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Parvovirus Nonstructural Proteins Induce an Epigenetic Modification through Histone Acetylation in Host Genes and Revert Tumor Malignancy to Benignancy

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Several malignant tumor cells become apoptotic and revert to the benign phenotype upon parvovirus infection. Recently, we demonstrated that the rat parvovirus RPV/UT also induces apoptosis in the rat thymic lymphoma cell line C58(NT)D. However, a minority of cells that escaped apoptosis showed properties different from the parental cells, such as resistance to apoptosis, enhanced cell adherence, and suppressed tumorigenicity. The present study was performed to determine the molecular mechanism of parvovirus-induced phenotypic modification, including oncosuppression. We demonstrated that the nonstructural (NS) proteins of RPV/UT induced apoptosis in C58(NT)D cells and suppressed tumor growth in vivo. Interestingly, NS proteins induced the expression of ciliary neurotrophic factor receptor alpha, which is up-regulated in revertant cell clones, and enhanced histone acetylation of its gene. These results indicate that parvoviral NS regulate host gene expression through histone acetylation, suggesting a possible mechanism of oncosuppression.

Autonomous parvoviruses are small, nonenveloped, nucleus-replicating viruses that contain a linear, single-stranded DNA genome of approximately 5 kb. The viral early and late promoters P4 and P38 encode the nonstructural proteins (NS-1 and NS-2) and the viral capsid proteins (VP-1 and VP-2), respectively (1). NS-1 is essential for viral replication and lytic infection and has multifunctional properties, such as ATPase activity, DNA helicase activity, and transactivation of the P38 promoter (10, 16, 33, 43). NS-2 is generated by alternative splicing and is expected to be correlated with translation of viral mRNA and capsid protein assembly through interaction with the nuclear export factor chromosomal region maintenance 1 (2, 26, 29). NS proteins show high sequence homology among parvoviruses and are considered to be correlated with cytotoxicity in permissive cells and parvoviral pathogenicity (28, 35).

Several autonomous parvoviruses, including H-1, Kilham rat virus, and minute virus of mice, have tumor-suppressing properties in vivo and in vitro (34). Recently, we showed that most cells of the malignant thymic lymphoma cell line C58(NT)D infected with an isolate of rat parvovirus (RPV/UT) underwent apoptotic cell death, while the revertant cell clone C58(NT)D/R derived from a minor population of cells that survived after virus propagation showed several different phenotypes and reduced tumorigenicity (40). Furthermore, modified phenotypes of C58(NT)D/R were maintained at least over 15 cell passages. These findings suggest that these parvoviruses may be promising candidate vectors for tumor gene therapy (3, 7), although the mechanism of parvovirus-induced oncosuppression is still unclear.

DNA methylation and histone modifications, such as deacetylation and methylation of specific lysine residues at the N termini of histones H3 and H4, play key roles in epigenetic gene regulation, resulting in inappropriate expression or silencing of genes without corresponding changes in their DNA sequence (15). The epigenetic regulation of gene expression is an important factor in tumorigenesis, and epigenetic transcriptional repression of tumor suppressor genes has been reported in a wide variety of tumors (4). Oncovirus-associated tumors, including Burkitt's lymphoma, which is transformed by Epstein-Barr virus, also show potential hypermethylation of the promoter regions of tumor suppressor genes (19), although it is unclear whether transcriptional repression by DNA methylation is a cause or a consequence of tumorigenesis. Epigenetic modification of the host cell by virus infection is an extremely interesting and important process that may provide a deeper understanding of tumor progression or inhibition. Therefore, we investigated whether epigenetic modification is involved in parvovirus-mediated oncosuppression.

In the present study, we first compared the gene expression profiles of the malignant C58(NT)D cells and the revertant cell clone C58(NT)D/R by representational difference analysis (RDA) to determine the molecular mechanism of parvoviral oncosuppression. Our results indicated up-regulated expression of several genes, including ciliary neurotrophic factor receptor alpha (CNTFRα), in C58(NT)D/R cells. Furthermore, parvoviral NS proteins modified the transcriptional regulation of CNTFRα through modulation of histone acetylation. Expression of NS induced phenotypic changes, such as enhanced

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TABLE 1. Primer sequences used for semiquantitative RT-PCR and ChIP assay

