombinant vaccinia virus: effects of mutations in the nucleotide-binding motif. Virology **191:**406–416.
32. **Op de Beeck, A., F. Anouja, S. Mousset, J. Rommelaere, and P. Caillet-Fauquet.** 1995. The nonstructural (NS) proteins of the autonomous parvovirus minute virus of mice interfere with the cell cycle, inducing accumulation in G2. Cell Growth Differ. **6:**781–798.
33. **Perros, M., L. Deleu, J.-M. Vanacker, Z. Kherrouch, N. Spruyt, S. Faisst, and J. Rommelaere.** 1995. Upstream CREs participate in the basal activity of minute virus of mice promoter P4 and in its stimulation in *ras*-transformed cells. J. Virol. **69:**5506–5515.
34. **Rhode, S. L., III, and S. M. Richard.** 1987. Characterization of the *trans*-activation-responsive element of the parvovirus H-1 P38 promoter. J. Virol. **61:**2807–2815.
35. **Rommelaere, J., and J. J. Cornelis.** 1991. Antineoplastic activity of parvoviruses. J. Virol. Methods **33:**233–251.
36. **Sakurai, A., T. Miyamoto, and L. J. DeGroot.** 1992. Cloning and characterization of the human thyroid hormone receptor β1 gene promoter. Biochem. Biophys. Res. Commun. **185:**78–84.
37. **Salomé, N., B. van Hille, N. Duponchel, G. Meneguzzi, F. Cuzin, J. Rommelaere, and J. Cornelis.** 1990. Sensitization of transformed rat cells to parvovirus MVMp is restricted to specific oncogenes. Oncogene **5:**123–130.
38. **Sap, J., A. Munoz, K. Damm, Y. Goldberg, J. Ghysdael, A. Leutz, H. Beug, and B. Vennström.** 1986. The c-erbA protein is a high affinity receptor for thyroid hormone. Nature (London) **324:**635–640.
39. **Spegelaere, P., J. J. Cornelis, M. Tuynder, and J. Rommelaere.** 1994. Lack of a detectable effect of capsid proteins on the cell-dependent activity of parvovirus MVMp promoters. Res. Virol. **145:**5–12.
40. **Tattersall, P., and E. M. Gardiner.** 1990. Autonomous parvovirus-host cell interactions, p. 111–121. *In* P. Tijssen (ed), Handbook of parvoviruses, vol. I. CRC Press, Inc., Boca Raton, Fla.
41. **Tsai, M.-J., and B. W. O'Malley.** 1994. Molecular mechanisms of action of steroid/thyroid hormone receptor superfamily. Annu. Rev. Biochem. **63:**451–486.
42. **Tsukiyama, T., H. Ueda, S. Hirose, and O. Niwa.** 1992. Embryonal long terminal repeat-binding protein is a murine homolog of FTZ-F1, a member of the steroid receptor superfamily. Mol. Cell. Biol. **12:**1286–1291.

42a. **Vanacker, J.-M.** Unpublished data.

43. **Vanacker, J.-M., V. Laudet, G. Adelmant, D. Stéhelin, and J. Rommelaere.** 1993. Interconnection between thyroid hormone receptor signalling pathways and parvovirus cytotoxic functions. J. Virol. **67:**7668–7672.
44. **Vanacker, J.-M., and J. Rommelaere.** 1995. Non-structural proteins of autonomous parvoviruses: from cellular effects to molecular mechanisms. Semin. Virol. **6:**291–297.

44a. **Vanacker, J.-M., and J. Rommelaere.** Unpublished results.

45. **van Hille, B., N. Duponchel, N. Salomé, N. Spruyt, S. Cotmore, P. Tattersall, J. Cornelis, and J. Rommelaere.** 1989. Limitations to the expression of parvoviral nonstructural proteins may determine the extent of sensitization of EJ-*ras* transformed rat cells to minute virus of mice. Virology **171:**89–97.
46. **Weinberger, C., C. C. Thompson, E. S. Ong, R. Lebo, D. J. Gruol, and R. M. Evans.** 1986. The c-erbA gene encodes a thyroid hormone receptor. Nature (London) **324:**641–646.
47. **Wilson, T. E., T. J. Fahrner, and J. Milbrandt.** 1993. The orphan receptors NGF-1B and steroiodogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. Mol. Cell. Biol. **13:**5794–5804.