[CANCER RESEARCH 61, 3969–3977, May 15, 2001]

Identification of the Transcriptional Regulatory Sequences of Human Calponin Promoter and Their Use in Targeting a Conditionally Replicating Herpes Vector to Malignant Human Soft Tissue and Bone Tumors[1]

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ABSTRACT

The *calponin* (basic or h1) gene, normally expressed in maturated smooth muscle cells, is aberrantly expressed in a variety of human soft tissue and bone tumors. In this study, we show that expression of the *calponin* gene in human soft tissue and bone tumor cells is regulated at the transcriptional level by the sequence between positions −260 and −219 upstream of the translation initiation site. A novel conditionally replicating herpes simplex virus-1 vector (*d*12.CALP) in which the calponin promoter drives expression of ICP4, a major *trans*-activating factor for viral genes was constructed and tested as an experimental treatment for malignant human soft tissue and bone tumors. In cell culture, *d*12.CALP at low multiplicity of infection (0.001 plaque-forming unit/cell) selectively killed calponin-positive human synovial sarcoma, leiomyosarcoma, and osteosarcoma cells. For *in vivo* studies, 10 animals harboring SK-LMS-1 human leiomyosarcoma cells were randomly divided and treated twice on days 0 and 9 intraneoplastically with either 1×10^7 plaque-forming units of *d*12.CALP/100 mm^3 of tumor volume or with medium alone. The viral treatment group showed stable and significant inhibition of tumorigenicity with apparent cure in four of five mice by day 35. Replication of viral DNA demonstrated by PCR amplification and expression of the inserted *LacZ* gene visualized by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside histochemistry was associated with oncolysis of *d*12.CALP-treated tumors, while sparing normal vascular smooth muscle cells. In mice harboring two SK-LMS-1 tumors, replication of *d*12.CALP was detected in a nontreated tumor distant from the site of virus inoculation. These results indicate that replication-competent virus vectors controlled by the calponin transcriptional regulatory sequence may be a new therapeutic strategy for treatment of malignant human soft tissue and bone tumors.

INTRODUCTION

Soft tissue and bone tumors represent ~1% of all primary tumors in Japan or 10,000 new cases per year in the United States (1). The overall prognosis of patients is poor because of the systemic metastasis. Despite intensive multimodal therapy such as surgery, chemotherapy, and radiotherapy, none of these have substantially changed the outcome of patients with the malignant tumors. Therefore, we are exploring a novel form of treatment using gene therapy techniques.

Calponin (h1 or basic) is an actin-associated protein originally isolated from smooth muscles of bovine aorta and chicken gizzard (2, 3). It has been suggested that the interaction of calponin with the glutamate residue at the COOH-terminus of the actin molecule inhibits a weak-to-strong transition of the actin-myosin binding, and, thus, plays a role in the reduction of cross-bridge cycling rate (4–8). The human *calponin* gene contains seven exons, spanning ~11.2 kb (9) on chromosome 19p13.2 in a region close to the *LDLR* and *MEF2B* genes (10). In normal tissues, calponin is most highly expressed in terminally differentiated and nonreplicating SMCs,[3] whereas proliferation of SMC down-regulates its expression (11). Recently, however, calponin protein and mRNA were shown to be aberrantly expressed in a variety of human soft tissue and bone tumors, including Kaposi's sarcoma (12), osteosarcoma (13), leiomyosarcoma (14–17), myofibroblastic tumor of bone (18), synovial sarcoma (15, 17), malignant fibrous histiocytoma (15, 17, 19), liposarcoma (15), malignant schwannoma (15), GISTs (17, 20, 21), malignant rhabdoid tumors (22), hemangioblastoma (23), parachordoma (24), and myoepithelial carcinoma of the salivary gland (25). Several reports demonstrated that calponin was expressed in 31% of GISTs ($n = 58$; 20), 91% of smooth muscle tumors ($n = 90$), and 32% of mesenchymal nonsmooth muscle tumors ($n = 131$; Ref. 17) at various levels in a diffuse or focal manner by immunohistochemistry. These observations suggest that, if expression of calponin in the tumor cells is regulated at the transcriptional level, the calponin promoter could be used to direct therapeutic genes or virus vectors to the human soft tissue and bone tumor cells expressing calponin. To date, no DNA sequences have been identified that can selectively drive expression of a gene in human soft tissue and bone tumors with sparing normal cells.

In the present study, we identified the transcriptional regulatory sequence of the calponin promoter in human soft tissue and bone tumor cells. We report here the construction of a novel replication-competent HSV vector, harboring the calponin promoter, which is highly effective in experimental gene therapy for the malignant human soft tissue and bone tumors.

MATERIALS AND METHODS

Cells, Culture Methods, Antibodies, and Viruses. Human leiomyosarcoma cell lines SK-LMS-1 (HTB-88), human osteosarcoma cell lines HOS (CRL-1543), MNNG-HOS (CRL-1547), and Vero cells (CCL-81) were purchased from American Type Culture Collection (Rockville, MD). Human leiomysarcoma cell line, SKN RCB0513, and cell line, OST/RCB0454, were from RIKEN GENE BANK (Tsukuba, Japan). Human synovial sarcoma and desmoid tumor cell lines were established from resected tumor samples from patients. Diagnosis of synovial sarcoma was confirmed by expression of the *SYT-SSX* fusion gene as described previously (15). Primary cultured HMCs, kindly provided by H. Yamabe (Hirosaki University School of Medicine,

Received 1/2/01; accepted 3/14/01.

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[1] Supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan; the Ministry of Health and Welfare, Japan; the Uehara Memorial Foundation (Tokyo); and Takeda Science Foundation (Osaka).

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[3] The abbreviations used are: SMC, smooth muscle cell; GIST, gastrointestinal stromal tumor; HSV, herpes simplex virus; HMC, human mesangial cell; HUVEC, human umbilical vein endothelial cell; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; MOI, multiplicity/multiplicities of infection; pfu, plaque-forming unit(s); FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR.

Hirosaki, Japan; Ref. 26), were prepared from the kidneys of human fetuses (16 and 18 weeks of gestation; established by Dr. M. R. Daha, University Hospital of Leiden) and used for experiments at passages 4–6. HUVECs (T200–05) were purchased from TOYOBO Biochemicals (Osaka, Japan). E5 cells, *ICP4* gene transfectants cloned from Vero cells, were kindly provided by N. Deluca (University of Pittsburgh School of Medicine, Pittsburgh, PA). SK-LMS-1 was cultured in Eagle's MEM supplemented with 1 mM sodium pyruvate. HOS, MNNG-HOS, OST, and Vero and E5 cells were cultured in DMEM. SKN cells were cultured in F12 medium. Synovial sarcoma cells and desmoid cells were cultured in RPMI 1640. HMCs were cultured in DMEM with 1 mg/ml D-glucose. All of the media were supplemented with 10%, 15% (for SKN) or 20% (for synovial sarcoma cells and desmoid cells) heat-inactivated FBS (Upstate Biotechnologies, Waltham, MA), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. HUVECs were cultured in the manufacturer's recommended medium. All of the cells were cultured at 37°C in humidified 5% CO_2.

A polyclonal antibody specific to mouse calponin (basic or h1) was prepared as described previously (27). A monoclonal antibody to HSV-1 and -2 ICP4 protein was purchased from Goodwin Institute for Cancer Research (clone no. 1101; Plantation, FL). Immunoblot analysis was carried out as described previously (13). Chemiluminescence (ECL; Amersham Pharmacia Biotech., Buckinghamshire, United Kingdom) was used to visualize the bound antibodies, according to the manufacturer's protocol.

ICP4 deletion mutant of HSV, *d*120 (28) and ICP6 (ribonucleotide reductase)-deletion mutant, hrR3 (29), provided by N. Deluca and S. Weller (University of Connecticut Health Center, Farmington, CT), respectively, were generated from low-multiplicity infections to E5 cells and Vero cells, respectively.

RNA Preparation and RT-PCR Analysis. Total RNA was extracted from cultured cells and tissues using the Isogene RNA extraction kit (Nippon Gene, Toyama, Japan), and subjected to the semiquantitative RT-PCR analysis as described previously (13). The parameters used for the PCR amplification were 30 cycles of denaturation (94°C, 40 s), annealing (60°C, 30 s), and polymerization (72°C, 90 s). Sequences of the selected forward and reverse 5′-to-3′ primers used, and predicted products size were as follows: human calponin (basic or h1), GAGTGTGCAGACGGAACTTCAGCC (forward) and GTCTGTGCCCAACTTGGGGTC (reverse), 671-bp; GAPDH, CCCATCACCATCTTCCAGGA (forward) and TTGTCATACCAGGAA ATGAGC (reverse), 731-bp.