Assay and gene	Primer sequence (5′ to 3′)	
	Forward	Reverse
Semiquantitative RT-PCR		
Cathepsin E	CTCTCAGACTTCTGGAGATC	TTCACAAAGGTCTGACCAGG
TARPP	TCTCCCAGCAGGTCCTGC	AGCCTTGGGTCATTGGCACC
DHCR24	ACATTCTGGAGGTGGACACC	CTCCACATGCTTGAAGAACC
PASS-1	CAAGACGGATGTTTTCCAGG	GACAGCACTGTTCAAGTAGC
CNTFRα	AGGAGGCACCCCATGTTCAG	CATGTCACCTCCAGTCGACG
GAPDH	ACCACAGTCGATGCCATCAC	TCCACCACCCTGTTGCTGTA
ChIP		
CNTFRα	CCCTCGCATTCGCCCTTTCTC	CCAAAGCTGAGAGTGTCATGAGC
Actin	GTTGCCTTTTCTGGCTCGAGTG	GACGTCCCTGCTTACCTGGTG

cell adherence, elongated microvilli, and suppressed tumorigenesis in C58(NT)D cells as well as features observed in C58(NT)D/R cells. These results indicated that parvoviral NS regulated host gene expression through histone acetylation, and this may have resulted in reverting tumor malignancy to benignancy.

MATERIALS AND METHODS

Cells and virus. The rat thymic lymphoma cell line C58(NT)D obtained from the American Type Culture Collection (ATCC) and the revertant clone C58(NT)D/R (40) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated horse serum in a 5% CO_2 atmosphere at 37°C. The retrovirus packaging cell line 293gp was cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum in a 5% CO_2 atmosphere at 37°C.

The rat parvovirus UT strain (RPV/UT) was isolated from an asymptomatic laboratory rat and serially passaged in C58(NT)D cells. The infectivity titer of the virus stock was assayed by the standard immunofluorescent antibody assay.

RNA preparation and cDNA synthesis. Total RNA was isolated by guanidinium isothiocyanate extraction followed by CsCl gradient purification. Polyadenylated RNA was purified from total RNA with a FastTrak 2.0 mRNA isolation kit (Invitrogen) and oligo(dT)-primed double-stranded cDNA was synthesized from 5 μg of polyadenylated RNA using the SuperScript Choice system (Invitrogen) according to the manufacturer's instructions.

Representational difference analysis of cDNA. RDA of cDNA was performed as described previously (14) with cDNA prepared from C58(NT)D and C58(NT)D/R cells. Briefly, cDNA was digested with DpnII and ligated to the adaptors. Amplicons were made by PCR amplification of the adaptor-ligated DpnII cDNA fragments as a primer, 5′-AGCACTCTCCAGCCTCTCACCGC A-3′. Driver DNA was prepared by digesting amplicons with DpnII, and tester DNA was prepared by gel purification of digested amplicons between 150 and 2,000 bp followed by ligation to other adaptors. The first subtractive hybridization was performed using a tester-driver ratio of 1:100. Subtractive hybridization was repeated twice using tester-driver ratios of 1:800 and 1:400,000 for the second and third rounds, respectively.

Preparation of recombinant retrovirus vector expressing NS proteins. We amplified the *NS* gene of RPV/UT with DNA extracted from infected C58(NT)D cells by PCR with specific primers (5′-ACC*GGATCC*CCATGGCTGGAAACG CTTAC-3′ and 5′-GAT*GGATCC*GTTGGTTCTCCTTGGTCAAG-3′); each end contains a BamHI restriction site (italic). A 2,210-bp BamHI fragment containing the *NS* gene was inserted into the BamHI site of pGCDNsam-IRES-EGFP as reported previously (37), designated DNEGFP/NS, which expresses enhanced green fluorescent protein (EGFP), NS-1, and NS-2 proteins. To produce the vesicular stomatitis virus G protein (VSV-G) pseudo-type retrovirus, DNEGFP/NS and the VSV-G expression vector were cotransfected into 293gp cells using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. The culture supernatant was collected and centrifuged at 6,000 × *g* and 4°C for 16 h followed by resuspension of the viral vector pellet in serum-free DMEM.

Western blotting analysis. Expression of specific proteins was detected by Western blotting analysis. Cells were washed once with phosphate-buffered saline (PBS) and then suspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 1% NP-40). Protein concentrations were quantified using the Bio-Rad protein assay (Bio-Rad Laboratories). Whole lysates were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon polyvinylidene difluoride membranes (Amersham Biosciences). Anti-rat CNTFRα antibody (Genzyme-Techne), anti-rat cathepsin E antibody (WAKO), anti-chicken actin antibody (Chemicon), and anti-NS antibody were used.

Semiquantitative reverse transcription-PCR. Total RNA was extracted using an Isogen RNA extraction kit (Nippongene) according to the manufacturer's instructions. First-strand cDNA was synthesized using SuperScript III RNase H^- reverse transcriptase (Invitrogen). Briefly, aliquots of 5 μg of total RNA were reverse-transcribed using oligo$(dT)_{12–18}$ primer (Invitrogen) in reaction mixtures with a total volume of 20 μl. PCR was carried out in a volume of 25 μl containing 1× PCR buffer (TaKaRa), 0.25 μM of each primer (Table 1), and 0.5 U of *Taq* polymerase (TaKaRa). Aliquots of 1 μl of cDNA were used for PCR amplification, and all of the genes were amplified by 25 to 40 cycles of PCR to determine the appropriate conditions for semiquantitative determination of differences in their expression levels.

