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(54) Titre : VECTEUR COMPETENT DE REPLICATION SPECIFIQUE AUX CELLULES
(54) Title: CELL-SPECIFIC REPLICATION-COMPETENT VECTOR

(57) **Abrégé/Abstract:**

The present invention provides a method for constructing a vector which expresses and replicates a gene specifically to particular cells; a DNA having a gene expression regulatory activity used for its construction; and further, a treatment method comprising the steps of introducing said vector into particular living tissues or cells such as malignant tumors and the like, and said vector expressing and replicating a gene. A cell-specific replication-competent vector which does not act to normal cells in an adult body is constructed by obtaining a transcriptional initiation regulatory region of human calponin gene which is expressed specifically in cells; by linking it upstream of a viral replication-related gene; and by integrating it into a virus DNA. Said constructed cell-specific replication-competent vector is introduced into malignant tumor cells to selectively injure tumor cells or proliferating smooth muscle cells of tumor neovascular.



ABSTRACT

The present invention provides a method for constructing a vector which expresses and replicates a gene specifically to particular cells; a DNA having a gene expression regulatory activity used for its construction; and further, a treatment method comprising the steps of introducing said vector into particular living tissues or cells such as malignant tumors and the like, and said vector expressing and replicating a gene. A cell-specific replication-competent vector which does not act to normal cells in an adult body is constructed by obtaining a transcriptional initiation regulatory region of human calponin gene which is expressed specifically in cells; by linking it upstream of a viral replication-related gene; and by integrating it into a virus DNA. Said constructed cell-specific replication-competent vector is introduced into malignant tumor cells to selectively injure tumor cells or proliferating smooth muscle cells of tumor neovascular.

DESCRIPTION

TITLE OF THE INVENTION

CELL-SPECIFIC REPLICATION-COMPETENT VECTOR

Technical Field

The present invention relates to a cell-specific replication-competent vector which does not act to normal adult cells and expresses a gene specifically to particular cells to self-replicate; a DNA having an activity to regulate gene expression, that can be used for a construction of said vector; a method for expressing a gene in particular living cells by using said vector; a method for disrupting particular cells, or the like.

More specifically, the present invention relates to a construction of a cell-specific replication-competent vector which does not act to normal adult cells wherein the vector can express a cell-specific gene, to disrupt specifically tumor cells in the field of gene therapy of tumor and the like, that is, to a gene introduction to cells for enabling treatment without injuring normal cells and to a construction of a cell disrupting vector.

Background Art

Recently, an ideal treatment method of cancer with minimal side effects, which does not affect normal cells but can injure selectively only cancer cells, have been required. For example, a gene therapy method can be exemplified, but as said treatment method can enhance the selectivity of cancer cells in various levels such as the cell selectivity of a gene to be inserted into cancer cells, the activity of an expression promoter, the method of introduction and infection of a virus vector, or the like, it is regarded as a promising treatment

method in the future. However, there is a common problem that the therapeutic gene cannot be introduced into all cancer cells.

On the other hand, concerning the immunocytic therapy of cancer, as a slight expression of tissue-specific differentiation antigen has been observed also in normal tissue, the side effect to normal cells has been a problem. A cancer antigen based on a mutation has a defect that said mutation is limited to each cancer, and it is not appropriate to generalize it as an immunocytic therapy for cancer as a molecular target.

Recently, a clinical study of gene therapy of malignant brain tumor using a replication-competent herpes simplex virus (HSV) vector, which injures selectively only the growth cells continuously by infection and replication, is conducted in the United States and in Great Britain (Gene Ther. 7, 859-866, 2000; Gene Ther. 7, 867-874, 2000). The replication-competent HSV vector is a vector wherein a ribonucleotide reductase (RR) or thymidine kinase (TK), essential to viral replication, is deleted. Said enzymes are expressed only at the time of proliferation in normal cells, but are expressed constitutively in tumor cells. Thus, when said HSV vector is infected to cells wherein proliferation is active whether it is a normal cell or a tumor cell, it is replicated by using cell-derived RR or TK, and shows cytolysis activity. On the other hand, in animal experiments in Japan, an anti-tumor effect of a replication-competent HSV vector against prostate cancer or pancreatic cancer has been reported (J. Surg. Oncol. 72, 136-141, 1999). However, these also do not have cell selectivity and the safety is not high. Therefore, it could be used for treating human, in brain which has a blood brain barrier, and where the vector does not diffuse into circulating blood, but there was a problem that it is not appropriate for treatment of organs other than the brain.