Isolation of the Human Calponin Promoter. Genomic clones containing the 5′ flanking region of the human *calponin* gene were isolated by screening a human genomic λEMBL3 phage library as reported previously (9). The 5′ side-deleted fragments, p-1159, -385, -343, -310, -299, -288, -260, -239, -219, -201, -176, and -153Luc constructs were generated by PCR amplification and subsequent subcloning into the pGL2-basic vector (Promega, Madison, WI). Numbers indicate the 5′ end of the DNA fragments upstream from the ATG translational initiation codon, which hereafter will be designated as +1. These deletion fragments had common 3′ end at position +73. The nucleotide sequence of the cloned fragments was determined on both strands using a DSQ-2000L DNA sequencer (SHIMADZU, Kyoto, Japan) according to the manufacturer's instructions, and it was confirmed that the sequence was identical to the published sequence (DDBJ/GenBank/EMBL database; accession no. D85611; Ref. 9).

Transfection and Luciferase Assays. Cells were cultured in the indicated medium, split and plated 24 h prior to transfection. Cells (5×10^4) at ~70% confluence in a 6-well dish were transfected with 1.2 μg of the promoter plasmid, 0.3 μg of the pCAGGS/β-gal reference plasmid and 3.75 μl of FuGENE 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) in each well according to the manufacturer's protocol. Twenty-two h after transfection, cells were harvested in 100 μl/well of the cell lysis buffer (PicaGene Luciferase Assay System, Toyo Ink, Tokyo). After centrifugation at 12,000 × *g* for 5 min at 4°C, aliquots (20-μl and 30-μl) of the supernatants were used for the luciferase and β-galactosidase assays, respectively. Luciferase activity was measured by a BLR-201 luminescence reader (Aloka, Tokyo, Japan). β-Galactosidase activity was measured using the β-galactosidase enzyme assay system (Promega) as described previously (30). All of the experiments were repeated at least three times to check reproducibility. Luciferase activities (light unit) were corrected for variations in transfection efficiencies as determined by assaying cell extracts for β-galactosidase activity. Transfection efficiency of different cell lines was estimated by comparing expression of the *pSV2-Luc* gene containing the SV40 enhancer and promoter. Data are expressed as percentage of normalized light units ± SE relative to the values of *pSV2-Luc*.

Virus Preparations. The 4.1-kb blunt-ended *Sal*I-*Mse*I fragment of pGH108 (28; kindly provided by G. Hayward, Johns Hopkins School of Medicine, Baltimore, MD), containing ICP4 coding sequence, was subcloned into the blunt-ended *Hin*dIII site of pAMP1 vector downstream of the 333-bp human calponin promoter (−260 to +73) at the cloning region and the 444-bp *Not*I fragment of the human 4F2 heavy-chain transcriptional enhancer (Ref. 31; kindly provided by J. M. Leiden, Harvard Medical School, Boston, MA) at *Sma*I site of the vector. The pAMP1/CALP-ICP4 vector was double digested with *Sal*I and *Hin*dIII, and the resulting 4.7-kb fragment was subcloned into the blunt-ended *Xba*I site of the pTKΔL recombination vector. The pTKΔL recombination vector contains the TK-coding sequence with deletion of the 0.5-kb *Bgl*II-*Kpn*I region and the *Escherichia coli lacZ* and SV40 polyadenylation site upstream of the TK sequence (+53 of TK; 32). Linearized pTKΔL-CALP-ICP4 at *Sal*I site in the plasmid backbone and *d*120 DNA were cotransfected into E5 cells by using Lipofectoamine (Life Technologies, Inc.) as described by the manufacturer's protocol. Recombinant viruses, identified as a single plaque and named *d*12.CALP, were stained blue with X-gal agarose overlay and were plaque- purified three times by infecting onto E5 cells in the presence of ganciclovir (1 μg/ml). DNA was purified and the recombination was verified by restriction enzyme digestion, Southern blot, and PCR analyses.

Viruses were prepared by infecting ~20 150 cm^2/tissue culture flasks (IWAKI, Funabashi, Japan) with E5 cells and harvesting the detached cells after 48 h. Cells were collected by centrifugation at 400 × *g* for 5 min at 4°C, and resuspended in 10 ml of the cold virus buffer [20 mM Tris-HCl (pH7.5) containing 150 mM NaCl]. The cells were lysed with three cycles of freezing and thawing in combination with sonication (six times for 1 min). After centrifugation at 1500 × *g* for 5 min at 4°C, the supernatant was centrifuged at 15,000 × *g* for 45 min at 4°C. The resulting pellet was resuspended in the cold virus buffer, and titers of purified *d*12.CALP were determined by plaque assays on E5 cells.

***In Vitro* Cytopathic Assay and Viral Replication Assay.** Viruses were infected onto subconfluent monolayers of cells in 6-well tissue culture plates at a MOI of 0.01 or 0.001 pfu/cell in 1% heat inactivated FBS/PBS. The infected cells were incubated at 37°C for 1 h, and then cultured in an appropriate culture medium containing 1% FBS and 11.3 μg/ml human IgG whole molecule (Jackson ImmunoResearch Lab., West Grove, PA). Forty-eight h after infection, numbers of plaques/well were counted. For virus replication assay, monolayer cultures of SK-LMS-1 or OST cells in 12-well tissue culture plates (2×10^5 cells/well) were infected with *d*12.CALP at a MOI of 0.1 in 1% FBS/PBS. The virus inoculum was removed after 1 h, and the cells were incubated in the indicated culture medium. At the times indicated in Fig. 4 (12 h, 24 h, and 48 h), the infected cells were harvested from the wells with 100 μl of the virus buffer. Aliquots (1 μl) of the cell lysates were subjected to dilution at 10^{-3}, 10^{-4} and 10^{-5}, and then titers of viruses were determined on E5 cells.

For immunoblot analysis of ICP4 expression, SK-LMS-1 and OST cells were infected with *d*12.CALP at MOI of 0.01 or the virus buffer alone, cultured for 22 h, and then harvested. Equal amounts of proteins were electrophoresed in a 9% SDS-PAGE gel, and transferred to a nitrocellulose membrane (Bio-Rad, Richmond, VA). Membranes were blocked with 5% skim milk (Difco Laboratories, Detroit, MI) for 2 h at room temperature and then incubated with anti-ICP4 antibody (1:500 dilution) at 4°C overnight.

***In Vivo* Treatment and Histological Analysis.** Tumors were established by s.c. injection of SK-LMS-1 cells or OST cells into the flanks of female athymic nude mice (6-week old, BALB/c Slc-*nu/nu*; Nihon SLC, Hamamatsu, Japan). Tumors, through several passages in nude mice, were allowed to grow to 7 mm in diameter. For intratumor injection, 50 μl (per tumor volume of 100 mm^3) of virus suspension containing 1×10^7 pfu *d*12.CALP or equal volume of the virus buffer alone were injected using a 30-gauge needle. Treatment was repeated in an identical fashion 9 days later. Tumors were measured at the indicated times after injection, and tumor volumes were calculated using the formula, 0.53 × length × width (2). In experiments to test whether *d*12.CALP can be directed to tumors at distant sites, SK-LMS-1 xenografts were established in the flanks at both left and right sides of a male nude mouse, age 6-week old, and the viruses were injected into the tumor at one side.

For histological studies, tumor-bearing mice were killed on the indicated days after single treatment with *d*12.CALP in a dose of 1×10^7 pfu/tumor volume of 100 mm^3. The s.c. tumors were removed and fixed with 2% paraformaldehyde, 0.5% glutaraldehyde in PBS containing 1 mM $MgCl_2$ overnight at 4°C. The tumors were then placed in a substrate solution, containing X-gal (1 mg/ml), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 1 mM $MgCl_2$ in PBS for 3 h at 37°C, and then washed with PBS containing 3% DMSO.

To assess viral distribution by PCR analysis, DNA was prepared from fresh tissues of infected or noninfected tumors, and remote organs including brain, lung, liver, kidney, heart, small intestine, and uterus or testis. The conditions for PCR amplification were 30 cycles of denaturation (94°C, 40 s), annealing (60°C, 30 s), and polymerization (72°C, 90 s). Sequences of the selected forward and reverse 5′-to-3′ primers used, and predicted products size were as follows (33): ICP6 (ribonucleotide reductase), GACAGCCATATCCTGAGC (forward), ACTCACAGATCGTTGACG ACCG (reverse), 221-bp; glycoprotein E, GAGATGCGAATATACGAAT (forward), GTGGGTGGG CTCGGCCAAAT (reverse), 320-bp; *E. coli LacZ*, GCGTTACCCAACTTAATCG (forward), TGTGAGCGAGTAACAACC (reverse), 320-bp; and GAPDH, CCCATCACCATCTTCC AGGA (forward), TTGTCATACCAGGAA ATGAGC (reverse), 731-bp.

All of the animal procedures were approved by the Animal Care and Use Committee of Osaka Medical Center for Cancer and Cardiovascular Diseases.