Scanning electron microscopy. Samples were washed twice with PBS and fixed in 2% glutaraldehyde and 1% osmium tetroxide, dehydrated, and critical-point dried with carbon dioxide. They were coated with platinum and examined by scanning electron microscopy (JSM-6320F microscope; JEOL).

ChIP assay. Chromatin immunoprecipitation (ChIP) was performed using an anti-acetyl-histone H3 (Lys9) antibody, which recognizes acetylated lysine 9 of histone H3, according to the manufacturer's instructions (Upstate Biotechnology). Briefly, cells were incubated in 1% formaldehyde for 10 min at 37°C. Cells were collected by centrifugation at 4°C and then rinsed twice in ice-cold PBS with protease inhibitor cocktail (Sigma). We resuspended cells in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) containing protease inhibitors followed by incubation on ice for 10 min. The DNA-protein complexes were sonicated to yield fragments ranging in size from 200 to 1,000 bp and immunoprecipitated with anti-acetyl-histone H3 (Lys9) antibody. The chromatin was recovered using protein A-Sepharose. PCR was then carried out in 25 μl of solution containing 1 μl of chromatin DNA. The PCR cycling profile consisted of one cycle of 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. DNA fragments were then visualized on 2% agarose gels stained with ethidium bromide. Pictures were taken with LAS-3000 (FUJIFILM).

Tumorigenicity in immunodeficient mice. Female immunodeficient mice (7 weeks old, BALB/$c^{nu/nu}$) purchased from CLEA Japan were inoculated subcutaneously with 10^5 or 10^6 cells. Mice were housed in an isolator controlled at 23 ± 2°C and 55 ± 10% relative humidity and had free access to food (NMF, Oriental Yeast, Tokyo, Japan) and autoclaved water. Mice were surveyed regularly, tumors were measured with a caliper, and tumor volumes were determined using the following formula: volume = 0.5 × (shorter diameter)^2× longer diameter. All animal experiments were approved by the Committee for Animal Experimentation, University of Tsukuba, and carried out in a humane manner based on Japanese law no. 105.

Nucleotide sequence accession number. The sequence of the RPV/UT *NS* gene has been deposited in GenBank under accession number AY764167.

RESULTS

Identification of differentially expressed genes between C58(NT)D and C58(NT)D/R by RDA. To identify genes that

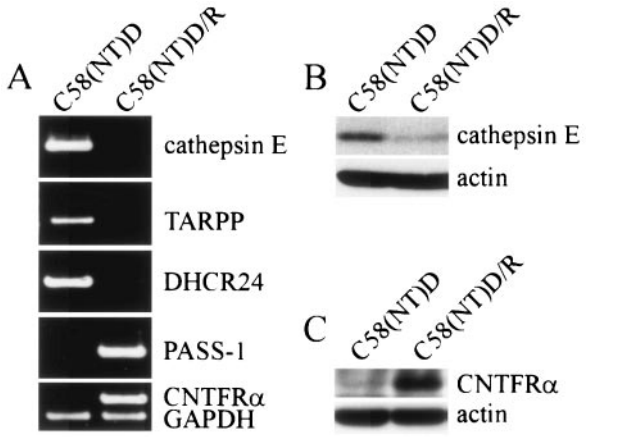

FIG. 1. Expression of isolated genes by RDA in C58(NT)D and C58(NT)D/R cells. The levels of expression of cathepsin E, TARPP, DHCR24, PASS-1, and CNTFRα were determined by semiquantitative RT-PCR (A) and Western blotting analysis (B and C). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and actin protein expression were used as internal controls.

