[CANCER RESEARCH 63, 2506–2512, May 15, 2003]

Identification of the Human IAI.3B Promoter Element and Its Use in the Construction of a Replication-selective Adenovirus for Ovarian Cancer Therapy[1]

Katsuyuki Hamada,[2] Shohei Kohno, Mari Iwamoto, Hiroko Yokota, Masato Okada, Masatoshi Tagawa, Susumu Hirose, Kenshi Yamasaki, Yuji Shirakata, Koji Hashimoto, and Masaharu Ito

Departments of Obstetrics and Gynecology [K. H., M. Iw., H. Y., M. It.], Neurosurgery [S. K.] and Dermatology [K. Y., Y. S., K. H.], School of Medicine, Ehime University, Ehime 791-0295; Division of Oncogene Research, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871 [M. O.]; Division of Pathology, Chiba Cancer Center Research Institute, Chiba 260-8717 [M. T.]; and Department of Developmental Genetics, National Institute of Genetics, Shizuoka 411-8540 [S. H.] Japan

ABSTRACT

Little is known concerning promoters or gene therapy specific for ovarian cancer. To explore the potential use of *IAI.3B* isolated from ovarian cancer cells in gene therapy for ovarian cancer, we identified the promoter region of the *IAI.3B* gene and created a replication-selective adenovirus, AdE3-*IAI.3B*, driven by the promoter. Transient transfection experiments showed that the DNA segment located between −1816 and −1 bp resulted in preferential expression in ovarian cancer cells with negligible expression in squamous cell carcinoma and normal cells. The promoter activity of *IAI.3B* was almost the same as that of *cytomegalovirus* and an order of magnitude higher than those of *midkine* and *cyclooxygenase-2* in ovarian cancer cells. AdE3-*IAI.3B* replicated as efficiently as the wild-type adenovirus and caused extensive cell killing in a panel of ovarian cancer cells *in vitro*. In contrast, squamous cell carcinoma and normal cells were not able to support AdE3-*IAI.3B* replication. In animal studies, AdE3-*IAI.3B* administered to flank and i.p. xenografts of ovarian cancer cells led to a significant therapeutic effect. These results demonstrate the usefulness of the *IAI.3B* promoter for generation of ovarian cancer-specific adenoviral vectors and provide a potential for the development of ovarian cancer-specific oncolytic viral therapies.

INTRODUCTION

Sufficient encouraging preclinical results have been obtained to justify initiation of clinical Phase I trials of gene therapy for ovarian cancer (1, 2). However, results from a recent clinical trial of intracavitary gene transfer indicate that gene delivery is restricted to a few superficial cell layers, and that treatment of larger three-dimensional tumors may still be inadequate (3). To confer specificity of infection and increase viral spread in the tumor mass, replication-selective adenoviruses are now being actively developed as cancer therapeutic agents (4–7).

Several promoters including *α-fetoprotein* (8), *osteocalcin* (9), *Muc-1* (10), *L-plastin* (11), *midkine* (12), and *cyclooxgenase-2* (*cox-2*; 12) are being evaluated in other laboratories to restrict viral replication to their cognate tumors. Although all of these vectors are severely replication-attenuated in normal cells and demonstrate high selectivity, their one drawback is that they are available for treatment of only a narrow range of ovarian cancer tumors, because only a limited number of such tumors express the targeted tumor markers. However, targeting a tumor marker broadly expressed in ovarian cancer tumors can increase the range of application of a single replication-selective adenovirus.

The *IAI.3B* gene was isolated using a polyclonal serum against a high molecular weight fraction derived from the pleural fluid of a patient with ovarian cancer (13). CA125 is overexpressed in ovarian cancer and widely used for monitoring this type of cancer. IAI.3B and CA125 exhibit very similar patterns of expression in a wide variety of normal and malignant tissues (13). The deduced peptide sequence of IAI.3B encompasses a B-box/coiled coil motif present in many genes with transformation potential. Although detailed sequence analysis of the *IAI.3B* gene has been finished, the promoter region of *IAI.3B* remained to be identified. In the present study, we characterize the promoter element of the *IAI.3B* gene and use it in a strategy for development of a novel replication-selective adenovirus named AdE3-*IAI.3B*. In this virus, the *E1A* gene is placed under the control of the human *IAI.3B* promoter element to target a variety of ovarian tumors.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The human ovarian teratocarcinoma PA-1 and lung squamous cell carcinoma EBC-1 cell lines were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The human ovarian clear cell carcinoma RMG-1 cell line was a gift from Dr. Shiro Nozawa, Keio University (Tokyo, Japan). Human ovarian adenocarcinoma OCC1, 420, OVCAR3, 429, and DOV13 cell lines were a gift from Dr. Gordon B. Mills, The University of Texas, M.D. Anderson Cancer Center (Houston, TX). The human ovarian clear cell carcinoma KK cell line, and human ovarian adenocarcinoma KF and MH cell lines were a gift from Dr. Yoshihiro Kikuchi, National Defense Medical College (Tokorozawa, Japan). The normal human keratinocyte K42 and skin fibroblast F27 cell lines were established by Dr. Koji Hashimoto, Ehime University (Ehime, Japan). The human skin squamous cell carcinoma HSC-5 cell line was a gift from Dr. Kazuo Aso, Yamagata University (Yamagata, Japan). Normal human ovarian epithelial NOE1, NOE2, and NOE3 cell lines, and human umbilical vein endothelial HUVEC cell line were established in our laboratory. The human cervical squamous cell carcinoma ME-180 cell line was obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in a humidified 5% CO_2/95% air incubator at 37°C. All of the cell lines except K42, NOE1, NOE2, NOE3, and HUVEC NOE3 were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. K42 was grown in MCDB153 (Nissui Co., Tokyo, Japan) with bovine hypothalamus extract. NOE1, NOE2, NOE3, and HUVEC were grown in MCDB153 with 5% heat-inactivated fetal bovine serum.

Real-Time Quantitative RT-PCR.[3] One hundred ng of RNA samples were used in reverse transcription and real-time PCR for RNA expression studies. A reverse transcription and real-time PCR reaction was carried out with the ABI prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) in a total volume of 50 μl that contained TaqMan one-step RT-PCR master mix (Applied Biosystems), 0.3 μM of each forward and reverse primer, and 0.21 μM of TaqMan probe. The forward and reverse primer and TaqMan probe were, respectively, 5′-CCACTTGTTCCATGTGACA-CAGA-3′, 5′-CCGTTTCGTTAACCACTTGTTCTC-3′ and 5′-AAGACAA-

Received 10/22/02; accepted 3/19/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by a grant from the Ministry of Education, Science, Sports and Culture, Japan, and by a Foundation of Industry-University Joint Research Grant from Primmune Corporation, Inc., Osaka, Japan.