Immunohistochemistry. The specimens were fixed in Bouin's solution [15% (v/v) saturated picric acid solution, 1.65% (v/v) formalin, and 1% (v/v) acetic acid/PBS] and were embedded in paraffin. Sections of 4-μm thickness were mounted on poly-L-lysin-coated microslides, deparaffinized in xylene, dehydrated through graded alcohol, and immersed in 70% methanol with H_2O_2 to block endogenous peroxidase. Then, antigen retrieval was performed using an autoclave at 121°C for 10 min in a 10-mM citrate buffer (pH 7.0). The sections were incubated with 1% (v/v) goat serum/PBS for 1 h at room temperature, washed in PBS, and incubated with a polyclonal antibody against mouse calponin (28) in 2% (w/v) BSA/PBS overnight at 4°C. They were then washed 5 times with 0.005% (v/v) Tween 20/PBS, followed by incubation with the biotinylated goat antirabbit IgG (TAGO Immunologicals, Camarillo, CA) in 2% (w/v) BSA/PBS for 1 h at room temperature and avidin-biotin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. After being washed in 0.005% (v/v) Tween 20/PBS, the final reaction product was visualized with diaminobenzidine (WAKO Chemicals, Osaka, Japan), and the sections were counterstained with hematoxylin. Tissue samples treated with goat serum were used to assess nonspecific stain.

Statistical Analysis. Statistical differences were determined by using the unpaired Student's *t* test. Differences were considered statistically significant with $P < 0.05$.

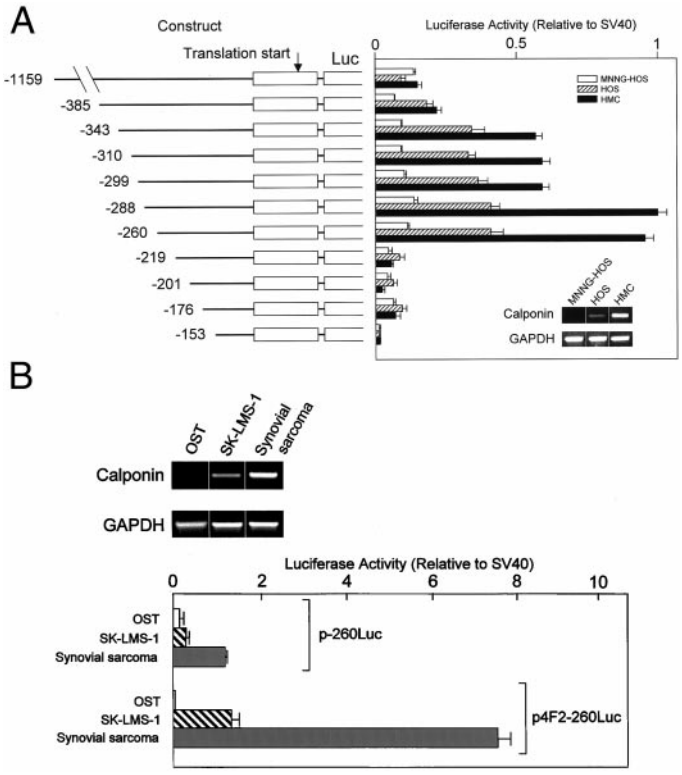

Fig. 1. Identification of transcriptional regulatory sequences of the proximal promoter of the human *calponin* gene by transient expression analysis of deletion constructs. *A*, MNNG-HOS and HOS cells and HMC mesangial cells were transfected with 5′-deleted promoter fragments linked to luciferase. Relative luciferase activities are expressed as values compared with that obtained with pSV2-Luc containing the SV40 enhancer and promoter (assigned a value of 1). The experiment was repeated three times and the data are means ± SE ($n = 6$ per group) from a representative experiment. *Inset*, expression of mRNAs for calponin (basic or h1) and GAPDH demonstrated by semiquantitative RT-PCR analysis. *B*, human calponin regulatory sequence can restrict luciferase expression to calponin-positive tumor cells. Expression of calponin mRNA in OST, SK-LMS-1 and synovial sarcoma cell lines is demonstrated by semiquantitative RT-PCR analysis. OST, SK-LMS-1, and synovial sarcoma cells were transfected with p-260Luc or p4F2–260Luc. Insertion of the 4F2 heavy-chain enhancer into the 5′ upstream region augmented transcriptional activity of the calponin promoter in the calponin-expressing cells. Relative luciferase activities are expressed as values compared with that obtained with pSV2-Luc containing the SV40 enhancer and promoter (assigned a value of 1). The experiment was repeated two times, and the data are means ± SE ($n = 6$ per group) from a representative experiment.

RESULTS

Identification of a Positive Regulatory Element of Human Calponin Promoter. To identify minimal positive regulatory element of the human calponin promoter, a series of 5′ deleted calponin promoter-luciferase constructs were transfected into human osteosarcoma cell lines MNNG-HOS and HOS, and HMC line, which stably displays smooth muscle-like phenotype such as a characteristic growth pattern of "hill and valley," and expresses smooth muscle-specific genes such as α-smooth muscle actin and SM22α.[4] The *calponin* gene was most highly expressed in HMCs (Fig. 1*A*). As reported previously (13), *calponin* was expressed in HOS cells at the intermediate level, but not in MNNG-HOS cells at all (Fig. 1*A*). Transient transfection assays of plasmids p-288Luc and p-260Luc into HOS and HMC cells resulted in luciferase activities that were four times (in HOS) and six times (in HMC) higher than that obtained with p-1159Luc, indicating that there is a negative regulatory element within −1159 to −288. There was a good correlation between expression of the *calponin* mRNA and transcriptional activities of the promoter region from −385 to −260. Further deletion from −260 to −219 was associated with a large decrease in the promoter activity in both HOS and HMC cells. Transfection of the more extensively deleted constructs (p-201Luc, p-176Luc, and p-153Luc) yielded relative luciferase activity comparable with that obtained with p-219Luc. These results indicate that the sequence between positions −260 and −219 includes elements involved in the positive control of *calponin* gene transcription in both HOS and HMC cells.

The region includes several sequence motifs similar to consensus binding sequences for Sox (AACAAT) at position −258 and GATA-1 (CACAATCAGC) at position −250. Deletion of p-260Luc to −239 was associated with a 50% decrease in transcriptional activity. To test whether the putative Sox and GATA-1 binding sites, and a region downstream to −239 were functionally active, mutations were introduced at positions −255/−254 (AA to GG), −246/−244 (A to G at −246 and C to T at −244), and −232/−231 (CC to TT) of the plasmid p-260Luc (data not shown). In transfection experiments in HMCs, p-260Luc containing each set of these mutations had 73 ± 0.2, 76 ± 0.2, and 39 ± 0.1%, respectively, of the activity of p-260Luc. These results suggest that the sequence encompassing −260 to −219 as a whole was required for the transcriptional activity of the calponin promoter.

[4] Y. Sugenoya, H. Yamamura, and K. Takahashi, submitted for publication.

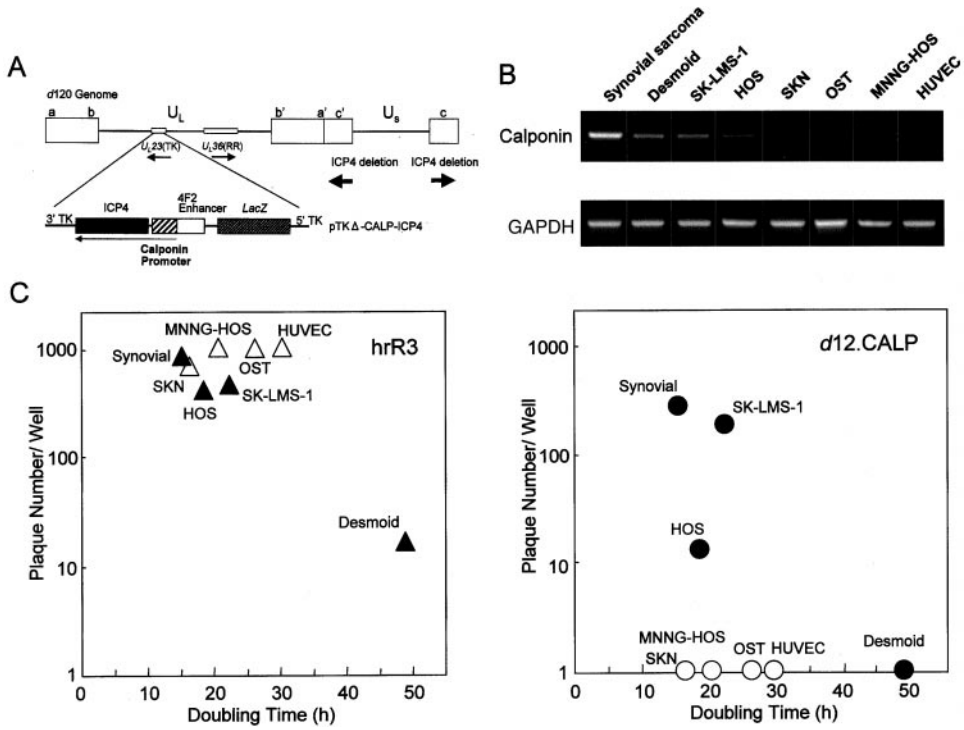

Fig. 2. Structure of *d*12.CALP and *in vitro* cytopathic assay. *A*, a general map of the ICP4-deficient strain *d*120 (28) demonstrates the unique long (U_L) and unique short (U_S) sequences of HSV-1 flanked by the long and short repeat sequences. *d*12.CALP carries a mutation in U_L23 gene encoding TK caused by the insertion of a targeting construct, pTKΔ-CALP-ICP4. *d*12.CALP expresses ICP4 under the control of calponin promoter and 4F2 heavy-chain enhancer and *LacZ* under the TK promoter. The U_L36 gene encodes a large subunit of ribonucleotide reductase (*RR*). *B* and *C*, calponin mRNA-positive (synovial sarcoma, desmoid, SK-LMS-1, and HOS) and mRNA-negative (SKN, OST and MNNG-HOS, and HUVEC endothelial cells) target cells ($2.5–5 \times 10^4$) as demonstrated by RT-PCR analysis (*B*) were plated in 6-well dishes 48–72 h before virus infection. The cells were infected with hrR3, an ICP6-deficient vector (29) that is not promoter regulated, or with *d*12.CALP at MOI of 0.001. hrR3 showed marked plaque formation irrespective of the expression of calponin but it was dependent on the rate of proliferation. In contrast, *d*12.CALP demonstrated cell destruction only in calponin-positive cells except for desmoid cells, which proliferate very slowly. ▲ and ●, cell lines expressing the calponin mRNA. The doubling time was calculated by counting the cell numbers from 24 to 72 h postplating. The data are means from triplicate cultures.