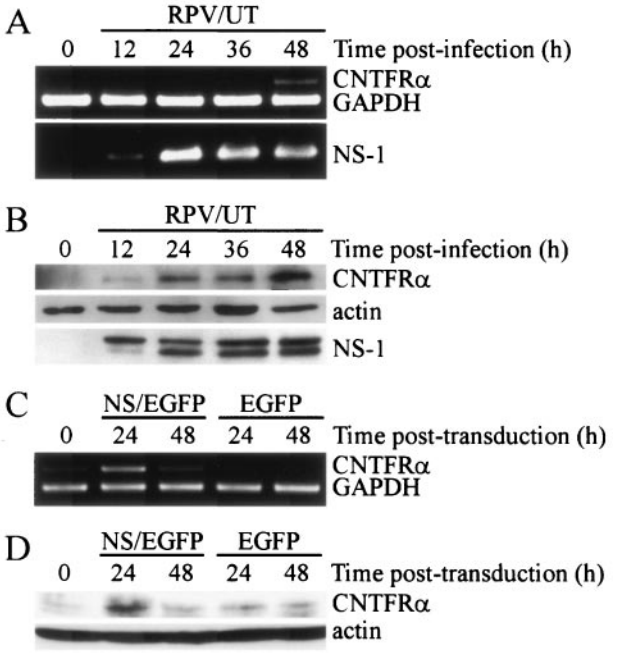

FIG. 2. Induction of CNTFRα expression by RPV/UT infection and NS protein transduction in C58(NT)D cells. C58(NT)D cells were infected with RPV/UT at an MOI of 1 for 48 h. Semiquantitative RT-PCR (A) and Western blotting analysis (B) for CNTFRα and NS-1 were performed with total RNA and cell lysate isolated from infected cells at the indicated time points. Semiquantitative RT-PCR (C) and Western blotting analysis (D) for CNTFRα were performed with cell lysate from C58(NT)D cells infected with NS/EGFP or EGFP retrovirus at an MOI of 3. GAPDH and actin served as internal controls.

are differentially expressed between the two cell clones, we performed RDA in which C58(NT)D cDNA was used as the tester cDNA with C58(NT)D/R cDNA as the driver cDNA, and vice versa. Two groups of seven DNA fragments were generated by RDA. Homology search indicated that six DNA fragments corresponded to five known genes, cathepsin E, thymocyte cyclic AMP-regulated phosphoprotein (TARPP), DHCR24, ciliary neurotrophic factor receptor alpha (CNTFRα), and protein associated with small stress 1 (PASS-1). Two other fragments showed no significant similarity to any known genes.

RT-PCR (Fig. 1A) and Western blotting analyses (Fig. 1B and C) were performed to confirm the differential expression of these genes in C58(NT)D and C58(NT)D/R cells. The results of these experiments clearly demonstrated that the levels of cathepsin E, TARPP, and DHCR24 gene expression were down-regulated, whereas PASS-1 and CNTFRα expression was up-regulated in C58(NT)D/R compared with C58(NT)D cells.

CNTFRα gene expression is induced by NS proteins. Previously, we reported that the clone C58(NT)D/R showed complete elimination of the virus through serial passages. Thus, it was unclear whether the up-regulation of CNTFRα was induced by virus infection. To address this question, C58(NT)D cells were infected with RPV/UT at a multiplicity of infection (MOI) of 1. Significant up-regulation was observed within 48 h postinfection (Fig. 2A and B), suggesting that RPV/UT induced expression of the CNTFRα.

Next, we examined whether NS was involved in the induction of CNTFRα expression by RPV/UT infection. To produce recombinant retrovirus expressing NS proteins, the *NS* gene of RPV/UT was amplified by PCR and subcloned into the retrovirus vector (see Materials and Methods). C58(NT)D cells were infected with the recombinant retrovirus expressing NS proteins and EGFP (NS/EGFP retrovirus) at an MOI of 3. Semiquantitative RT-PCR and Western blotting analysis demonstrated that NS induced up-regulation of CNTFRα expression (Fig. 2C and D). NS was also shown to induce morphological changes, such as increased cell adherence and elongated microvilli, which were observed in RPV/UT-infected C58(NT)D and C58(NT)D/R cells (Fig. 3).

DNMT and HDAC inhibitors induce CNTFRα expression. To examine the involvement of epigenetic regulation in CNTFRα expression, C58(NT)D cells were treated with the DNA methyltransferase (DNMT) inhibitor 5-azacytidine for 6 days and the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) and sodium butyrate (NaB) for 12 h. RT-PCR (Fig. 4A and B) and Western blotting analysis (Fig. 4C) showed that 5-azacytidine and both HDAC inhibitors induced CNTFRα expression in C58(NT)D cells. These observations indicated that CNTFRα expression is regulated directly and/or indirectly through DNA methylation and histone acetylation.

NS proteins modulate histone acetylation on the CNTFRα gene. Parvovirus NS-1 binds to the coactivator cyclic AMP response element-binding protein (CREB) binding protein (CBP), known as histone acetyltransferase, and activates both viral and cellular promoters. Therefore, we investigated whether NS modulates histone acetylation in the 5′ upstream region of the *CNTFR*α gene. We examined the patterns of acetylation of specific lysine residues in the N-terminal tail of histone H3 associated with the *CNTFR*α gene in C58(NT)D and C58(NT)D/R cells. The results of the ChIP assay indicated that lysine 9 of histone H3 was hyperacetylated in C58(NT)D/R cells and hypoacetylated in C58(NT)D cells (Fig. 5A).