According to the above-mentioned, it is considered that if it is possible to control the injury activity of the HSV vector specifically to target cells, it could be a more effective and safe treatment method. By now, Miyatake, one of the present inventors, and Martuza et al. of the United States have reported a replication-competent HSV vector selective to liver tumor using an albumin promoter (J. Virol. 71, 5124-5132, 1997). However, when such vector is used, the expression of the albumin gene decreases in liver cell carcinoma, and also injures normal regenerated liver cells, it is considered that it is not appropriate for clinical application in human. Furthermore, Martuza and Miyatake, one of the present inventors, have reported the possibility of application to mesothelioma in the US Patent No. 5728379 ("Tumor- or cell-specific herpes simplex virus replication") which has been patented in March 1998, but there is no description of a possible application to human sarcoma in general, such as leiomyosarcoma, osteosarcoma and the like.

By gene analysis of the cause of disease and the pathology of sarcoma, a mutation of p53 and Rb and the existence of a fusion gene in some of the tumors have been reported, but they are not yet in a stage to be applied widely for treatment. In an animal experiment using nude mice, Milas et al. have introduced p53 gene into leiomyosarcoma cells, by using an adenovirus vector not that does not have a replication ability, and have reported that there was an effect to delay tumor proliferation (Cancer Gene Ther. 7, 422-429, 2000). Furthermore, a method for introducing and expressing thymidine kinase, which is a suicide gene, into osteosarcoma by using a promoter of osteocalcin gene, has been reported (Cancer Gene Ther. 5, 274-280, 1988). However, as it is a method using a virus vector wherein the replication ability is deleted, the efficiency of introducing gene is low and cannot be applied to sarcoma other than osteosarcoma.

Especially, in the report of Milas et al., an example using human smooth muscle cell line SK-LMS-1, which is the same as that described in the example of the present invention, is described. However, the amount of viral particles used is more than 100 to 1000 times compared to the amount of particles of virus vector used in the present invention, and the effect is inferior than the example of the present invention. Therefore, the results of Milas et al. are not preferable from the point of view that the amount of viral particles to be injected into the body should be as less as possible to hold down side effects.

Moreover, as for a therapy by suppressing vascularization of cancer, the group of Folkman of the United States have reported a dramatic anti-tumor effect of peptidergic inhibiting factors such as angiostatin, endostatin or the like, in mice experiments (Cell 79, 315-328, 1994, Cell 88, 277-285, 1997). In Japan also, Nakamura et al. have reported the action to suppress vascularization of NK₄ which is a fragment in a molecule of a liver cell growth factor (Biochem. Biophys. Res. Commun. 279, 846-852, 2000). However, these methods have problems such as: a great number of peptides are necessary; it is reported that the reproducibility is low regarding endostatin; the mechanism of the action is unknown; and the efficacy in human is not yet confirmed, or the like. The inhibitor of vascularization, now being under clinical test does not have cell selectivity and the inhibition efficiency is low. The peptide inhibiting the action of integrin on the surface of endothelial cells, which Cheresh et al. of the United States have reported, similarly do not have cell selectivity and the inhibition efficiency is low (J. Clin. Invest. 103, 1227-1230, 1999). All of these studies are treatment wherein the target is a vascular endothelial cell, but the therapeutic agent having cell selectivity wherein the target is proliferating vascular

smooth muscle cells composing tumor vessel remains unknown. Actually, the antagonist of a platelet-derived growth factor receptor which promotes the proliferation and the migration of smooth muscle cells has been reported to have a strong tumor neovascular suppressing action (Cancer Res. 60, 4152-4160, 2000), and the significance of attacking the vascular smooth muscle to suppress tumor vascularization is estimated, but as this method is cell non-selective, side effects are also predicted.

On the other hand, the present inventors have found that a calponin gene which is said to be a differentiated marker of smooth muscle is expressed in the tumor cells of human-derived sarcoma and reported it for the first time (Int. J. Cancer 79, 245-250, 1998, Sarcoma 3, 107-113, 1999, Intern. J. Cancer 82, 678-686, 1999). Thereafter, many reports have been reported subsequently in and out of Japan, that the calponin gene is expressed abnormally in almost 20 kinds of human malignant tumors derived from mesenchymal cells, such as gastrointestinal stromal tumor (GIST), salivary gland sarcoma, fibrosarcoma, malignant neurilemmoma and the like, in addition to sarcoma to bone and soft parts. The calponin mentioned above (h1 or basic) has been shown by an X-ray crystal structure and a mechanism analysis in vitro and in vivo, that it suppresses the sliding movement of actin/miosin by binding to C-terminus of actin molecule (Biochem. Biophys. Res. Commun. 279, 150-157, 2000; J. Physiol. 529, 811-824, 2000). The calponin gene is considered to express selectively in smooth muscle cells in an adult body and to be a differentiated marker of the blood vessels (Physiol. Rev. 75, 487-517, 1995).