Expression of the *Calponin* Gene in Human Soft Tissue and Bone Tumor Cells Is Regulated at the Transcriptional Level. To further assess whether there is a correlation between *calponin* expression and transcriptional activity of the calponin promoter in human soft tissue and bone tumor cells, various human cell lines with or without *calponin* expression were transfected with p-260Luc or a construct containing the human 4F2 heavy-chain transcriptional enhancer (31) inserted upstream of p-260Luc (p4F2–260Luc). Expression of *calponin* mRNA was detected by RT-PCR analysis in synovial sarcoma and SK-LMS-1 leiomyosarcoma cells. By contrast, OST osteosarcoma cells exhibited little if any calponin expression (Fig. 1*B*). As shown in Fig. 1*B*, transcriptional activities of p-260Luc and p4F2–260Luc were correlated with expression levels of the transcripts of *calponin* mRNA in all of the cells examined. These observations indicate that the *calponin* gene expression in human soft tissue and bone tumor cells may be regulated at the transcriptional level by a 260-bp sequence upstream of the translation initiation site. Moreover, insertion of the heterologous 4F2 enhancer upstream of the calponin promoter increased transcriptional activity of p-260Luc by 3- to 5-fold in calponin-positive synovial sarcoma and SK-LMS-1 cells. Therefore, we used the 4F2 enhancer/-260 calponin promoter sequence to regulate expression of the HSV *ICP4* gene in other human soft tissue and bone tumor cells.

Selective Replication of a Recombinant HSV Vector in Calponin-positive Cells *in Vitro*. To construct a HSV vector that replicates selectively in calponin-positive and proliferating cells, *d*12.CALP was generated by inserting the DNA fragment containing 4F2 enhancer/-260 calponin promoter/ICP4 (pTKΔ-CALP-ICP4) into the *TK* locus (U_L23) of an ICP4$^-$ HSV mutant *d*120 (28). The plasmid pTKΔ-CALP-ICP4 contains two chimeric transgenes expressing ICP4 protein and β-galactosidase from *E. coli LacZ* insertion (Fig. 2*A*). Human cell lines with or without calponin expression were used to assess the selectivity of *d*12.CALP viral replication (Fig. 2*B*). The cells were infected with *d*12.CALP or hrR3 at a MOI of 0.001 for 48 h. Plaque formation was assessed to evaluate viral replication (Fig. 2*C*). In calponin-positive synovial sarcoma, SK-LMS-1, and HOS cells, *d*12.CALP had cytopathic effects comparable with hrR3. In contrast, there was no apparent cell lysis by *d*12.CALP on calponin-negative SKN, OST, MNNG-HOS and HUVEC cells. Although desmoid cells, which had the slowest replication rate, expressed calponin mRNA at levels comparable with SK-LMS-1 cells, there was no apparent plaque formation by *d*12.CALP. The results demonstrate that the cytopathic effect of *d*12.CALP is dependent on both calponin expression and the rate of proliferation of the cells. As shown in Fig. 3, infection of SK-LMS-1 and synovial sarcoma cells with *d*12.CALP at low MOI (0.001) resulted in complete oncolysis of the cultures in 10-cm dishes by 96 h after infection. We also noted a cell-to-cell spreading of cytolysis of synovial sarcoma cells (Fig. 3*A*). Some of the infected SK-LMS-1 cells became multinucleated before lysis (Fig. 3*B*, *arrow*).

Viral titers were assessed by single-step growth assays. *d*12.CALP replicated in calponin-positive SK-LMS-1 cells but the titers of *d*12.CALP were reduced by 6–7 logs in calponin-negative OST cells at 48 h postinfection (Fig. 4*A*). Consistent with the viral replication assay, immunoblot of cell extracts at 22 h postinfection demonstrated expression of ICP4 protein in SK-LMS-1 cells, but not in OST cells (Fig. 4*B*). In contrast, *d*120 viral vector did not yield viral progenies at all in either SK-LMS-1 or OST cultures (data not shown).

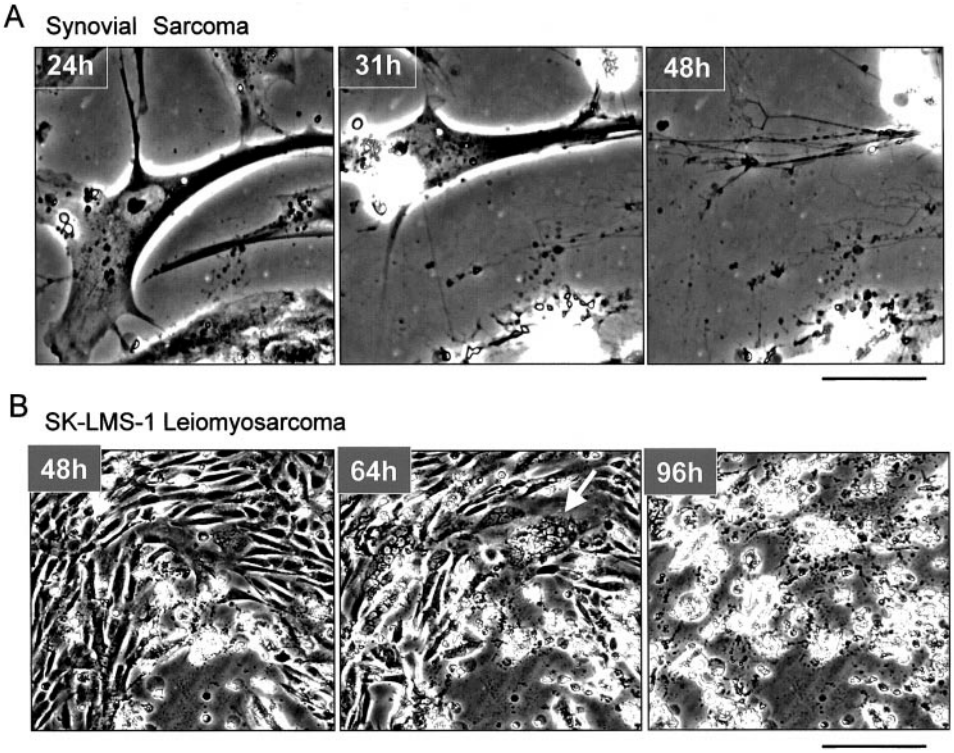

Fig. 3. Destruction of tumor cells by *d*12.CALP *in vitro*. *A*, destruction of synovial sarcoma cells; *bar*, 10 μm. *B*, extensive destruction of SK-LMS-1 cells. *Arrow*, multinucleated cells immediately before cytolysis; *bar*, 100 μm. Infected cells were photographed at the indicated time (*h*) postinfection.

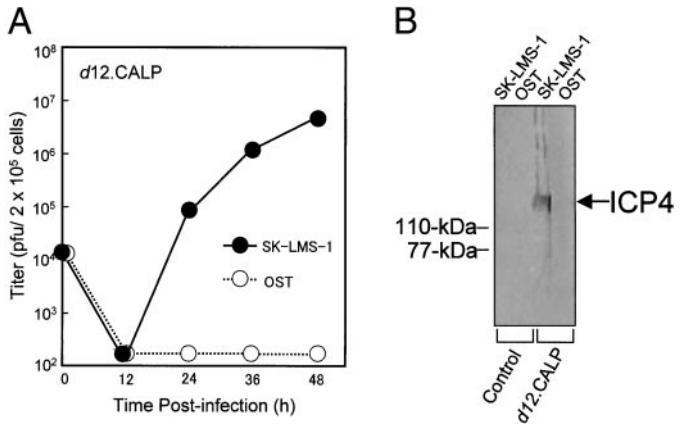

Fig. 4. Selective replication of *d*12.CALP in calponin-positive cells *in vitro*. *A*, single-step growth curves for *d*12.CALP in calponin-positive SK-LMS-1 cells and calponin-negative OST cells. Monolayers of cells (2×10^4) in 12-well dishes were infected with *d*12.CALP at MOI of 0.1 at 24 h postplating. At the times indicated postinfection, virus was harvested and titrated on E5 ($ICP4^+$) cells. The virus yield (*pfu*) per well of infected cells was determined. The data are means from triplicate cultures. *B*, cells were infected with *d*12.CALP at a MOI of 0.01. Lysates from control (noninfected) and infected cells at 22 h postinfection were subjected to immunoblot analysis with anti-ICP4 antibody. Molecular weight markers (Bio-Rad, Hercules, CA) used are phosphorylase b [M_r 110,000 (110-kDa)] and BSA [M_r 77,000 (77-kDa)].