[2] To whom requests for reprints should be addressed, at Department of Obstetrics and Gynecology, School of Medicine, Ehime University, Shitsukawa, Shigenobu, Onsen-gun, Ehime 791-0295 Japan. Phone: 81-899-60-5378; Fax: 81-899-60-5381; E-mail: hamakatu@m.ehime-u.ac.jp.

[3] The abbreviations used are: RT-PCR, reverse transcription-PCR; MOI, multiplicity of infection; PFU, plaque forming unit.

GCCCCCAGACTGGTTCACAAG-3′. The reaction was performed with the following thermal cycling method: 30 min at 48°C for reverse transcription, 5 min at 95°C for AmpliTaq Gold activation, 15 s at 95°C and 60 s at 60°C for 40 cycles. *GAPDH* was chosen as a housekeeping gene to be tested as an endogenous control.

Cloning of Genomic DNA of the *IAI.3B* Gene. A human genomic library (EMBL3 SP6/T7; Clontech Laboratories, Inc., Palo Alto, CA) was screened with the ^{32}P-labeled PCR-amplified human *IAI.3B* partial DNA fragment between exon 1A and exon 1B. Five positive clones were obtained after the second screening. Genomic DNA encompassing the 5-kb 5′-flanking region, and exon 1B of *IAI.3B* was directly sequenced by an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The transcription start site of *IAI.3B* mRNA was determined by a CapFinder method (Clontech).

Assay for *IAI.3B* Promoter Activity. Various lengths of DNA fragments upstream from the exon 1B of *IAI.3B* were PCR-amplified and inserted into the luciferase reporter vector PicaGene Basic, a promoterless and enhancerless vector (Toyo Ink MFG Co., Tokyo, Japan) in sense orientation relative to the luciferase coding sequence between *Mlu*I and *Bgl*II sites. The sequence of each insert was confirmed by an ABI PRISM 310 Genetic Analyzer. Constructs containing *IAI.3B* 5′-flanking sequences, which were fused to the Luciferase gene, were transfected into cells in the presence of *N*-[1-(2,3-dioleoyloxyl)propyl]-*N,N,N*-trimethylammoniummethyl sulfate Liposomal transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). Dual luciferase assays were performed according to the manufacturer's protocol (Promega). The 561-bp human *midkine* and 883-bp human *cox-2* promoters were cloned by PCR and subjected to dual luciferase assays.

Construction of the AdE3-*IAI.3B* Vector. The pXC1 plasmid has adenovirus 5 sequences from 22 bp to 5790 bp containing the *E1* gene (Microbix Biosystems Inc., Toronto, Ontario, Canada). A unique *Age*I site was introduced at nucleotide position 552, essentially as described by Rodriguez *et al.* (14), to generate the plasmid pXC1-*Age*I. The *IAI.3B* promoter was ligated to pXC1-*Age*I plasmid to obtain pXC1-IAI.3B-1816. To construct the AdE3-*IAI.3B* virus, homologous recombination was performed between pXC1-IAI.3B-1816 plasmid and the right-hand side of pBHGE3 adenovirus DNA containing the *E3* region in 293 cells by a standard technique (15). To construct the wild-type adenovirus AdE3, homologous recombination was performed between pXC1 and pBHGE3 in 293 cells. The replication-defective *E1*-deleted Ad5CMV-*LacZ* virus was used to determine transduction efficiencies. The 50% transduction efficiency was assessed by scoring 500 X-Gal-positive cells in each of three replicate dishes and then determining the 50% value for β-galactosidase-positive blue cells. All of the viruses were purified with double cesium chloride gradients using standard methods, and titered with standard spectrophotometry and plaque assay.

Western Blot Analysis. Total cell lysates were prepared by lysing cell monolayers in plates with SDS-PAGE sample buffer. Each lane was loaded with 5 μg of cell lysate protein as determined by BCA protein assay (Pierce, Rockford, IL). After electrophoresis at 25 mA for 2.5 h, the proteins in the gels were transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore Corp., Bedford, MA). Then, the membranes were probed with two primary antibodies, rabbit antiadenovirus-2 E1A polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and human anti-β-actin monoclonal antibody (Sigma-Aldrich Fine Chemicals, St. Louis, MO), and secondary antibodies, horseradish peroxidase-conjugated sheep antirabbit IgG (Dako, Glostrup, Denmark) and horseradish peroxidase-conjugated sheep antimouse IgG (Amersham, Piscataway, NJ), respectively. The membranes were developed according to the Amersham ECL protocol.

Adenoviral Replication Assay. Each cell line was infected for 48 h with AdE3-*IAI.3B* or AdE3 at various MOIs that allow infection of all of the cells. Medium and cells were scraped into 1-ml medium, subjected to three freeze-thaw cycles, and centrifuged to collect the supernatant. Serial dilutions of the supernatant were assayed for live virus particles for 12–14 days by standard plaque assays under semisolid agarose on 293 cells. The efficiency of replication of the AdE3-*IAI.3B* or AdE3 virus in each cell line was compared with that in 293 cells.

Cell Count Assay. Cells were plated at a density of 2×10^4 cells/well in 12-well plates. Cells were infected with AdE3, AdE3-*IAI.3B*, or the Ad5CMV-*LacZ* viral control. Culture medium alone was used as a mock infection control. After 10 days, cells were harvested and counted to determine the IC_{50}.

Inhibition of s.c. Tumor Growth. To determine inhibition of s.c. tumor growth, AdE3-*IAI.3B* was injected into s.c. tumors in female nude (*nu/nu*) mice (Charles River Laboratories, Tsukuba, Japan). In brief, 5×10^6 PA-1, RMG-1, or ME-180 cells in 100 μl of RPMI 1640 were injected into the right posterior flank of each mouse through an insulin syringe with a 27 1/2-gauge needle. Seven animals were used for each group. After 15 (ME-180), 20 (PA-1), and 25 (RMG-1) days, tumors with a diameter of 5–8 mm were established. Then, 100 μl of AdE3 (1×10^9 PFU), AdE3-*IAI.3B* (1×10^9 PFU), Ad5CMV-*LacZ* (1×10^9 PFU), or medium alone were injected intratumorally on days 0, 2, and 4. The tumors were measured every day with calipers in two perpendicular diameters. Tumor volume was calculated by assuming a spherical shape, with the average tumor diameter calculated as the square root of the product of cross-sectional diameters.