The object of the present invention is to construct a cell-specific replication-competent vector to use for treating malignant tumors and the like, which expresses and replicates a gene specifically in particular cells such as malignant tumors and the

like, and does not injure normal cells; to provide a DNA having a gene expressionregulatory activity used for its construction; and to provide a method of treatment by introducing and expressing said vector into particular living cells such as malignant tumors and the like, etc.

The present inventors have made a keen study to elucidate the objects mentioned above, and constructed a cell-specific replication-competent vector which does not act to normal adult cells that can induce a viral replication, by the following: by obtaining a transcriptional initiation regulatory region in particular tumor cells or smooth muscle cells of the human calponin gene, which is expressed specifically in said cells; by integrating upstream of the gene that encodes a transcription factor necessary to start replication of a viral replication-related gene; by substituting it with a TK gene which is an enzyme essential to the replication of virus DNA; and by expressing said gene in particular cells such as malignant tumor cells or proliferating smooth muscle cells in new blood vessels in tumors and the like. By introducing said cell-specific replication-competent vector constructed into a malignant tumor tissue, they have found that it injures selectively tumor cells or tumor neovascular proliferating smooth muscles, and the present invention has been completed.

Disclosure of the Invention

The present invention relates to a cell-specific replication-competent vector which does not act to normal adult cells, wherein a transcriptional initiation regulatory region of a gene specifically expressed in cells is integrated upstream of a predetermined gene;

the cell-specific replication-competent vector which does not act to normal adult cells, wherein the transcriptional initiation regulatory region of a gene specifically

expressed in cells is a region including a base sequence shown in Seq. ID No.1; the cell-specific replication-competent vector which does not act to normal adult cells, wherein the region including the base sequence shown in Seq. ID No.1 is a region including a human calponin gene promoter comprising a base sequence shown in Seq. ID No.2; the cell-specific replication-competent vector which does not act to normal adult cells, wherein the region including the base sequence shown in Seq. ID No.2 is a region including a base sequence shown in Seq. ID No.3; the cell-specific replication-competent vector which does not act to normal adult cells, wherein the transcriptional initiation regulatory region of a gene specifically expressed in cells comprises a base sequence wherein one or a few bases are deleted, substituted or added in the base sequence shown in Seq. ID No.1, Seq. ID No.2, or Seq. ID No.3, and is a region including a base sequence having an activity to regulate transcriptional initiation; the cell-specific replication-competent vector which does not act to normal adult cells, wherein an enhancer is integrated upstream of the transcriptional initiation regulatory region; the cell-specific replication-competent vector which does not act to normal adult cells, wherein the enhancer is an 4F2 enhancer; the cell-specific replication-competent vector which does not act to normal adult cells, wherein the predetermined gene is a viral replication-related gene; the cell-specific replication-competent vector which does not act to normal adult cells, wherein the viral replication-related gene is a gene that encodes a transcription factor essential to start viral replication (ICP4); the cell-specific replication-competent vector which does not act to normal

adult cells, wherein an apoptosis-related gene is linked further downstream of the predetermined gene, and expressed under the control of said transcriptional initiation regulatory region and the enhancer;

the cell-specific replication-competent vector which does not act to normal adult cells, wherein a DNA that encodes a protein having action to suppress vascularization is linked further downstream of the predetermined gene, and expressed under the control of said transcriptional initiation regulatory region and the enhancer; the cell-specific replication-competent vector which does not act to normal adult cells,

wherein a DNA that encodes a protein having action to suppress cancer metastasis is linked further downstream of the predetermined gene, and expressed under the control of said transcriptional initiation regulatory region and the enhancer;

the cell-specific replication-competent vector which does not act to normal adult cells, wherein a DNA that encodes a protein having action to suppress cancer is linked further downstream of the indicated gene, and expressed under the control of said transcriptional initiation regulatory region and the enhancer; the cell-specific replication-competent vector which does not act to normal adult cells,

wherein the replication-competent vector is a virus vector; the cell-specific replication-competent vector which does not act to normal adult cells,

wherein the virus vector is a herpes simplex virus vector (HSV vector) or an adenovirus vector; the cell-specific replication-competent vector which does not act to normal adult cells,

wherein the vector is a replication-competent vector specific to tumor cells and specific to proliferating

smooth muscles of new tumor blood vessels; the cell-specific replication-competent vector which does not act to normal adult cells, wherein a DNA that encodes ribonucleotide reductase and/or a DNA that encodes thymidine kinase is deleted.