Treatment of Human Leiomyosarcoma Xenografts with a Recombinant HSV Vector. To evaluate the therapeutic efficacy of *d*12.CALP *in vivo*, SK-LMS-1 leiomyosarcoma xenografts were established in nude mice, and then 1×10^7 pfu *d*12.CALP per tumor volume of 100 mm^3 were injected into the xenografts twice. As a control, virus buffer alone was injected into tumors. There was no significant difference in tumor volumes (138 ± 20 *versus* 139 ± 28 mm^3; $n = 5$) nor in the expression levels of immunoreactive calponin between *d*12.CALP-treated and control tumors before treatment (data not shown). Infection with *d*12.CALP was associated with the inhibition of SK-LMS-1 tumor growth but not with the inhibition of calponin-negative OST tumor (Fig. 5*A*). In contrast, treatment of SK-LMS-1 xenografts with virus buffer alone was associated with the progressive tumor growth and death of all of the animals ($n = 5$) by 89 days after treatment (Fig. 5*B*). By 5 weeks after the initial *d*12.CALP infection, tumors were completely regressed in four of five mice (Fig. 6). One animal exhibited tumor regrowth. Retreatment of the recurrent tumor with *d*12.CALP resulted in stable suppression of tumor growth.

Expression of β-galactosidase from the *LacZ* insertion in the *TK* locus was identified in *d*12.CALP-treated SK-LMS-1 tumors (Fig. 7, *A* and *B*), but not control tumors by histochemical staining with X-gal. This identifies a region of active *d*12.CALP viral spread *in vivo*. Necrosis was evident at day 8, and this region was devoid of *LacZ* expression (Fig. 7*A*, *arrows*). At higher magnification, some of the blue-stained tumor cells appeared multinucleated as observed in cytopathic assays *in vitro* (Fig. 7*C*, *arrows*), losing the typical morpho-

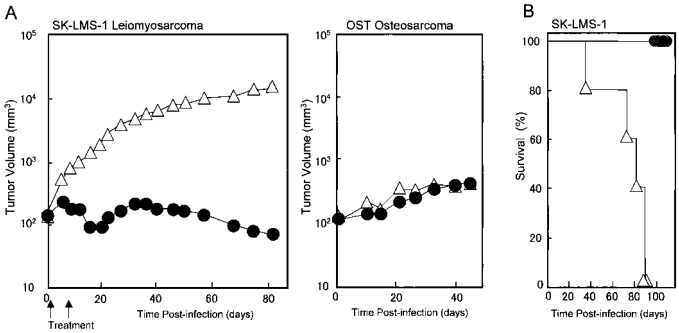

Fig. 5. *d*12.CALP inhibits tumor growth in vivo. *A*, effects of *d*12.CALP on growth of SK-LMS-1 and OST tumor xenografts in nude mice. Groups of mice (n = 5) were treated with 1×10^7 pfu/100 mm^3 tumor volume of *d*12.CALP (●) by intratumoral injection on days 0 and 9. An equal volume of virus buffer (see "Materials and Methods") was injected as a control (△). Calponin-expressing SK-LMS-1 human leiomyosarcoma grown in nude mice were effectively treated by two doses of d12.CALP (left), whereas calponin-nonexpressing OST tumors were unaffected (*right*). The data are means from 5 animals. *B*, survival of the mice with SK-LMS-1 tumors ($n = 5$ per group). All of the mice treated with *d*12.CALP (●) were alive as of April, 15, 205 days after treatment.

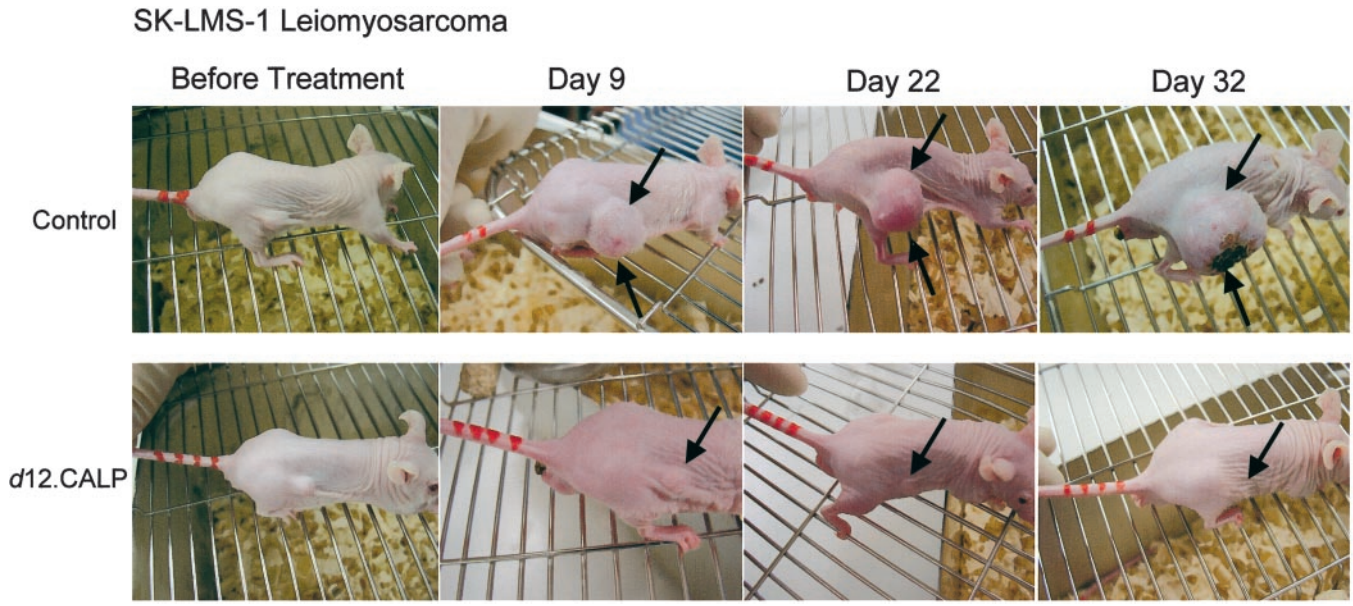

Fig. 6. Complete regression of a SK-LMS-1 xenograft in *d*12.CALP-treated nude mice. The *d*12.CALP-treated mice is one of the four cured cases described in Fig. 5. The control (buffer-injected) mice died at day 36.

logical appearance of SK-LMS-1 cells. As shown in Fig. 7*D*, however, SMCs surrounding normal blood vessels in virus-infected mice were negative for *LacZ* expression. Furthermore, on the PCR analysis, the *d*12.CALP-specific *LacZ* sequence was not detected in DNA prepared from brain, lung, liver, kidney, heart, small intestine, or uterus at day 8 after intratumor injection of *d*12.CALP (Fig. 7*E*). Histologically, there was no evidence of virus replication and *LacZ* expression in remote organs, including aortic and gastrointestinal smooth muscles (data not shown).

Spread of a Recombinant HSV Vector in Distant Tumors. To assess whether *d*12.CALP, which was injected and replicating in a SK-LMS-1 xenograft, could target tumor cells at distant sites via blood vessels, we investigated viral distribution in a SK-LMS-1 xenograft in the left flank after intratumoral inoculation of *d*12.CALP into a SK-LMS-1 xenograft in the right flank. As shown in Fig. 8*A*, there was detectable β-galactosidase expression at day 20 in the nontreated tumor on the opposite flank as well as in the site of inoculation. Histologically, there was extensive tumor necrosis in both treated and nontreated tumors (data not shown). As shown in Fig. 8*B*, however, calponin-positive SMCs surrounding normal blood vessels were not affected by *d*12.CALP infection. Spreading of viral DNA from *d*12.CALP in the tumor tissues in both flanks but not in brain or testis was demonstrated by PCR amplification, using primers specific to the sequence from ribonucleotide reductase (ICP6), glycoprotein E, and *E. coli LacZ* inserted into the *TK* locus (Fig. 8*C*).

DISCUSSION

We report here a novel therapeutic strategy targeting malignant human soft tissue and bone tumors, using the human calponin promoter. We showed that aberrant expression of the *calponin* gene in human soft tissue and bone tumor cells is transcriptionally regulated. Driving the ICP4 expression by the calponin promoter/4F2 heavy-chain transcriptional enhancer confers a high degree of specificity to an engineered replication-competent HSV mutant *d*12.CALP. The virus vector replicates selectively in calponin-positive and proliferating human soft tissue and bone tumors and is oncolytic both in cell culture and in nude mice.