Inhibition of i.p. Ovarian Tumor Growth. To simulate a clinical trial of gene therapy for ovarian cancer, the orthotopic i.p. carcinomatosis model was used, because ovarian cancer remains localized within the peritoneal cavity in a large proportion of patients, ultimately causing local morbidity and lethal complications. Female nude (*nu/nu*) mice (Charles River) were injected i.p. with 5×10^6 PA-1 cells on day 0. Ten animals were used for each group. On days 4, 5, and 6, mice were injected i.p. with AdE3-*IAI.3B* (1×10^7 PFU), Ad5CMV-*LacZ* (1×10^7 PFU), or no virus. The virus was diluted with 100 μl RPMI 1640 in each case. Survival data were plotted on Kaplan-Meier curves, and using the LIFETEST procedure the AdE3-*IAI.3B* group was compared with the other groups with the log-rank test.

RESULTS

Ovarian Cancer Cells Express High Levels of *IAI.3B* mRNA. The mRNA level of the *IAI.3B* gene was measured using real-time RT-PCR. We demonstrated the mRNA expressions of the *IAI.3B* gene relative to that of *GAPDH* in ovarian cancer cells (PA-1, RMG-1, 420, OCC1, OVCAR3, KK, KF, 429, DOV13, and MH), squamous cell carcinoma cells (ME-180, EBC-1, and HSC-5), normal ovarian epithelial cells (NOE1, NOE2, and NOE3), normal keratinocyte cells (K42), normal fibroblast cells (F27), and normal endothelial cells (HUVEC; Fig. 1). All of the ovarian cancer cells examined had at least 10–100-fold higher levels of *IAI.3B* mRNA than other cells ($P < 0.05$, unpaired *t* test).

***IAI.3B* Promoter Is Activated in Ovarian Cancer Cells.** A 5-kb promoter element of the *IAI.3B* gene was cloned after screening of a human genomic DNA library. The transcription start site of *IAI.3B* mRNA was 6-bp upstream of the sequence of exon 1B, which was reported by Brown *et al.* (16). Exon 1A, also reported by Brown *et al.*

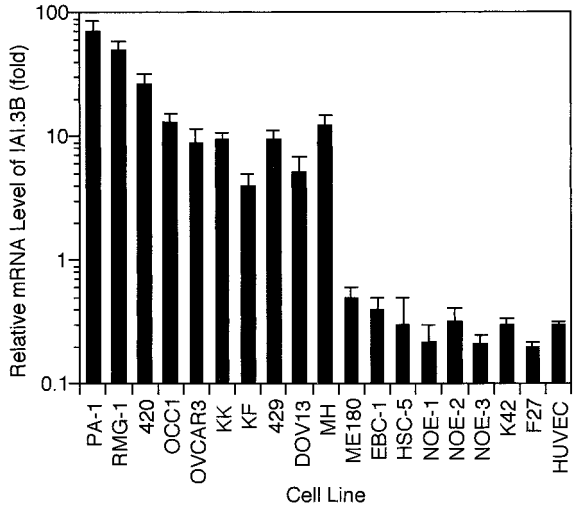

Fig. 1. Relative mRNA levels of *IAI.3B* in various cell lines as measured by real-time quantitative RT-PCR. The mRNA level of *IAI.3B* was normalized to that of the endogenous housekeeping gene control *GAPDH* in ovarian cancer, squamous cell carcinoma, and normal cells. *Bars*, ±SDs.