Furthermore, the present invention relates to a DNA comprising a base sequence shown in Seq. ID No.1 or its complementary sequence;

a DNA comprising a base sequence shown in Seq. ID No.2 or its complementary sequence; a DNA comprising a base sequence shown in Seq. ID No.3 or its complementary sequence;

a DNA comprising a base sequence wherein one or a few bases are deleted, substituted or added in a base sequence shown in Seq. ID No.1, Seq. ID No.2 or Seq. ID No.3 and comprises a base sequence having an activity regulating transcriptional initiation or its complementary sequence; a DNA comprising a base sequence shown in Seq. ID No.1, Seq. ID No.2 or Seq. ID No.3, or a base sequence wherein one or a few bases are deleted, substituted or added in a base sequence shown in Seq. ID No.1, Seq. ID No.2 or Seq. ID No.3 and comprises a base sequence wherein an enhancer sequence is integrated upstream of a base sequence having an activity to regulate transcriptional initiation or its complementary sequence; a DNA, wherein the enhancer is a 4F2 enhancer.

Moreover, the present invention relates to a method for expressing and replicating a gene, a protein or a peptide of a cell-specific replication-competent vector which does not act to normal adult cells, by introducing the cell-specific replication-competent vector which does not act to normal adult cells

into a living cell tissue, to express and replicate; the method for expressing and replicating a gene, a protein or a

peptide of the cell-specific replication-competent vector which does not act to normal adult cells, wherein the living cell tissue is a tumor tissue; a therapeutic drug comprising the cell-specific replication-competent vector which does not act to normal adult cells; the therapeutic drug characterized by being a therapeutic drug against malignant tumor; a method for treating malignant tumors wherein the cell-specific replication-competent vector which does not act to normal adult cells

is introduced into a tumor tissue, to express a predetermined gene, protein or peptide; a method for treating malignant tumors wherein the cell-specific replication-competent vector which does not act to normal adult cells

is introduced into a tumor tissue, to express a predetermined gene, protein or peptide; and selectively disrupts only tumor cells; a method for treating malignant tumors wherein the cell-specific replication-competent vector which does not act to normal adult cells is introduced into a tumor tissue, to express a predetermined gene, protein or peptide, and selectively disrupts only proliferating smooth muscles cells surrounding blood vessels of new tumor blood vessels.

Brief Description of Drawings

Figure 1 is a figure that shows the results of the transcriptional activity when a 5'deleted F mutant of calponin gene promoter is transfected.

Figure 2 is a figure that shows the effect of an enhancer at a transcriptional level in a regulatory region controlling human calponin expression in calponin-positive tumor cells.

Figure 3 is a figure that shows the structure of d12. CALP and

the results of the cytopathic assay in vitro.

Figure 4 is a figure that shows the injury effect to tumor cells by d12.CALP in vitro.

Figure 5 is a figure that shows the selective injury activity of d12.CALP in calponin-positive cells in vitro.

Figure 6 is a figure that shows the suppressing effect of tumor formation of d12.CALP in vivo.

Figure 7 is a picture that shows nude mice treated with d12.CALP.

Figure 8 is a picture that shows a replication of d12.CALP in vivo.

Figure 9 is a picture showing that d12.CALP diffuses and replicates at a site distant from the infection site in vivo.

Figure 10 is a picture showing an explosion of a tumor vessel by a selective injury activity toward tumor neovascular smooth muscle cells of d12.CALP in vivo; and the reduction of the tumor due to hemorrhagic necrosis of the tumor cells.

Best Mode of Carrying Out the Invention

As for the cell-specific replication-competent vector which does not act to normal adult cells of the present invention, there is no specific limitation as long as it is a vector in which a transcriptional initiation regulatory region of a gene specifically expressed in cells is integrated upstream of a predetermined gene. However, it is preferable for it to be a replication-competent vector specific to tumor cells and specific to proliferating smooth muscle of new tumor blood vessels. As for the transcriptional initiation regulatory region of said gene specifically expressed in cells, a promoter region of a gene specifically expressed in cells or a region of a part of said promoter can be exemplified, and more concretely,

the examples include: a region including a base sequence from -260 to -219 of a calponin gene promoter shown by Seq. ID No.1; preferably a human calponin gene promoter comprising a base sequence shown in Seq. ID No.2; more preferably a human calponin gene promoter comprising a base sequence shown by Seq. ID No.3 and a region including a part of its structural gene. Furthermore, as for the transcriptional initiation regulatory region of a gene specifically expressed in cells, a base sequence wherein one or a few bases are deleted, substituted or added in the above-mentioned base sequence shown in Seq. ID No.1, Seq. ID No.2 or Seq. ID No.3, having a regulating activity of transcriptional initiation, for example a region including a homologous region to a calponin promoter derived from mouse, rat and pig can be exemplified.