Soft tissue and bone tumors are a heterogeneous group of tumors that are classified on a histogenetic basis according to the adult tissue they resemble (1) and are thought to originate from pluripotent mesenchymal cells. In previous studies, we demonstrated that calponin was expressed in the multipotential mesenchymal cells in embryos and undifferentiated osteoblasts (27). Shah *et al.* (34) reported that calponin-positive mesenchymal cells could be generated from the neural crest stem cells by stimulation with TGF-β superfamily members. Terminal differentiation of osteoblast-lineage cells into bone cells was associated with down-regulation of the calponin expression (27). A subset of human soft tissue and bone tumors, transformed mesenchymal cells, may be blocked in a proto-differentiated state and, thus, may constitutively synthesize the *calponin* gene products. Transient expression assays using a series of deleted constructs of the calponin promoter uncovered a minimal positive regulatory element from position -260 that could faithfully discriminate calponin-positive cells from negative cells (Fig. 1). The finding that deletions at either end of this region are associated with decreased or complete loss of transcriptional activity suggested regulation by at least two distinct negative (-385 and -288) and positive (-260 and -219) *cis*-acting elements. Insertion of 4F2 heavy-chain enhancer into the calponin promoter displayed enhancement of transcriptional activity in calponin-positive SK-LMS-1 cells and synovial sarcoma cells. By contrast, the 4F2 enhancer suppressed the transcriptional activity in calponin-negative OST cells by currently unknown mechanisms, and, thus, it increased the target cell specificity.

A major challenge of cancer gene therapy for solid tumors is to increase the efficacy of intratumoral distribution of vectors. Although replication-competent virus vectors hold promise in an attempt to overcome this problem (35) and are being tested clinically (36, 37), one of the limitations of this vector system for cancer therapy in the extracranial tissues may be the nonspecific cytopathic effects on both tumor cells and nontarget proliferating cells. To circumvent this limitation, the albumin promoter has been used with a HSV mutant that could target hepatoma cells (38). Insertion of the PSA promoter and glandular kallikrein promoter into adenovirus to drive E1A and E1B, respectively, has resulted in selective replication in prostate cancer cells (39). Kurihara *et al.* (40) reported that an adenovirus vector in which the *DF3/MUC1* promoter drives expression of *E1A*

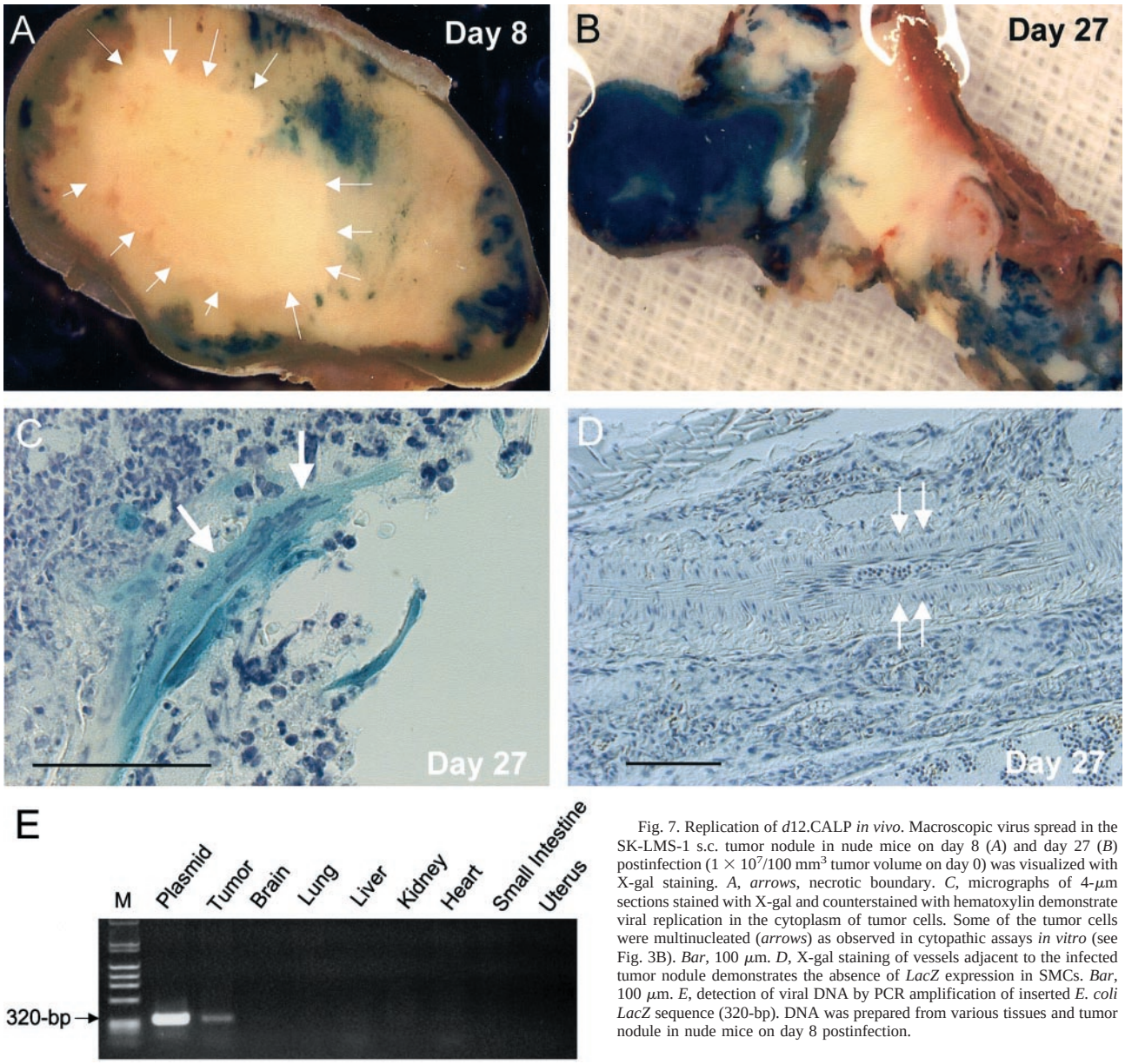

Fig. 7. Replication of *d*12.CALP *in vivo*. Macroscopic virus spread in the SK-LMS-1 s.c. tumor nodule in nude mice on day 8 (*A*) and day 27 (*B*) postinfection (1×10^7/100 mm^3 tumor volume on day 0) was visualized with X-gal staining. *A*, *arrows*, necrotic boundary. *C*, micrographs of 4-μm sections stained with X-gal and counterstained with hematoxylin demonstrate viral replication in the cytoplasm of tumor cells. Some of the tumor cells were multinucleated (*arrows*) as observed in cytopathic assays *in vitro* (see Fig. 3B). *Bar*, 100 μm. *D*, X-gal staining of vessels adjacent to the infected tumor nodule demonstrates the absence of *LacZ* expression in SMCs. *Bar*, 100 μm. *E*, detection of viral DNA by PCR amplification of inserted *E. coli LacZ* sequence (320-bp). DNA was prepared from various tissues and tumor nodule in nude mice on day 8 postinfection.

gene selectively replicates in MUC1-positive breast cancer cells. Other studies with an *E1B* gene-deleted adenovirus mutant have demonstrated selective replication in *p53*-mutant tumor cells (41). In this context, using the calponin promoter to direct cancer-specific gene expression of replication-competent viruses seems to be suitable for sparing nontarget cells, because most of the calponin-positive SMCs are nonreplicating, and calponin expression is down-regulated concomitantly with proliferation of SMCs (42). In fact, normal vessels containing calponin-positive SMCs adjacent to the infected tumor masses showed loss of *LacZ* expression and no significant toxicity (Figs. 7*D* and 8*B*).

In human sarcomas, to our knowledge, there are no previous reports of using a tumor tissue-specific or -selective DNA sequences to selectively destroy tumor cells. Milas *et al.* (43) demonstrated that adenovirus-mediated *p53* gene delivery into SK-LMS-1 leiomyosarcoma cells *in vivo* resulted in a 35-day tumor growth delay and tumor regression of nearly 40% of mice. In our present study with *d*12.CALP, stable and complete tumor regression lasting for more than 200 days was obtained in four of five mice with virus dose per mouse by some 2 logs lower than the replication-defective adenovirus vector expressing *p53* (6×10^9 pfu *versus* 3×10^7 pfu; Figs. 5 and 6). In our preliminary experiments, when tumor mass before treatment was smaller than 100 mm^3, a single intratumoral inoculation of *d*12.CALP at a virus dose of 1×10^6 pfu/100 mm^3 of tumor volume was sufficient for complete regression of SK-LMS-1 tumors within 3 weeks (data not shown). The cytopathic effects of *d*12.CALP was not caused by nonspecific viral toxicity because *d*12.CALP had no effects on growth of calponin-negative OST tumors in cell culture and in nude mice. Moreover, we found that *d*12.CALP replicated within tumors and spread to distant tumor sites most probably via s.c. blood vessels (Fig. 8). We also presented evidence of viral replication even in the distant tumors. These findings indicate that *d*12.CALP, with the capacity to selectively replicate in the calponin-positive tumors while sparing nontarget cells such as calponin-negative proliferating cells and calponin-positive nonproliferating SMCs, has the potential for greater efficacy as an antineoplastic agent than that achieved with replication-defective viruses, and, more importantly, it has the potential for targeting metastatic tumors.