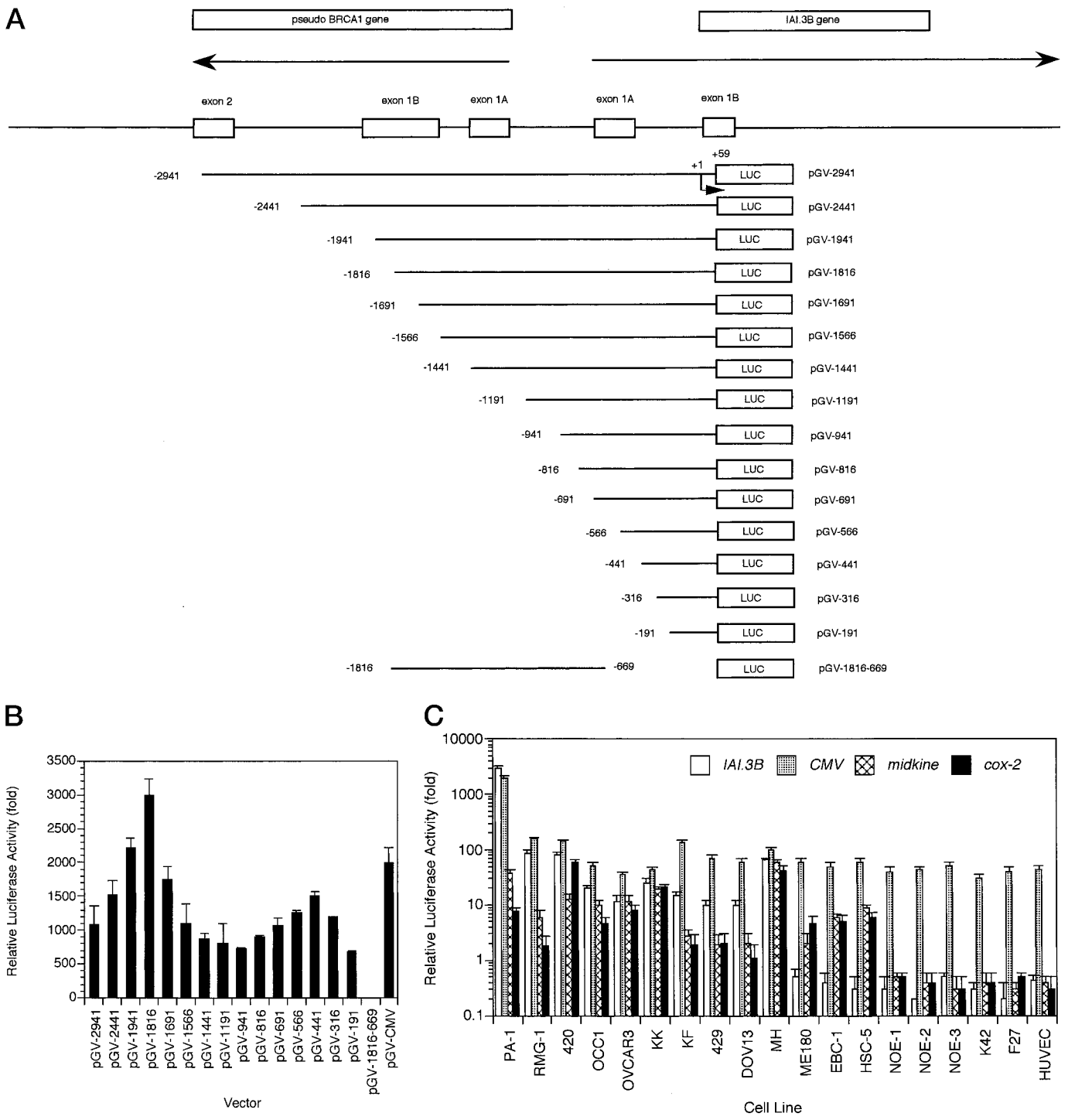

Fig. 2. Transcriptional activity of *IAI.3B* promoter. *A*, a schematic representation of reporter plasmids. The 5′-truncated fragments of the promoter region upstream of the *IAI.3B* gene were inserted into luciferase (LUC) reporter vector in sense orientation. The *arrow* indicates the transcription start site. *Numbers* indicate numbers of bases upstream (−) and downstream (+) of the transcription start site. The name of each reporter construct was assigned according to the 5′-end nucleotide numbers of inserted promoter sequences, upstream of the transcription start site. *B*, luciferase activities of reporter plasmids of the *IAI.3B* promoter in ovarian cancer PA-1 cells. The plasmid (pGV-CMV) driven by the *CMV* enhancer/promoter was used for comparison with the *IAI.3B* promoter. Luciferase activity in each plasmid was plotted as a ratio to that in the control plasmid (pGV-SV40) driven by the *SV40* enhancer/promoter. *Bars*, ±SDs. *C*, transcriptional activities of the 1816-bp region of *IAI.3B* promoter in ovarian cancer cells and other type of cells (squamous cell carcinoma and normal cells). Luciferase activity in each plasmid was plotted as the ratio to that in the control plasmid (pGV-SV40) driven by the *SV40* enhancer/promoter. The plasmids driven by the *CMV* enhancer/promoter, *midkine* promoter, and *cox-2* promoter were used for comparison with the *IAI.3B* promoter. *Bars*, ±SDs.

(16), was not transcribed in ovarian cancer cells. No TATA box was found. We examined 16 different lengths of 5′-upstream *IAI.3B* genomic DNA in searching for promoter activity (Fig. 2*A*). The transcriptional activities of the *IAI.3B* promoter were extremely high in ovarian cancer PA-1 cells, when *SV40* promoter activity was estimated as 1 (Fig. 2*B*). Deletion of the 5′-flanking sequences from −2941 bp to −1816 bp resulted in gradual increase in transcriptional activity, suggesting the presence of a negative regulatory element in the region. A 1816-bp upstream region of *IAI.3B* (pGV-1816) showed the highest transcriptional activity, at 1.5 times that of control reporter plasmid (pGV-CMV) driven by the *CMV* enhancer/promoter and 3000 times that of control reporter plasmid (pGV-SV40) driven by the

SV40 enhancer/promoter. Additional deletion to −941 bp (pGV-941) resulted in significant decrease in the activity, suggesting the presence of a positive element between −1816 bp and −941 bp. Deletion from −941 bp to −441 bp region revealed gradual increase in transcriptional activity, suggesting the presence of another negative element in the region. These results indicate that distal and proximal promoter elements of *IAI.3B* reside in the upstream regions between −1816 bp and −941 bp, and between −441 bp and −1 bp, respectively. The region between −1816 bp and −669 bp of the *IAI.3B* promoter (pGV-1816−669) demonstrated extremely low transcriptional activity, at 7% of that of control reporter plasmid (pGV-SV40). Thus, the sequence 5′-upstream from exon 1A exhibited no significant transcriptional activity.

To compare the transcriptional activity of the *IAI.3B* promoter between ovarian cancer cells and various other types of cells, *IAI.3B*, *CMV*, *midkine*, and *cox-2* promoter activities were estimated taking *SV40* promoter activity to be 1 (Fig. 2*C*). *IAI.3B* promoter activity correlated well with mRNA levels ($r = 0.8$; $P < 0.001$; regression analysis). The *IAI.3B* promoter activity in ovarian cancer cells was 950 times that in squamous cell carcinoma and normal cells ($P < 0.001$, unpaired *t* test), 12 times *midkine* promoter activity in ovarian cancer cells ($P < 0.01$, paired *t* test), and 48 times *cox-2* promoter activity in ovarian cancer cells ($P < 0.05$, paired *t* test). The promoter activities of *midkine* and *cox-2* were not significantly different. Furthermore, the promoter activities of *midkine* and *cox-2* were up-regulated in only 6 of 10 and 5 of 10 ovarian cancer cells used in this study, respectively.

Adenoviral Infection in Ovarian Cancer Cells. To determine adenovirus E1A protein expression in PA-1, RMG-1, and ME-180 cells after infection with AdE3-*IAI.3B* and AdE3, Western blot analyses were performed using rabbit antiadenovirus-2 E1A polyclonal antibodies. E1A protein was not detected in PA-1, RMG-1, and ME-180 cells before infection in contrast to 293 cells used as a positive control. AdE3 expressed E1A at significant levels in PA-1, RMG-1, and ME-180 cells. AdE3-*IAI.3B* expressed E1A at significant levels in PA-1and RMG-1 cells but not in ME-180 cells (Fig. 3*A*). Furthermore, AdE3 expressed E1A at significant levels in 8 other ovarian cancer cell lines, 2 other squamous cell carcinoma cell lines,