As for the transcriptional initiation regulatory region of a gene specifically expressed in cells, other than the above mentioned, when proliferating smooth muscle cells are made the target to attack, the promoter region of SM22 α gene (the sequence from -480 to -26 for human SM22 α gene; its homologous region for SM22 α gene derived from GenBank accession #D84342-D84344 mouse, rat or other mammals) can be used, and when endothelial cells are made the target to attack, a promoter region of Flk-1 or a promoter region of Flk-1 gene can be used. In these cases, a region including a part of a structural gene can also be made the transcriptional initiation regulatory region.

It is preferable to link an enhancer which activates significantly the transcription at the upstream of a transcriptional initiation regulatory region of a gene specifically expressed in cells mentioned above. As for said enhancer, there is no specific limitation as long as it is an enhancer such as an enhancer of an adenovirus early gene, an enhancer of Moloney murine leukemia virus long terminal repeat, an enhancer of histone H2A gene, an enhancer of

immunoglobulin, an enhancer of insulin gene, a enhancer of c-fos gene, a enhancer of T-cell antigen receptor gene, an enhancer of myopathic creatine kinase gene, a transcriptional enhancer of human 4F2 heavy-chain and the like. However, in the case where the transcriptional initiation regulatory region of a gene specifically expressed in cells is a region including a sequence from -260 to +73 of a promoter of a calponin gene, a 4F2 enhancer such as human 4F2 heavy-chain transcriptional enhancer (Seq. ID No.4) which is an enhancer of a 4F2 heavy-chain gene which is a membrane type-II glycoprotein which carry only once the transmembrane structure which is believed to be an activating factor of an amino acid transporter, is preferable from the point of view that it enhances the transcription efficiency significantly.

As for the predetermined gene to be used for the construction of the cell-specific replication-competent vector which does not act to normal adult cells of the present invention, there is no specific limitation as long as it is a gene necessary to start or maintain viral replication. As for examples, viral replication-related gene such as E1A gene of adenovirus, ICP6 (ribonucleotide reductase) gene and the like can be exemplified, and a gene that encodes a transcription factor necessary to start the replication of herpes virus (ICP 4) can be preferably exemplified. Furthermore, as for these genes, it may be a gene wherein a part or all of the original structural gene located in the downstream of the transcriptional initiation regulatory region is bound with the above mentioned predetermined gene inframe, and a DNA that encodes a fusion protein of a part of the N-terminal side of calponin protein with ICP4 protein can be exemplified concretely.

As for the cell-specific replication-competent vector which does not act to normal adult cells of the present invention, a cell-

specific replication-competent vector wherein one or more than two apoptosis-related gene, DNA that encodes a protein having an action to suppress vascularization, DNA that encodes a protein having an action to suppress cancer metastasis, or DNA that encodes a protein having an action to suppress cancer and the like are linked further downstream of the predetermined gene, and can express under control of said transcriptional initiation regulatory region and the enhancer can be used. Examples of the apoptosis-related gene mentioned above include: apoptosis-promoting gene such as Bcl-xs, Bok/Mtd, Bcl-Gs/Bra, Bcl-GL, Bcl-Rambo, Hrk/DP5, Bik/Nbkblkbad, Bid, BimL, S, EL/BodL, M, S, Noxa/APR, Puma and the like; examples of DNA that encodes a protein having an action to suppress vascularization include: DNA that encodes a protein such as angiostatin, endostatin, FLK1, FLT1, FLT4, Tie1, Tie2 and the like; examples for the DNA that encodes a protein having an action to suppress cancer metastasis include: DNA that encodes a protein such as matrix metalloprotease (MMP) inhibitor, bovine lactoferrin (bLF) and the like; examples of DNA that encodes a protein having an action to suppress cancer include DNA that encodes materials suppressing cell cycle such as p21, p16, p15, p53 and the like or materials suppressing cell proliferation such as p53, Rb, IRF-1, APC and the like, but they are not limited to these examples.

As for the backbone of the virus vector used for the construction of the cell-specific replication-competent vector which does not act to normal cells in adult body of the present invention, it is preferable for it to be a vector that can be expressed by being infected to tumor cells such as sarcoma to bone and soft parts, leiomyosarcoma, gastrointestinal stromal tumor (GIST), malignant mesothelioma, malignant fibrous histiocytoma, fibrosarcoma, malignant meningioma, neurilemmoma and the like, or proliferating smooth muscle cells or cells around vessels of new tumor blood vessels, or

introducing a gene. As for said vector, an expression vector derived from chromosome, episome, liposome and virus can be exemplified. However, virus vector including papovavirus such as SV40, vaccinia virus, adenovirus, adeno-associated virus vector, vector derived from fowl pox virus, pseudorabies virus, retrovirus, herpes simplex virus vector (HSV vector) and the like are preferable, and among these, HSV vector and adenovirus vector, especially a conditionally replication-competent HSV vector or a conditionally replication-competent adenovirus vector is preferable from the point of view of the high efficiency of gene expression, the cytotoxic activity specific to proliferating cell, or the like. By using for example, a DNA that encodes ribonucleotide reductase, a DNA that encodes thymidine kinase or a vector wherein both of these are deleted as the conditionally replication-competent HSV vector mentioned above, the cell-specific replication-competent vector which does not act to normal adult cells of the present invention can be preferably constructed.