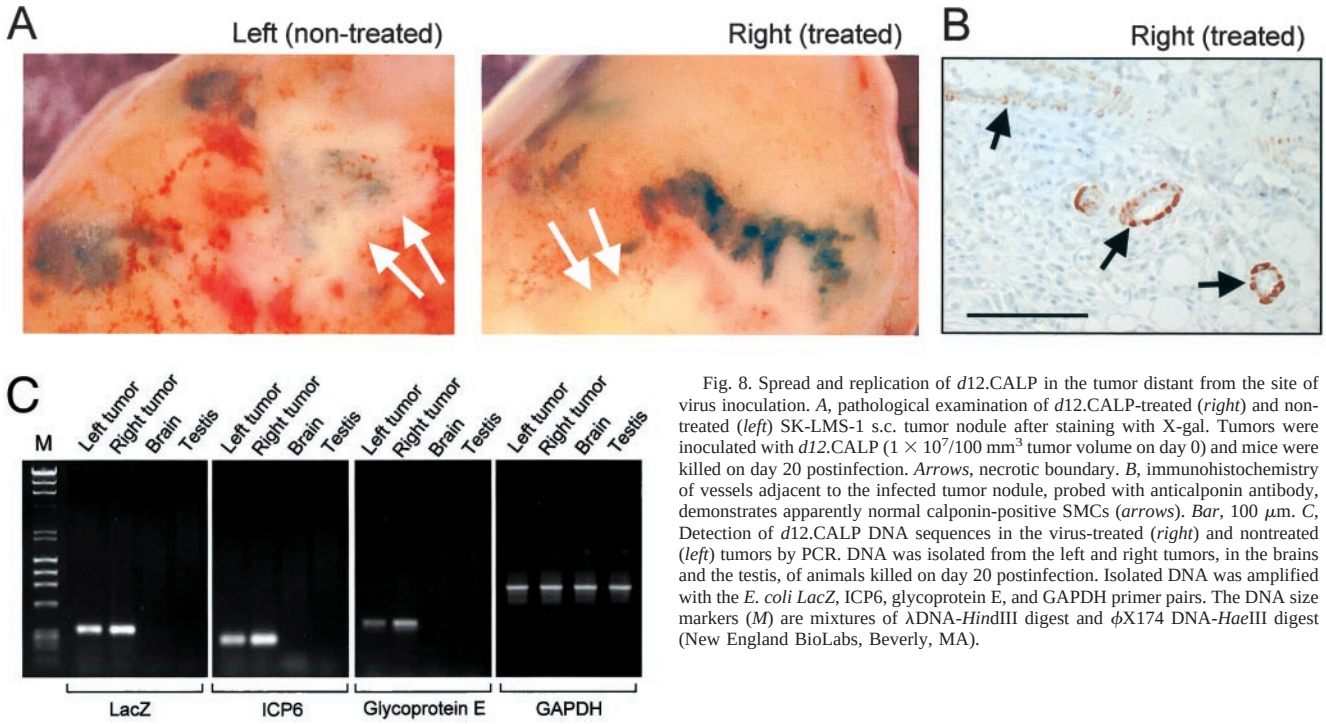

Fig. 8. Spread and replication of *d*12.CALP in the tumor distant from the site of virus inoculation. *A*, pathological examination of *d*12.CALP-treated (*right*) and nontreated (*left*) SK-LMS-1 s.c. tumor nodule after staining with X-gal. Tumors were inoculated with *d12*.CALP (1×10^7/100 mm^3 tumor volume on day 0) and mice were killed on day 20 postinfection. *Arrows*, necrotic boundary. *B*, immunohistochemistry of vessels adjacent to the infected tumor nodule, probed with anticalponin antibody, demonstrates apparently normal calponin-positive SMCs (*arrows*). *Bar*, 100 μm. *C*, Detection of *d*12.CALP DNA sequences in the virus-treated (*right*) and nontreated (*left*) tumors by PCR. DNA was isolated from the left and right tumors, in the brains and the testis, of animals killed on day 20 postinfection. Isolated DNA was amplified with the *E. coli LacZ*, ICP6, glycoprotein E, and GAPDH primer pairs. The DNA size markers (*M*) are mixtures of λDNA-*Hin*dIII digest and ϕX174 DNA-*Hae*III digest (New England BioLabs, Beverly, MA).

Nearly 90% of primary human smooth muscle tumors (17), 30% of GIST (17, 20), 40 to 60% of synovial sarcoma (15, 17) and 60% of osteosarcoma (13) express calponin at various levels. A major implication of this study is that replication-competent viruses, when replication is controlled by the tumor-responsive calponin regulatory sequence, offer a novel therapeutic strategy for these malignancies, which have fewer choices for effective treatment as compared with other cancers. To improve efficacy and safety for preclinical and clinical testing, we are constructing new conditionally replicating oncolytic HSV vectors, in which the calponin regulatory sequence drives the *ICP4* gene and a gene with antineoplastic activity via bicistronic expression. These strains of HSV carry intact *TK* gene and a mutation in the U_L39 gene, which encodes the large subunit of HSV ribonucleotide reductase (ICP6), and show hypersensitivity to acyclovir and ganciclovir, an attractive safety feature for clinical use (44).

ACKNOWLEDGMENTS

We are grateful to Drs. Robert Martuza, Samuel Rabkin, and Tomoki Todo (Harvard Medical School), and Dr. Izumu Saito (Tokyo University) for valuable discussion and advice. We thank Drs. Tadao Serikawa and Nobuo Hashimoto (Kyoto University) for giving us permission to use the Animal Center at Kyoto University, and Tomoko Nogami (Osaka Medical Center) for technical assistance. We also thank Dr. Yasuji Oshima (Japan Science and Technology Corporation, Kansai University) for valuable advice and encouragement.

REFERENCES

1. Eizinger, F. M., and Weiss, S. W. (eds.), Soft Tissue Tumors, Ed. 3, pp. 1–16. St. Louis: Mosby Year Book, Inc., 1994.
2. Takahashi, K., Hiwada, K., and Kokubu, T. Vascular smooth muscle calponin: a novel troponin T-like protein. Hypertension, *11:* 620–626, 1988.
3. Takahashi, K., and Nadal-Ginard, B. Molecular cloning and sequence analysis of smooth muscle calponin. J. Biol. Chem., *266:* 13284–13288, 1991.
4. Haeberle, J. R. Calponin decreases the rate of cross-bridge cycling and increases maximum force production by smooth muscle myosin in an *in vitro* motility assay. J. Biol. Chem., *269:* 12424–12431, 1994.
5. EL-Mezgueldi, M., and Marston, S. B. The effects of smooth muscle calponin on the strong and weak myosin binding sites of F-actin. J. Biol. Chem., *271:* 28161–28167, 1996.
6. Hodgkinson, J. L., El-Mezgueldi, M., Craig, R., Vibert, P., Marston, S. B., and Lehman, W. 3-D image reconstruction of reconstituted smooth muscle thin filaments containing calponin: visualization of interactions between F-actin and calponin. J. Mol. Biol., *273:* 150–159, 1997.
7. Matthew, J. D., Khromov, A. S., McDuffie, M. J., Somlyo, A. V., Somlyo, A. P., Taniguchi, S., and Takahashi, K. Contractile properties and proteins of a calponin knockout mouse. J. Physiol. (Lond.), *529:* 811–824, 2000.
8. Takahashi, K., Yoshimoto, R., Fuchibe, K., Fujishige, A., Mitsui-Saito, M., Hori, M., Ozaki, H., Yamamura, H., Awata, N., Taniguchi, S., Katsuki, M., Tsuchiya, T., and Karaki, H. Regulation of shortening velocity by calponin in intact contracting smooth muscles. Biochem. Biophys. Res. Commun., *279:* 150–157, 2000.
9. Takahashi, K., Tazunoki, T., Okada, T., Ohgami, K., Miwa, T., Miki, A., and Shibata, N. The 5′-flanking region of the human smooth muscle cell *calponin* gene contains a *cis*-acting domain for interaction with a methylated DNA-binding transcription repressor. J. Biochem. (Tokyo), *120:* 18–21, 1996.
10. Miano, J. M., Krahe, R., Garcia, E., Elliott, J. M., and Olson, E. N. Expression, genomic structure and high resolution mapping to 19p13.2 of the human smooth muscle cell *calponin* gene. Gene (Amst.), *197:* 215–224, 1997.
11. Owens, G. Regulation of differentiation of vascular smooth muscle cells. Physiol. Rev., *75:* 487–517, 1995.
12. Lebbe, C., de Cremoux, P., Millot, G., Podgorniak, M. P., Verola, O., Berger, R., Morel, P., and Calvo, F. Characterization of *in vitro* culture of HIV-negative Kaposi's sarcoma-derived cells. *In vitro* responses to α interferon. Arch. Dermatol. Res., *289:* 421–428, 1997.
13. Yamamura, H., Yoshikawa, H., Tatsuta, M., Akedo, H., and Takahashi, K. Expression of the smooth muscle *calponin* gene in human osteosarcoma and its possible association with prognosis. Int. J. Cancer, *79:* 245–250, 1998.
14. Horiuchi, A., Nikaido, T., Ito, K., Zhai, Y-L., Orii, A., Taniguchi, S., Toki, T., and Fujii, S. Reduced expression of calponin h1 in leiomyosarcoma of the uterus. Lab. Investig., *78:* 839–846, 1998.
15. Ono, H., Yoshikawa, H., Ueda, T., Yamamura, H., Kudawara, I., Manou, M., Ishiguro, S., Funai, H., Koyanagi, Y., Araki, N., Hashimoto, N., Sonobe, H., Tatsuta, M., and Takahashi, K. Expression of smooth muscle calponin in synovial sarcoma. Sarcoma, *3:* 107–113, 1999.
16. Jenson, H. B., Montalvo, E. A., McClain, K. L., Ench, Y., Heard, P., Christy, B. A., Dewalt-Hagan, P. J., and Moyer, M. P. Characterization of natural Epstein-Barr virus infection and replication in smooth muscle cells from a leiomyosarcoma. J. Med. Virol., *57:* 36–46, 1999.
17. Miettinen, M. M., Sarlomo-Rikka, M., Kovatich, A. J., and Lasota, J. Calponin and h-caldesmon in soft tissue tumors: consistent h-caldesmon immunoreactivity in gastrointestinal stromal tumors indicates traits of smooth muscle differentiation. Mod. Pathol., *12:* 756–762, 1999.
18. Folpe, A. L., Wirganowicz, P. Z., O'Donnel, R. J., and Gown, A. M. Myofibroblastic tumour of bone with abundant intra- and extracellular actin: report of a unique tumour, with immunohistochemical and ultrastructural characterization. Histopathology, *33:* 485–494, 1998.
19. Fanburg-Smith, J. C., and Miettinen, M. M. Angiomatoid. "malignant" fibrous histiocytoma: a clinicopathologic study of 158 cases and further exploration of the myoid phenotype. Hum. Pathol., *30:* 1336–1343, 1999.