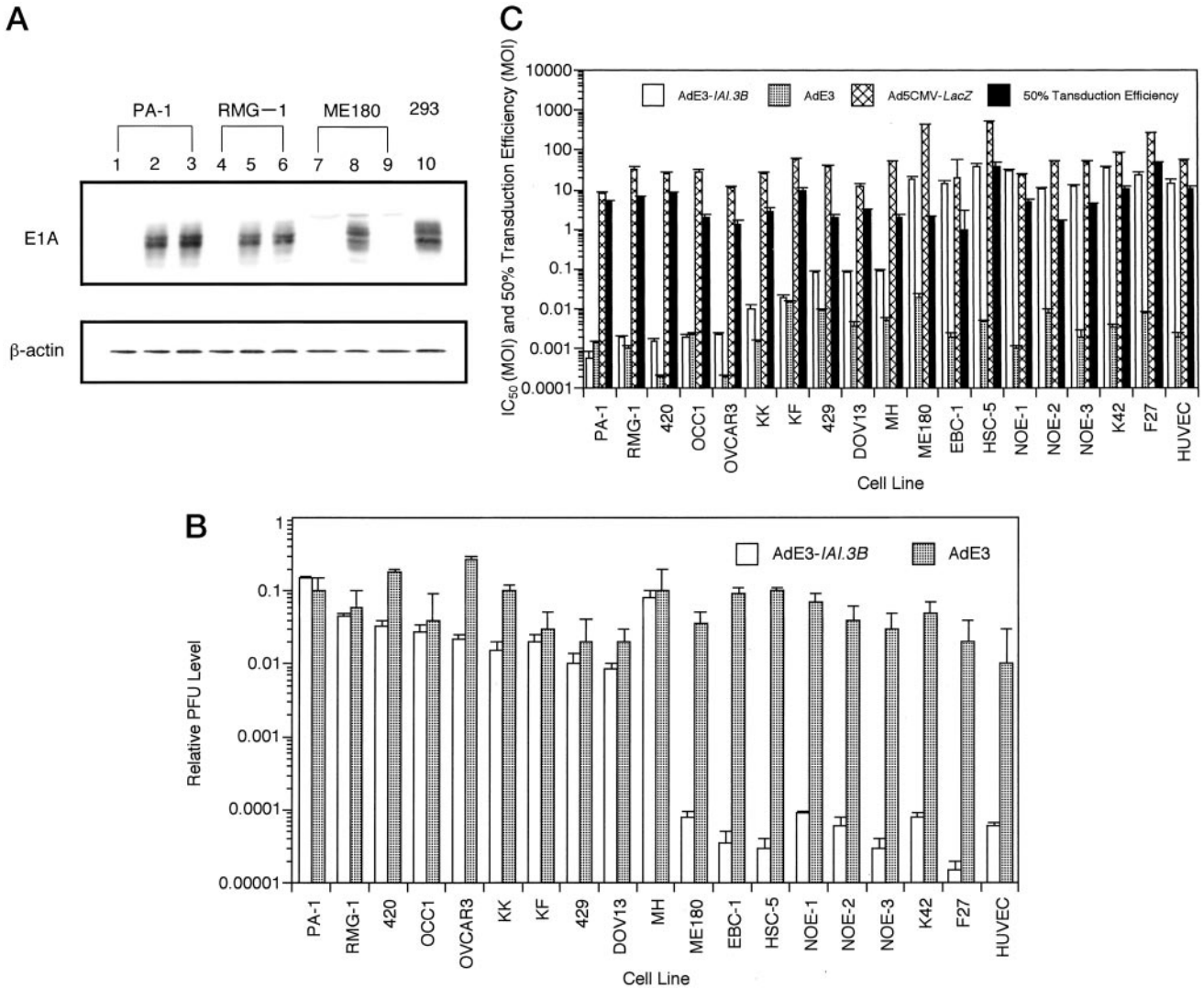

Fig. 3. Effects of AdE3-*IAI.3B* infection *in vitro*. *A*, expression of E1A by AdE3-*IAI.3B*. Western blots were probed with antiadenovirus-2 E1A (*top*) and anti-β actin antibodies (*bottom*). Cellular extracts were subjected to SDS-PAGE (*Lanes 1–3*, PA-1 cells; *Lanes 4–6*, RMG-1 cells; *Lanes 7–9*, ME-180 cells; *Lane 10*, 293 cells). Each cell line was infected for 24 h with AdE3-*IAI.3B* at 25 MOI (*Lanes 3, 6*, and *9*), AdE3 at 25 MOI (*Lanes 2, 5*, and *8*), or mock infection (*Lanes 1, 4, 7*, and *10*). *B*, relative PFU levels of AdE3-*IAI.3B* and AdE3. Cells were infected for 48 h with AdE3-*IAI.3B* and AdE3 at various MOIs that allow infection of all cells. Plaque assay was tested for virus production for 12–14 days under semisolid agarose on 293 cells. The PFU level in each cell line was plotted as the ratio to that in 293 cells. *Bars*, ±SDs. *C*, cytotoxicity of virus and transduction efficiency in ovarian cancer cells and other type of cells. To compare the cytotoxicity of each virus, cells were infected with AdE3-*IAI.3B*, AdE3, and Ad5CMV-*LacZ*. Ten days later, cell viability was determined by cell count assay. IC_{50} (MOI) was calculated. To compare the transduction efficiency of adenovirus, cells were infected with Ad5CMV-*LacZ*. *Bars*, ±SDs.

and 6 normal cell lines. AdE3-*IAI.3B* expressed E1A at significant levels in 8 other ovarian cancer cell lines but not in 2 other squamous cell carcinoma cell lines or 6 normal cell lines (data not shown). Thus, the *IAI.3B* promoter insertion in AdE3-*IAI.3B* virus yielded selective E1A expression in IAI.3B-producing cells (ovarian cancer cells) but not in non-IAI.3B-producing cells (squamous cell carcinoma and normal cells).

To compare the replicative activity of AdE3-*IAI.3B* between ovarian cancer cells and various other types of cells, relative amounts of live virus particles were estimated taking that in 293 cells to be 1. AdE3 replicated in ovarian cancer, squamous cell carcinoma, and normal cells. On the other hand, AdE3-*IAI.3B* replicated as efficiently as AdE3 in ovarian cancer cells but not in squamous cell carcinoma or normal cells (Fig. 3*B*).

Transcriptionally Targeted AdE3-*IAI.3B* Has a Potent Antiproliferative Effect in Ovarian Cancer Cells but not in Squamous Cell Carcinoma or Normal Cells. The extent of the antiproliferative effect of AdE3-*IAI.3B* was determined by comparing the rate of growth of AdE3-*IAI.3B*, AdE3, and Ad5CMV-*LacZ*-infected cells. The IC_{50} of each adenovirus was determined in adenovirus-infected cells (Fig. 3*C*). AdE3-*IAI.3B* virus exhibited a strong antiproliferative effect in ovarian cancer cells at an IC_{50} of 0.0006–0.09 MOI with a mean value of 0.03 MOI, which was 700 times lower than those in squamous cell carcinoma and normal cells ($P < 0.01$, unpaired *t* test). On the other hand, the IC_{50} of AdE3 and Ad5CMV-*LacZ* in ovarian cancer cells did not differ significantly from those in squamous cell carcinoma and normal cells. In ovarian cancer cells, the IC_{50} of AdE3-*IAI.3B* did not differ significantly from that of AdE3 but was 1000 times lower than that of Ad5CMV-*LacZ* ($P < 0.001$, paired *t* test). In squamous cell carcinoma and normal cells, the IC_{50} of AdE3-*IAI.3B* did not differ significantly from that of Ad5CMV-*LacZ* but was 4000 times higher than that of AdE3 ($P < 0.001$, paired *t* test). Adenoviral infectivity estimated by 50% transduction efficiency did not differ significantly between ovarian cancer cells and other cells (Fig. 3*B*; unpaired *t* test). Thus, AdE3-*IAI.3B* virus exhibited a strong antiproliferative effect in ovarian cancer cells. In contrast, AdE3-*IAI.3B* had minimum effect on the growth of squamous cell carcinoma and normal cells, and appeared to behave in the same manner as the replication-defective adenovirus Ad5CMV-*LacZ* in these cells.