Next, we performed the ChIP assay with genomic DNA

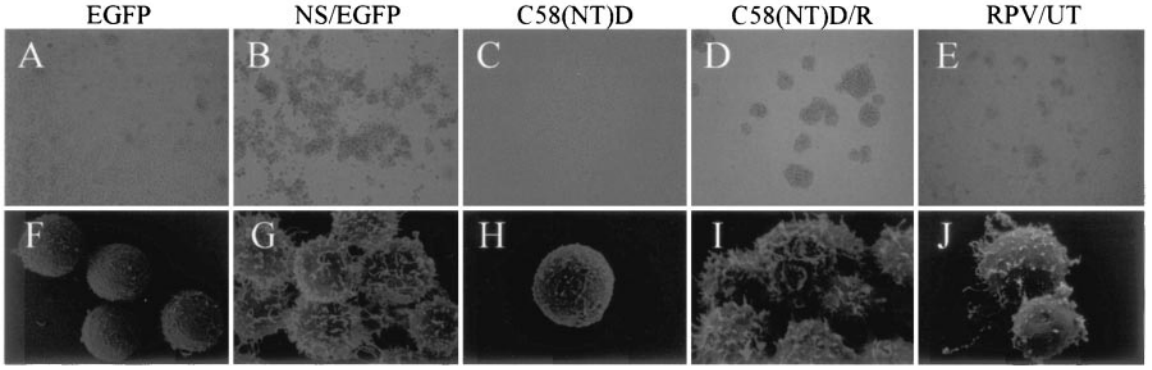

FIG. 3. Phenotypic changes in C58(NT)D cells induced by NS proteins. Increased cell adherence was observed in C58(NT)D/R cells (D) and C58(NT)D cells infected with RPV/UT (E) and NS/EGFP retrovirus (B) for 24 h, compared with C58(NT)D cells (C) and EGFP retrovirus-infected C58(NT)D cells (A), respectively. Scanning electron microscopy showed elongated microvilli in NS/EGFP retrovirus-infected C58(NT)D cells (G) at 12 h postinfection, which was observed in C58(NT)D/R cells (I) and RPV/UT infected-C58(NT)D cells (J).

extracted from C58(NT)D 48 h after infection with NS/EGFP- and EGFP-expressing retroviruses, respectively. In NS/EGFP retrovirus-infected cells, histone H3 in the upstream region of the *CNTFR*α gene was hyperacetylated, in contrast to EGFP retrovirus-infected cells, in which histone H3 was shown to be hypoacetylated (Fig. 5B). Together with the above results, these observations suggested that NS proteins may up-regulate CNTFRα expression through hyperacetylation of lysine residues in histone H3.

NS proteins inhibit tumor growth in immunodeficient mice. As the results of our previous studies indicated that RPV/UT induced suppression of tumorigenicity of C58(NT)D cells, we examined whether NS proteins also suppress tumorigenicity. First, we injected aliquots of 10^5 C58(NT)D cells subcutaneously into BALB/c$^{nu/nu}$ mice 12 h after infection with the NS/EGFP retrovirus at an MOI of 3. For comparison, cells were infected in parallel with the EGFP retrovirus at an MOI of 3 and then injected into BALB/c$^{nu/nu}$ mice. Seven of the 10 animals injected with EGFP-transduced cells developed tumors, with an average diameter of 1,909 mm^3, within 28 days postinfection (Fig. 6A, B, C, and D). In contrast, only four of the 10 animals injected with NS/EGFP-transduced cells developed tumors with an average diameter of 335 mm^3 (Fig. 6A, B, C, and D).

In the second experiment, to determine the ability of NS to induce reversion of the malignant phenotype, we subcloned