20. Tazawa, K., Tsukada, K., Makuuchi, H., and Tsutsumi, Y. An immunohistochemical and clinicopathological study of gastrointestinal stromal tumors. Pathol. Int., *49:* 786–798, 1999.
21. Tsutsumi, Y., Tazawa, K., and Shibuya, M. Type VI collagen immunoreactivity in skenoid fibers in small intestinal stromal tumors. Pathol. Int., *49:* 836–839, 1999.
22. Sugimoto, T., Hosoi, H., Horii, Y., Ishida, H., Mine, H., Takahashi, K., Abe, T., Ohta, S., and Sawada, T. Malignant rhabdoid-tumor cell line showing neural and smooth-muscle-cell phenotypes. Int. J. Cancer, *82:* 678–686, 1999.
23. Fanburg-Smith, J. C., Gyure, K. A., Michal, M., Katz, D., and Thompson, L. D. R. Retroperitoneal peripheral hemangioblastoma: a case report and review of the literature. Ann. Diagn. Pathol., *4:* 81–87, 2000.
24. Folpe, A. L., Agoff, S. N., Willis, J., and Weiss, S. W. Parachordoma is immunohistochemically and cytogenetically distinct from axial chordoma and extraskeletal myxoid chondrosarcoma. Am. J. Surg. Pathol., *23:* 1059–1067, 1999.
25. Savera, A. T., Sloman, A., Huvos, A. G., and Klimstra, D. S. Myoepithelial carcinoma of the salivary glands: a clinicopathologic study of 25 patients. Am. J. Surg. Pathol., *24:* 761–774, 2000.
26. Yamabe, H., Osawa, H., Inuma, H., Kaizuka, M., Tamura, N., Tunoda, S., Baba, Y., Shirato, K., and Onodera, K. Thrombin stimulates production of transforming growth factor-β by cultured human mesangial cells. Nephrol. Dial. Transplant., *12:* 438–442, 1997.
27. Yoshikawa, H., Taniguchi, S., Yamamura, H., Mori, S., Sugimoto, M., Miyado, K., Nakamura, K., Nakao, K., Katsuki, M., Shibata, N., and Takahashi, K. Mice lacking smooth muscle calponin display increased bone formation that is associated with enhancement of bone morphogenetic protein responses. Genes Cells, *3:* 685–695, 1998.
28. DeLuca, N. A., MacCarthy, A. M., and Schaffer, P. A. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. J. Virol., *56:* 558–570, 1985.
29. Goldstein, D. J., and Weller, S. K. Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: isolation and characterization of an ICP6 *lac*Z insertion mutant. J. Virol., *62:* 196–205, 1988.
30. Yamamura, H., Masuda, H., Ikeda, W., Tokuyama, T., Takagi, M., Shibata, N., Tatsuta, M., and Takahashi, K. Structure and expression of the human *SM22*α gene, assignment of the gene to chromosome 11, and repression of the promoter activity by cytosine DNA methylation. J. Biochem. (Tokyo), *122:* 157–167, 1997.
31. Karpinski, B. A., Yang, L. H., Cacheris, P., Morle, G. P., and Leiden, J. M. The first intron of the 4F2 heavy-chain gene contains a transcriptional enhancer element that binds multiple nuclear proteins. Mol. Cell. Biol., *9:* 2588–2597, 1989.
32. Miyatake, S., Iyer, A., Martuza, R. L., and Rabkin, S. D. Transcriptional targeting of herpes simplex virus for cell-specific replication. J. Virol., *71:* 5124–5132, 1997.
33. Sundaresan, P., Hunter, W. D., Martuza, R. L., and Rabkin, S. D. Attenuated, replication-competent herpes simplex virus type 1 mutant G207: safety evaluation in mice. J. Virol., *74:* 3832–3841, 2000.
34. Shah, N. M., Groves, A. K., and Anderson, D. J. Alternative neural crest cell fate are instructively promoted by TGF β superfamily members. Cell, *85:* 331–343, 1996.
35. Martuza, R. L., Malick, A., Markert, J. M., Ruffner, K. L., and Coen, D. M. Experimental therapy of human glioma by means of a genetically engineered virus mutant. Science (Wash. DC), *252:* 854–856, 1991.
36. Rampling, R., Cruickshank, G., Papanastassiou, V., Nicoll, J., Hadley, D., Brennan, D., Petty, R., MacLean, A., Harland, J., McKie, E., Mabbs, R., and Brown, M. Toxicity evaluation of replication-competent herpes simplex virus (ICP 34.5 null mutant 1716) in patients with recurrent malignant glioma. Gene Ther., *7:* 859–866, 2000.
37. Markert, J. M., Medlock, M. D., Rabkin, S. D., Gillespie, G. Y., Todo, T., Hunter, W. D., Palmer, C. A., Feigenbaum, F., Tornatore, C., Tufaro, F., and Martuza, R. L. Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: results of a Phase I trial. Gene Ther., *7:* 867–874, 2000.
38. Miyatake, S., Tani, S., Feigenbaum, F., Sundaresan, P., Toda, H., Narumi, O., Kikuchi, H., Hashimoto, N., Hangai, M., Martuza, R. L., and Rabkin, S. D. Hepatoma-specific antitumor activity of an albumin enhancer/promoter regulated herpes simplex virus *in vivo*. Gene Ther., *6:* 564–572, 1999.
39. Yu, D-C., Sakamoto, G. T., and Henderson, D. R. Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of carydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. Cancer Res., *59:* 1498–1504, 1999.
40. Kurihara, T., Brough, D. E., Kovesdi, I., and Kufe, D. W. Selectivity of a replication-competent adenovirus for human breast carcinoma cells expressing the MUC1 antigen. J. Clin. Investi., *106:* 763–771, 2000.
41. Kirn, D., Hermiston, T., and McCormick, F. ONYX-015: clinical data are encouraging. Nat. Med., *4:* 1341–1342, 1998.
42. Gimona, M., Herzog, M., Vandekerckhove, J., and Small, J. V. Smooth muscle specific expression of calponin. FEBS Lett., *274:* 159–162, 1990.
43. Milas, M., Yu, D., Lang, A., Ge, T., Feig, B., El-Nagger, A. K., and Pollack, R. E. Adenovirus-mediated *p53* gene delivery inhibits human sarcoma tumorigenicity. Cancer Gene Ther., *7:* 422–429, 2000.
44. Martuza, R. L. Conditionally replicating herpes vectors for cancer therapy. J. Clin. Investi., *105:* 841–846, 2000.