Replication-selective Virus AdE3-*IAI.3B* Suppresses s.c. Tumor Growth of Ovarian Cancer Cells. To evaluate the antitumor effect of AdE3-*IAI.3B*, s.c. tumors were established in flanks of nude mice using ovarian cancer PA-1 and RMG-1 cells, and squamous cell carcinoma ME-180 cells. By 30 days, there were significant reductions in tumor size in the AdE3-*IAI.3B* and AdE3-treated groups compared with the medium alone, and Ad5CMV-*LacZ*-treated groups for both the PA-1 and RMG-1 tumor models. AdE3-*IAI.3B* and AdE3 had completely eradicated all of the PA-1 tumors ($P < 0.001$, χ^2 test; Fig. 4*A*). In the RMG-1 tumor model, AdE3-*IAI.3B* and AdE3 significantly reduced tumor sizes by 96–98% compared with medium alone and Ad5CMV-*LacZ* ($P < 0.001$, unpaired *t* test; Fig. 4*B*). AdE3-*IAI.3B* eradicated 1 of 7 RMG-1 tumors, but AdE3 eradicated none of them. In contrast, AdE3-*IAI.3B* did not significantly reduce the sizes of ME-180 tumors compared with Ad5CMV-*LacZ* (Fig. 4*C*). However, AdE3 significantly reduced the size of these squamous cell carcinoma tumors by 96% compared with Ad5CMV-*LacZ* ($P < 0.001$, unpaired *t* test). Neither AdE3-*IAI.3B* nor AdE3 eradicated any ME-180 tumors.

Replication-selective Virus AdE3-*IAI.3B* Suppresses i.p. Tumor Growth of Ovarian Cancer Cells. We used a well-established murine model of i.p. carcinomatosis and treated mice with three i.p. doses of AdE3-*IAI.3B*, the nonreplicative Ad5CMV-*LacZ*, or no virus on days 4, 5, and 6 (Fig. 5). In the PA-1 tumor model, the median survival was not reached for AdE3-*IAI.3B*. For control with no virus and Ad5CMV-*LacZ*, the median survivals were 56 and 72 days, respectively. The mean survival times for control with no virus, Ad5CMV-*LacZ*, and AdE3-*IAI.3B* were 59, 70, and 137 days, respectively. All of the mice in the control group died before day 74. All of the mice treated with AdE3-*IAI.3B* survived until at least day 85. Survival was significantly better in animals treated with AdE3-*IAI.3B* ($P < 0.001$, log-rank test).

DISCUSSION

It has been reported that the *IAI.3B* gene has exons 1A and 1B (16). However, we found that exon 1A was not transcribed and that the sequence 5′-upstream from exon 1A exhibited no significant promoter activity. In this study, we for the first time demonstrated the promoter region of the *IAI.3B* gene and its up-regulation in all 10 of the ovarian cancer cells tested. Campbell *et al.* (13) reported no significant difference in level of *IAI.3B* mRNA among seven normal tissues such as heart, brain, and lung in their Northern analyses. However, our quantitative analyses using real-time RT-PCR revealed significant differences in *IAI.3B* mRNA levels between ovarian cancer cells and other cells tested. The promoter activity of *IAI.3B* correlated well with mRNA levels in all of these types of cells. The promoter activity of *IAI.3B* in ovarian cancer cells was extremely high and almost the same as that of *CMV*. Before this study, ovarian cancer-specific promoter had not been identified, and the *midkine* and *cox-2* promoters were therefore considered best for driving the *E1A* and *E1B* genes for oncolytic adenovirus to treat ovarian cancer (12). However, the promoter activity of *IAI.3B* was an order of magnitude higher than those of *midkine* and *cox-2* in ovarian cancer cells. Furthermore, the *midkine* and *cox-2* promoters were up-regulated in only half of the ovarian cancer cells used in this study. Altogether, these findings suggest that the *IAI.3B* promoter is a good candidate for a promoter to drive oncolytic adenovirus for ovarian cancer therapy.

AdE3-*IAI.3B* has a construction design similar to that of the adenovirus Ad*E2F-1*^{RC}, because both have an intact *E1A* promoter upstream of their respective heterologous promoters (17). On infection with the Ad*E2F-1*^{RC}, small cell lung cancer A549 cells expressed E1B55-kDa and E1B 19-kDa proteins, whereas normal cells expressed a low level of only E1B 55-kDa protein (17). This might reflect the differential activation of the *E1B* promoter by E1A protein (18). Although the activity of heterologous promoter composed of *E1A* and *IAI.3B* was twice as much as that of the *IAI.3B* promoter, this heterologous promoter retained the specificity of ovarian cancer cells. On the basis of these results, we used the *E1A-IAI.3B* composite promoter for construction of a replication-selective adenovirus.

Our study is the first to report a replication-selective adenovirus in which the cell-type specificity of the *IAI.3B* promoter is used to target ovarian cancer cells. We demonstrated that AdE3-*IAI.3B* selectively killed ovarian cancer cells expressing high levels of IAI.3B, but did not kill squamous cell carcinoma or normal cells expressing low levels of IAI.3B. Additionally, in mouse flank and i.p. xenograft models, AdE3-*IAI.3B* exhibited significant therapeutic effects. Replicating incompetent virus (Ad5CMV-*LacZ*) had minimum effect suggesting that the therapeutic effect of AdE3-*IAI.3B* was associated with viral replication, cell lysis, and viral spread. At the doses tested, three injections of AdE3-*IAI.3B* into small flank tumors eradicated all of the PA-1 tumors but only 1 of 7 RMG-1 tumors. This finding may be related to the *IAI.3B* promoter activities present in both tumor cells. Replication-selective adenoviruses using *E2F* promoter (17), *AFP* promoter (19), *PSA* promoter (14), or without *E1B 55-kDa* sequence