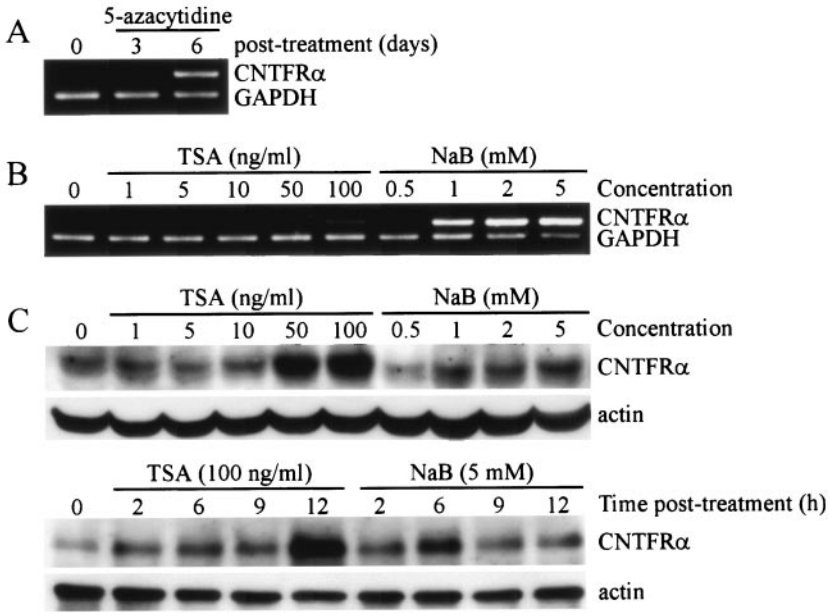

FIG. 4. Epigenetic regulation of CNTFRα expression. (A) Semiquantitative RT-PCR was performed with total RNA extracted from C58(NT)D cells treated with 5-azacytidine for 3 and 6 days. Semiquantitative RT-PCR (B) and Western blotting analysis (C top) with cell lysate from C58(NT)D cells treated with trichostatin A (TSA) and sodium butyrate (NaB) at the indicated concentrations for 12 h using specific primer and antibody for CNTFRα. (C, bottom) Time course experiment of *CNTFR*α gene expression induced by trichostatin A (100 ng/ml) and sodium butyrate (5 mM) treatment in C58(NT)D cells. GAPDH and actin served as internal controls.

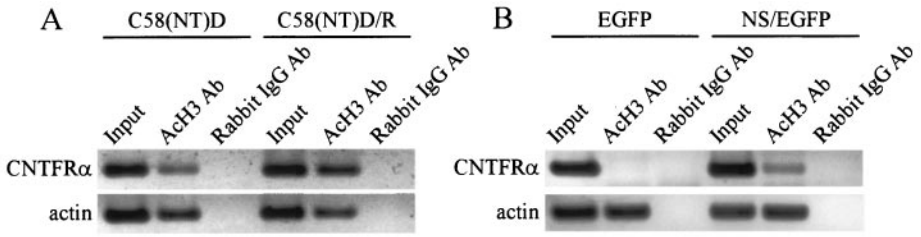

FIG. 5. Hyperacetylation of histone H3 in *CNTFR*α gene induced by NS. The ChIP assay was carried out with genomic DNA extract from C58(N)D and C58(NT)D/R cells (A) and EGFP and NS/EGFP retrovirus-infected C58(NT)D cells (B) using anti-acetyl-histone H3 (Lys9) antibody (AcH3 Ab) and anti-rabbit IgG antibody (negative control).

EGFP-positive cells from the surviving cells by limiting dilution after infection with either recombinant NS/EGFP or EGFP retrovirus in vitro. Three identical subclones, designated C58 (NT)D/NSEGFP1, C58(NT)D/NSEGFP2, and C58(NT)D/NSEGFP3, were injected into BALB/$c^{nu/nu}$ mice. For comparison, two identical subclones, C58(NT)D/EGFP1 and C58(NT)D/EGFP2, isolated from EGFP-transduced cells were also injected into BALB/$c^{nu/nu}$ mice. Statistically significant differences ($P < 0.05$) were observed between the average sizes of tumors from C58(NT)D/NSEGFP and C58(NT)D/EGFP clones at 16, 19, and 22 days postinoculation, excluding between C58(NT)D/NSEGFP2 and C58(NT)D/EGFP1 at 19 and 22 days postinoculation (Fig. 6E). These results indicated that NS inhibited tumor growth in immunodeficient mice.

DISCUSSION

Autonomous parvoviruses are unique in that they selectively kill spontaneous and chemically and virally induced tumor cells (34). This characteristic makes these viruses potentially attrac-