As for a DNA which can be the object of the present invention, there is no specific limitation as long as it is a DNA comprising a base sequence shown in Seq. ID No.1 or its complementary sequence; a DNA comprising a base sequence shown in Seq. ID No.3 or its complementary sequence; or a DNA comprising a base sequence wherein one or a few bases are deleted, substituted, or added in a base sequence shown in Seq. ID No.1, Seq. ID No.2 or Seq. ID No.3, and comprising a base sequence having a regulating activity of transcriptional initiation, for example, a homologous sequence of a calponin gene derived from mammals such as mouse, rat or pig and the like, or its complementary sequence. However, preferably, a DNA comprising: a base sequence shown in Seq. ID No.1; a base sequence shown in Seq. ID No.2; a base sequence shown in Seq. ID No.3; or a sequence comprising a base sequence wherein one or a few bases are

deleted, substituted or added in a base sequence shown in Seq. ID No.1, Seq. ID No.2 or Seq. ID No.3, and an enhancer sequence is integrated upstream of a base sequence and the like, having a regulating activity of transcriptional initiation, or their complementary sequence can be exemplified. Furthermore, as for the enhancer sequence, said known enhancer sequences, preferably a 4F2 enhancer sequence such as a human 4F2 heavy-chain transcriptional enhancer and the like comprising a base sequence shown in Seq. ID No.4 can be exemplified.

As for the method for expressing and replicating the cell-specific replication-competent vector which does not act to a normal cell in an adult body of the present invention, there is no specific limitation as long it is a method for expressing and replicating by introducing directly said cell-specific replication-competent vector which does not act to normal adult cells into a living cell tissue, preferably to a tissue or organ wherein a tumor such as sarcoma to bone and soft parts, leiomyosarcoma, gastrointestinal stromal tumor (GIST), malignant mesothelioma, malignant fibrous histiocytoma, fibrosarcoma, malignant meningioma, neurilemmoma and the like are generated or by injecting it from a vascular system that nourishes the tumors; or in case the proliferating smooth muscle of new tumor blood vessels is made the target to attack, a method for expressing and replicating by introducing directly or injecting it from a vascular system that nourishes the tumors regardless of the type of malignant solid tumor. Moreover, as for the therapeutic drug of the present invention, it can be of any kind as long as it comprises said cell-specific replication-competent vector which does not act to normal adult cells of the present invention as an active ingredient, and it is preferable that said therapeutic drug is a therapeutic drug against living cell tissues, preferably against malignant tumors mentioned

above.

Moreover, as for the treatment method of malignant tumor of the present invention, there is no specific limitation as long as it is a method for introducing said cell-specific replication-competent vector which does not act in normal adult cells of the present invention into a tumor tissue to express a predetermined gene, protein or peptide. Among these, a method for disrupting selectively only a tumor cell or a method for disrupting selectively only a proliferating smooth muscle or cells surrounding blood vessels of new tumor blood vessels is preferable. As for the method for introducing into a tissue wherein a malignant tumor is generated, a method for introducing directly the cell-specific replication-competent vector mentioned above into malignant cells, or a method for injecting into a vascular system that perfuses in a tumor can be preferably exemplified.

The present invention will be explained more specifically in the following with reference to the examples, while the scope of the invention will not be limited to these examples.

Example A [Methods and materials]

A-1 (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. Human leiomyosarcoma cell line SKN (RCB0513) and human osteosarcoma cell line OST (RCB0454) were purchased from RIKEN GENE BANK. Human synovial sarcoma and desmoid tumor cell lines were established from resected tumor samples from patients of each tumor. Diagnosis of synovial sarcoma was conducted as described previously (Sarcoma 3, 107-113, 1999), by confirming the expression of the SYT-SSX fusion gene. Primary cultured human mesangium cells (HMC; provided by Dr.