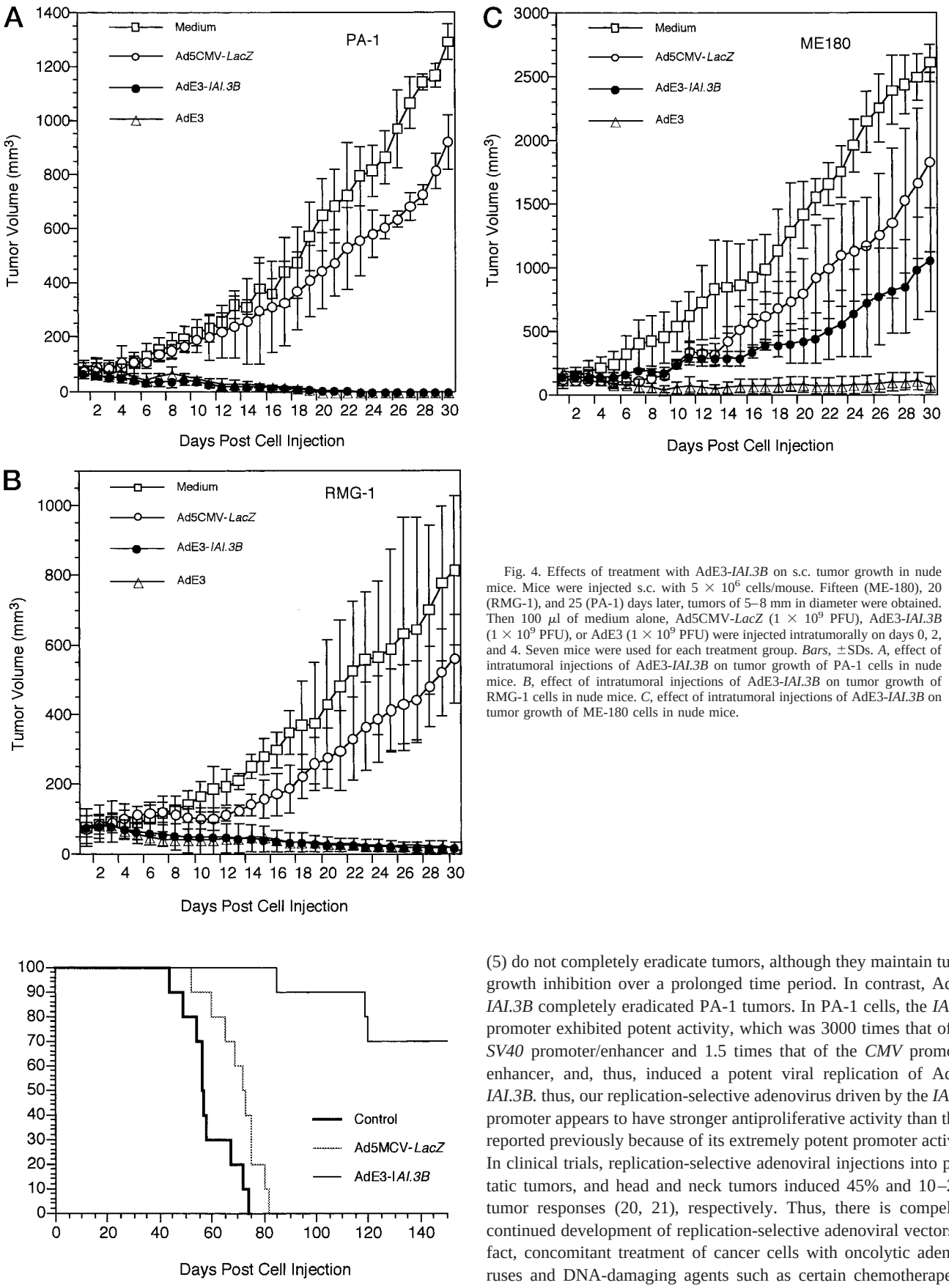

Fig. 4. Effects of treatment with AdE3-*IAI.3B* on s.c. tumor growth in nude mice. Mice were injected s.c. with 5×10^6 cells/mouse. Fifteen (ME-180), 20 (RMG-1), and 25 (PA-1) days later, tumors of 5–8 mm in diameter were obtained. Then 100 μl of medium alone, Ad5CMV-*LacZ* (1×10^9 PFU), AdE3-*IAI.3B* (1×10^9 PFU), or AdE3 (1×10^9 PFU) were injected intratumorally on days 0, 2, and 4. Seven mice were used for each treatment group. *Bars*, ±SDs. *A*, effect of intratumoral injections of AdE3-*IAI.3B* on tumor growth of PA-1 cells in nude mice. *B*, effect of intratumoral injections of AdE3-*IAI.3B* on tumor growth of RMG-1 cells in nude mice. *C*, effect of intratumoral injections of AdE3-*IAI.3B* on tumor growth of ME-180 cells in nude mice.

Fig. 5. Therapeutic effect of AdE3-*IAI.3B* in an i.p. model of ovarian cancer. Carcinomatosis was induced with 5×10^6 PA-1 i.p. cells. Four, 5, and 6 days later, AdE3-*IAI.3B*, Ad5CMV-*LacZ* (nonreplicative control), or no virus was injected i.p. at doses of 1×10^7 PFU/day.

(5) do not completely eradicate tumors, although they maintain tumor growth inhibition over a prolonged time period. In contrast, AdE3-*IAI.3B* completely eradicated PA-1 tumors. In PA-1 cells, the *IAI.3B* promoter exhibited potent activity, which was 3000 times that of the *SV40* promoter/enhancer and 1.5 times that of the *CMV* promoter/enhancer, and, thus, induced a potent viral replication of AdE3-*IAI.3B*. thus, our replication-selective adenovirus driven by the *IAI.3B* promoter appears to have stronger antiproliferative activity than those reported previously because of its extremely potent promoter activity. In clinical trials, replication-selective adenoviral injections into prostatic tumors, and head and neck tumors induced 45% and 10–20% tumor responses (20, 21), respectively. Thus, there is compelling continued development of replication-selective adenoviral vectors. In fact, concomitant treatment of cancer cells with oncolytic adenoviruses and DNA-damaging agents such as certain chemotherapeutic agents can result in supra-additive cell killing (22). Thus, considering the safety and activity of AdE3-*IAI.3B*, strong reasons exist for additional laboratory and clinical investigation of AdE3-*IAI.3B* in

conjunction with chemotherapy for the treatment of human ovarian cancer.

ACKNOWLEDGMENTS

We thank Keizo Oka for help in preparing culture medium and Shuzo Sawada for support of this project.