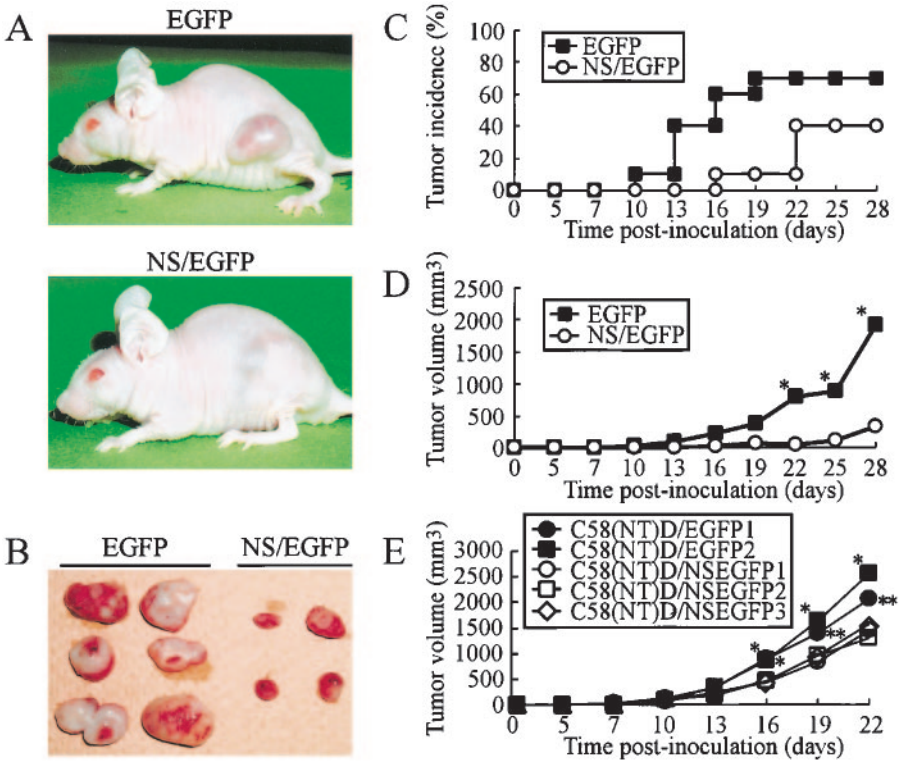

FIG. 6. Suppression of tumor growth by NS proteins in immunodeficient mice. We infected 10^5 C58(NT)D cells with NS/EGFP or EGFP retrovirus at an MOI of 3 for 12 h and then subcutaneously injected it into athymic BALB/$c^{nu/nu}$ mice. (A) Tumor-bearing BALB/$c^{nu/nu}$ mice at 19 days postinoculation. (B) Tumors derived from EGFP-transduced C58(NT)D cells (left) and NS/EGFP-transduced C58(NT)D cells (right) at 28 days postinoculation. Time course of tumor incidence (C) and the means of tumor volume (D) were determined in mice inoculated with EGFP-transduced C58(NT)D cells (■; $n = 10$) and NS/EGFP-transduced C58(NT)D cells (○; $n = 10$). *, $P < 0.05$ compared with tumors from NS/EGFP-transduced C58(NT)D. (E) Time course of changes in the means of tumor volume from 10^6 C58(NT)D/EGFP1 (●; $n = 8$) and C58(NT)D/EGFP2 (■; $n = 6$) cell clones, and C58(NT)D/NSEGFP1 (○; $n = 8$), C58(NT)D/NSEGFP2 (□; $n = 8$), and C58(NT)D/NSEGFP3 (◇; $n = 10$) cell clones. *, $P < 0.05$ compared with tumors from C58(NT)D/NSEGFP1, C58(NT)D/NSEGFP2, and C58(NT)D/NSEGFP3; **, $P < 0.05$ compared with tumors from C58(NT)D/NSEGFP1 and C58(NT)D/EGFP3.

tive tools for use in cancer gene therapy. In fact, a recombinant parvovirus vector expressing NS proteins and various cytokines or chemokines driven by its own P4 promoter was shown to suppress tumor growth and metastases when used to infect malignant tumor cells in vitro and in vivo (3, 7). However, the molecular mechanism of parvovirus-mediated oncosuppression is poorly understood, although the cytotoxic effect of NS-1 is considered to play a key role. Here, we reported that parvoviral NS contributed to epigenetic modification, which may be linked to host phenotypic changes, including resistance to apoptotic cell death and suppressed tumorigenicity.

The results of our previous study indicated that RPV/UT induced phenotypic changes in C58(NT)D cells, and the virus-resistant revertant subclone, C58(NT)D/R, was obtained (40). The majority of infected cells underwent apoptosis. However, a minor cell population escaped apoptotic cell death and showed altered properties, such as resistance to viral infection and apoptosis, enhanced cell adherence, and suppressed tumorigenicity, compared with the parental cells. Similar revertant subclones have been reported previously in several malignant tumors, including the human erythroleukemia cell line K562 and human monocytic cell line U937, infected with the H-1 virus (22, 38). These findings suggest that the oncosuppressive effect of parvovirus infection is responsible for two distinct phenotypic changes, apoptosis and reversion. In the present study, we showed that NS induced similar phenotypic changes in C58(NT)D cells and C58(NT)D/R cells (Fig. 3), indicating that NS is the main effector of phenotypic modification of host cells.

To clarify the molecular mechanism of the observed phenotypic modification, we identified genes that showed differential expression between C58(NT)D and the revertant C58(NT)D/R by RDA. CNTFRα, one of the genes isolated by RDA, was up-regulated in the C58(NT)D/R clone and also induced by RPV/UT infection and NS transduction in the C58(NT)D cells (Fig. 2). CNTFRα is an alpha subunit of CNTFR and binds specifically to its ligands ciliary neurotrophic factor (CNTF) and a complex of cardiotrophin-like cytokines and the soluble receptor cytokine-like factor 1 (8, 12, 20, 32). Binding of CNTFRα to CNTF leads to activation of NF-κB mediated by gp130 and leukemia inhibitory factor receptor beta subunits. Previous studies showed that NF-κB activation through the CNTF/CNTFR signaling pathway promoted the survival of neuronal cells and prevented apoptosis (23, 25, 39). Therefore, we speculated that the expression of CNTFRα is correlated with the apoptosis-resistant characteristics of C58(NT)D/R cells and may promote the survival of RPV/UT-infected C58(NT) cells, resulting in the appearance of resistant cells.