Yamabe, Hirosaki University School of Medicine; Nephrol. Dial. Transplant. 12, 438-442, 1997) were prepared from the kidneys of human fetuses (16 or 18 weeks of gestation) (established by Dr. M. R. Daha, University Hospital of Leiden), subcultured 4 to 6 times, and then used in the following example. Human umbilical vein endothelial cell line HUVEC (T200-05) were purchased from TOYOBO Biochemicals. Vero cells wherein ICP4 gene was introduced and E5 cells were provided by N. Deluca (University of Pittsburgh School of Medicine, Pittsburgh) for use. SK-LMS-1 was cultured in Eagle's MEM supplemented with 1mM sodium pyruvate. HOS, MNNG-HOS, OST, Vero cells and E5 cells were cultured in DMEM. SKN cells were cultured in F12 medium. Synovial sarcoma cells and desmoid tumor cells were cultured in RPMI 1640 medium. Human mesangium cells were cultured in DMEM added with 1 mg/ml D-glucose. All media contain the following respectively: 10%, 15% (for SKN) or 20% (for synovial sarcoma cells and desmoid cells) in final concentration of heat-inactivated fetal bovine serum (Upstate Biotechnologies); 2mM L-glutamine; 100 units/mL penicillin; and 100 µg/mL streptomycin. HUVEC were cultured in a medium according to the manufacturer's instructions. Furthermore, all cells mentioned above were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

A polyclonal antibody specific to mouse calponin (basic or h1) was prepared in the same manner as described previously (Genes Cells 3, 685-695, 1998). A monoclonal antibody to HSV-1 or HSV-2 ICP4 protein (clone No. 1101) was purchased from Goodwin Institute for Cancer Research. Immunoblot analysis was carried out in the same manner as described previously (Int. J. Cancer 79, 245-250, 1998). Chemiluminescence (ECL; Amersham Pharmacia Biotech) was used to visualize the bound antibodies, according to the manufacturer's protocol. Moreover, ICP4 deletion mutant of HSV, d120 (J. Virol 56, 558-570, 1985) and ICP6 (ribonucleotide reductase)-deletion mutant of

HSV, hrR3, provided by N. Deluca or Dr. S. Weller (University of Connecticut Health Center, Farmington), respectively were generated from low-multiplicity infections to E5 cells or Vero cells, respectively.

A-2 (RNA preparation and RT-PCR analysis)

Total RNA was extracted respectively from cells or tissues cultured using the Isogene™ RNA extraction kit (Nippon Gene), and subjected to semi-quantitative RT-PCR analysis as described previously (Int. J. Cancer 79, 245-250, 1998). The conditions for the PCR amplification were: denaturation at 94°C for 40 seconds, annealing at 60°C for 30 seconds, extension reaction at 72°C for 90 seconds; and the cycle was repeated 30 times. As a human calponin primer, 5'-gagtgtgcagacggaacttcagcc-3' [forward primer 1 (FP1); nt# 10-33 GenBank™ D17408; Seq. ID No.5] and 5'-gtctgtgcccagcttggggtc-3' [reverse primer 1 (RP1); nt#660-680; Seq. ID No.6] were used; as a primer of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as a control, 5'-cccatcaccatcttccagga-3' [forward primer 2 (FP2); nt#342-360; Seq. ID No.7] and 5'-ttgtcataccaggaaatgagc-3' [reverse primer 2 (RP2); nt# 1052-1070; Seq. ID No. 8] were used to amplify the DNA of 671bp and 731bp, respectively.

A-3 (Isolation of the human calponin promoter)

Genomic clones containing the 5' upstream side of the human calponin gene were isolated by screening a human genomic λEMBL3 phage library as described previously (J. Biochem. 120, 18-21, 1996). The 5' side-deleted fragments, p-1159Luc, p-385Luc, p-343Luc, p-310Luc, p-299Luc, p-288Luc, p-260Luc, p-239Luc, p-219Luc, p-201Luc, p-176Luc, p-153Luc were generated by PCR amplification, and then subcloned into pGL2-Basic vector (Promega). Numbers indicate the 5' end of the DNA

fragments upstream from the ATG translational initiation codon, hereinafter referred as +1. These deletion fragments have common 3' end at position +73. The nucleotide sequence of the cloned fragments was determined by using a DQS-2000L DNA sequencer (SHIMADZU) according to the manufacturer's protocol, and it was confirmed that the sequence was identical to the sequence (DDBJ/GenBankTM/EMBL database; accession No. D85611) described previously (J. Biochem. 120, 18-21, 1996).

A-4 (Transfection and luciferase analysis)

Cells cultured beforehand were divided and were sprayed on a plate 24 hours before transfection. Cells (5×10^4) were transfected by injecting in a 6-well dish, 1.2 μ g of the promoter plasmid, 0.3 μ g of the pCAGGS/ β -gal reference plasmid, 3.75 μ l of FuGENETM6 transfection reagent (Roche) in each well, according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were harvested in 100 μ l/well of the cell lysis buffer (PicaGeneTM Luciferase Assay System, Toyo Ink). After centrifugation at 4°C at 12000 g for 5 minutes, the supernatant (20 μ l or 30 μ l) were used for luciferase assay and β -galactosidase assay, respectively. Luciferase activity was measured by using a BLR-201 luminescence reader (Aloka). β -galactosidase assay was carried out by using β -galactosidase enzyme assay system (Promega) as described previously (J. Biochem. (Tokyo) 122, 157-167, 1997). All experiments were repeated at least three times to check reproducibility. By assaying cell extracts for β -galactosidase activity, the transfection efficiency was determined, and luciferase activities (light units) were corrected according to the value. By comparing expression of the pSV2-Luc gene containing the SV40 enhancer and SV40 promoter, transfection efficiency of different cell lines was estimated. Data are expressed as % normalized light units $\pm$ S.E. relative to the

values of pSV2-Luc.