REFERENCES

1. Link, C. J., Moorman, D., Seregina, T., Levy, J., and Schabold, K. A Phase I trial of *in vivo* gene therapy with the herpes simplex thymidine kinase/ganciclovir system for the treatment of refractory or recurrent ovarian cancer. Hum. Gene Ther., *7:* 1161–1179, 1996.
2. Alvarez, R. D., and Curiel, D. T. A Phase I study of recombinant adenovirus vector-mediated intraperitoneal delivery of herpes simplex virus thymidine kinase (HSV-tk) gene and intravenous ganciclovir for previously treated ovarian and extraovarian cancer patients. Hum. Gene Ther., *8:* 597–613, 1997.
3. Sterman, D. H., Treat, J., Elshami, A. A., Amin, K., Molnar-Kimber, K., Coonrod, L., Recio, A., Wilson, J. M., Roberts, J. R., Litzky, L. A., Albelda, S. M., and Kaiser, L. R. Adenovirus mediated herpes simplex virus thymidine kinase/ganciclovir gene therapy in patients with localized malignancy: results of a Phase I clinical trial in malignant mesothelioma. Hum. Gene Ther., *9:* 1083–1092, 1998.
4. Alemany, R., Balague, C., and Curiel, D. T. Replicative adenoviruses for cancer therapy. Nat. Biotechnol., *18:* 723–727, 2000.
5. Kirn, D., Martuza, R. L., and Zwiebel, J. Replication-selective virotherapy for cancer: biological principles, risk management and future directions. Nat. Med., *7:* 781–787, 2001.
6. Hawkins, L. K., Lemoine, N. R., and Kirn, D. Oncolytic biotherapy: a novel therapeutic platform. Lancet Oncol., *3:* 17–26, 2002.
7. Kruyt, F. A., and Curiel, D. T. Toward a new generation of conditionally replicating adenoviruses: pairing tumor selectivity with maximal oncolysis. Hum. Gene Ther., *4:* 485–495, 2002.
8. Hallenbeck, P. L., Chang, Y. N., Hay, C., Golightly, D., Stewart, D., Lin, J., Phipps, S., and Chiang, Y. L. A novel tumor-specific replication-restricted adenoviral vector for gene therapy of hepatocellular carcinoma. Hum. Gene Ther., *10:* 1721–1733, 1999.
9. Wada, Y., Gardner, T. A., Egawa, M., Park, M. S., Hsieh, C. L., Zhau, H. E., Kao, C., Kamidono, S., Gillenwater, J. Y., and Chung, L. W. A conditional replication-competent adenoviral vector. Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. Cancer Res., *61:* 6012–6019, 2001.
10. 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. Investig., *106:* 763–771, 2000.
11. Pang, X. Y., Won, J. H., Rutherford, T., Fuji, T., Peterman, D., Piston, G., Sapid, E., Leavitt, J., Kaminski, B., Crystal, R., Schwartz, P., and Dessert, A. The use of the L-plastin promoter for adenoviral-mediated, tumor-specific gene expression in ovarian and bladder cancer cell lines. Cancer Res., *61:* 4405–4413, 2001.
12. Casado, E., Gomez-Navarro, J., Yamamoto, M., Adachi, Y., Coolidge, C. J., Arafat, W. O., Barker, S. D., Wang, M. H., Mahasreshti, P. J., Hemminki, A., Gonzalez-Baron, M., Barnes, M. N., Pustilnik, T. B., Siegal, G. P., Alvarez, R. D., and Curiel, D. T. Strategies to accomplish targeted expression of transgenes in ovarian cancer for molecular therapeutic applications. Clin. Cancer Res., *7:* 2496–2504, 2001.
13. Campbell, I. G., Nicolai, H. M., Foulkes, W. D., Senger, G., Stamp, G. W., Allan, G., Boyer, C., Jones, K., Bast, R. C., Jr., and Solomon. E. A novel gene encoding a B-box protein within the BRCA1 region at 17q21.1. Hum. Mol. Genet., *3:* 589–594, 1994.
14. Rodriguez, R., Schuur, E. R., Lim, H. Y., Henderson, G. A., Simons, J. W., and Henderson, D. R. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. Cancer Res., *57:* 2559–2563, 1997.
15. Hamada, K., Sakaue, M., Alemany, R., Zhang, W. W., Horio, Y., Roth, J. A., and Mitchell, M. F. Adenovirus-mediated transfer of HPV 16 E6/E7 antisense RNA to human cervical cancer cells. Gynecol. Oncol., *63:* 219–227, 1996.
16. Brown, M. A., Xu, C. F., Nicolai, H., Griffiths, B., and Chambers, J. A. The 5′ end of BRCA1 lies within a duplicated region of human chromosome 17q21. Oncogene, *12:* 2507–2513, 1996.
17. Tsukuda, K., Wiewrodt, R., Molnar-Kimber, K., Jovanovic, V. P., and Amin, K. M. An E2F-responsive replication-selective adenovirus targeted to the defective cell cycle in cancer cells: potent antitumoral efficacy but no toxicity to normal cell. Cancer Res., *62:* 3438–3447, 2002.
18. Wu, L., Rosser, D. S. E., Schimdt, M. C., and Berk, A. A TATA box implicated in E1A transcriptional activation of a simple adenovirus 2 promoter. Nature (Lond.), *326:* 512–515, 1987.
19. Li, Y., Yu, D. C., Chen, Y., Amin, P., Zhang, H., Nguyen, N., and Henderson, D. R. A hepatocellular carcinoma-specific adenovirus variant. CV890, eliminates tumor cells in combination with doxorubicin. Cancer Res., *61:* 6428–6436, 2001.
20. DeWeese, T. L., van der Poel, H., Li, S., Mikhak, B., Drew, R., Goemann, M., Hamper, U., DeJong, R., Detorie, N., Rodriguez, R., Haulk, T., DeMarzo, A. M., Piantadosi, S., Yu, D. C., Chen, Y., Henderson, D. R., Carducci, M. A., Nelson, W. G., and Simons, J. W. A phase I trial of CV706, a replication-competent. PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. Cancer Res., *61:* 7464–7472, 2001.
21. Kirn, D. Clinical research results with dl520 (Onyx-015), a replication-selective adenovirus for the treatment of cancer: what have we learned? Gene Ther., *8:* 89–98, 2001.
22. Yu, D. C., Chen, Y., Dilley, J., Li, Y., Embry, M., Zhang, H., Nguygen, N., Amin, P., Oh, J., and Henderson, D. R. Antitumor synergy of CV787, a prostate cancer-specific adenovirus, and paclitaxel and docetaxel. Cancer Res., *61:* 517–525, 2001.