The expression of CNTFRα in C58(NT)D/R cells was inherited by daughter cells, at least over the course of 15 passages, despite the lack of detectable viral antigen, viral DNA, and RNA in these cells as determined by immunofluorescent assay, PCR, and RT-PCR analysis, respectively (data not shown). This phenomenon is likely to be a form of epigenetic inheritance that defines meiotically and mitotically heritable changes in gene expression without this information being encoded in the nucleotide sequence itself. Indeed, the DNMT inhibitor and HDAC inhibitors induced CNTFRα expression in C58(NT)D cells in this study (Fig. 4). Moreover, the lysine 9 residue of histone H3 on the flanking region of the *CNTFR*α gene was shown to be hyperacetylated in C58(NT)D/R cells compared with the parental cell line C58(NT)D (Fig. 5A). This evidence suggests that epigenetic regulation, especially histone acetylation and probably also DNA demethylation, contributes to the expression of CNTFRα directly and/or indirectly.

With respect to correlation between histone acetylation and DNA demethylation, Kishikawa et al. have recently suggested that the maintenance DNA methylase DNMT1, which is constitutively expressed and is required for the maintenance of global methylation after cell divisions, is regulated by histone acetylation (18). Furthermore, several investigators showed that the HDAC inhibitor enhanced DNA demethylation (5) and that valproic acid induced histone acetylation, DNA demethylation, and expression of an ectopically methylated reporter gene plasmid (9). Taken together, DNA demethylation directed by histone acetylation is suggested. NS stimulated acetylation of histone H3 lysine 9 in the *CNTFR*α gene, suggesting that NS modifies the epigenetic regulation of host gene expression. In addition, 5-azacytidine induced apoptotic cell death in C58(NT)D cells, indicating the possibility that DNA demethylation cause apoptosis (unpublished data).

Thus, we thought of the following course in parvovirus-infected cells: NS proteins induce epigenetic modification through histone acetylation followed by DNA demethylation on several host genes. These modifications and/or other cytotoxic activity, such as *trans*-inhibition of heterologous promoter, of NS cause apoptotic cell death in most of the infected cells, however, some of them survive by modulation of the apoptotic signal pathway(s), and the other modified characteristics of surviving cells are also maintained even after cell division lacking NS expression due to epigenetic inheritance.

Previously, we reported that minute virus of mice NS-1 transactivates the parvovirus P38 promoter via direct interaction with the coactivator CBP, which possesses histone acetyltransferase activity (30). CBP also binds to other histone acetyltransferases, such as the p300 and CREB-binding protein associated factor and the steroid receptor coactivator 1 (36), and various viral proteins, such as adenovirus E1A, simian virus 40 large T antigen, and Kaposi's sarcoma-associated herpesvirus vIRF-3 (6, 21). The interaction of CBP and viral proteins modulates cellular histone acetylation and transcriptional activity of specific promoters (24, 41). These reports suggest that CBP and/or a CBP-associated protein(s) may be involved in histone acetylation induced by parvovirus NS proteins.

Recent molecular studies demonstrated that epigenetic regulation contributes to tumor development and progression. In particular, tumor suppressor genes were shown to be silenced by DNA methylation of their promoter regions (4). Inhibitors of DNA methylation and histone deacetylation induced reactivation of several tumor suppressor genes, including p21 (also known as WAF1 and CIP1), resulting in cell cycle arrest, apoptosis, or differentiation (17, 27). Therefore, these agents are expected to be useful as drugs for epigenetic cancer therapy. Indeed, several HDAC inhibitors and DNMT inhibitors are currently in clinical trials as intravenous or oral dosage forms (11, 42). NS-1 of human parvovirus B19 and minute virus of mice significantly induced p21 expression in human kidney, monkey epithelial, and rat fibroblast cell lines (13, 31, 44). In the case of RPV, NS proteins may also induce a tumor

suppressor gene(s), such as p21, as a consequence of histone acetylation on their promoter region, leading to apoptosis or tumor suppression.

In conclusion, the results of the present study indicated that RPV NS induces phenotypic changes, including increased cell adhesion, elongated microvilli, and suppressed tumorigenicity, and modulates host gene expression, such as CNTFRα, which is a potent anitiapoptotic protein, through histone acetylation in a malignant T-lymphoma cell line. The possibility that epigenetic modification by NS contributes, at least in part, to the phenotypic changes of host cells is suggested, although whether alteration of epigenetic status is sufficient to cause phenotypic changes remains to be determined. Studies for screening of epigenetically regulated genes associated with phenotypic changes are in progress. These findings will provide new insight into the oncosuppressive effect of parvovirus and may facilitate the development of a parvoviral vector(s) for use in cancer gene therapy. In addition, the virus-mediated epigenetic modification of the host genome may also provide a novel conception for understanding of virus-host interaction and pathogenicity.

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