A-5 (Virus preparation)

A 4.1kb blunt-ended SalI-MseI fragment (provided by Dr. Hayward, Johns Hopkins School of Medicine) derived from pGH108 (J. Virol. 56, 558-570, 1985) containing ICP4 coding region, was subcloned into the blunt-ended Hind III site of pAMP1 vector downstream of the 333bp human calponin promoter (-260 to +73) at the cloning region and the 444bp NotI fragment of human 4F2 heavy-chain transcriptional enhancer (Mol. Cell Biol. 9, 2588-2597, 1989) (provided by Mr. Leiden, Harvard Medical School) at SmaI site of said vector. The pAMP1/CALP-ICP4 vector was double digested with SalI and Hind III, and the resulting 4.7kb fragment was subcloned into the blunt-ended XbaI site of the pTKAL recombination vector. The pTKAL recombination vector contains the TK coding sequence with deletion of the 0.5kb BglII-KpnI region, Escherichia coli-derived LacZ, and SV40-derived poly A signal site upstream of the TK sequence (+53 of TK) (J. Virol. 71, 5124-5132, 1997). Linearized pTKAL-CALP-ICP4 at SalI site in the plasmid backbone and d120 DNA were co-transfected into E5 cells by using Lipofectamine™ (GIBCO/BRL), according to the manufacturer's protocol. Recombinant virus vectors d12.CALP identified as a single plaque, were stained blue with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) agarose overlay, and were plaque purified three times by infecting to E5 cells in the presence of gancyclovir (1 μ /l). DNA was purified and resolved with restriction enzyme, and the recombination was confirmed by Southern blot and PCR analysis.

Viruses were prepared by infecting 10 to 20 150cm²/tissue culture flasks (IWAKI CLASS) of E5 cells, and harvesting the detached cells after 48 hours. Cells were collected by centrifugation at 4°C for 5 minutes at 400 × g, and were suspended in 10ml of cold virus

buffer (20mM Tris-HCl, pH 7.5 containing 150 mM NaCl). The cells were lysed with three cycles of freezing and thawing in combination with sonication (6 times for 1 minute). After centrifugation at 4°C for 5 minutes at 1500 × g, the supernatant was further centrifuged at 4°C for 45 minutes at 15000 × g. The resulting pellet was suspended in the cold virus buffer, and titers of purified d12.CALP were determined by plaque assay on E5 cells.

A-6 (In vitro cytopathic assay and viral replication assay)

Viruses were infected onto subconfluent monolayers in a 6-well tissue culture plates at a multiplicity of infection (MOI) of 0.01 or 0.001 pfu/cell in 1% heat inactivated FBS/PBS. Said infected cells were incubated at 37°C for 1 hour, and then cultured in said medium containing 1% FBS and 11.3 µg/ml human IgG (Jackson ImmunoResearch Lab.). Forty-eight hours after infection, numbers of plaques/well were counted. For viral replication assay, monolayer cultures of SK-LMS-1 cells or OST cells in 12-well tissue culture plates (2×10^5 cells/well) were infected with d12.CALP at a multiplicity of infection (MOI) of 0.1 in 1% FBS/PBS. The virus inoculum was removed after 1 hour, and the above-mentioned cells were incubated in said medium. The infected virus was harvested from the wells at the predetermined time (12 hours, 24 hours and 48 hours) with 100 µl of the virus buffer. The cell lysates (1 µl) were diluted to 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 cells and OST cells were infected with d12.CALP at a multiplicity of infection (MOI) of 0.01 or the virus buffer alone, and was harvested after 22 hours of culture. Equal amounts of proteins were electrophoresed in a 9% SDS-PAGE gel, and transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked with 5% skim milk (DIFCO Laboratories)

for 2 hours at room temperature, and then incubated with anti-ICP4 antibody (dilution ratio 1 : 500) at 4°C overnight.

A-7 (In vivo treatment and histological analysis)

SK-LMS-1 cells or OST cells were injected subcutaneously into the flanks of 6 week-old female athymic nude mice (BALB/c SLC-nu/nu) (Nihon SLC), to settle the tumors. The tumors were grown in the nude mice to about 6 to 7 mm in diameter. 50 μ l (per tumor volume of 100mm³) of virus suspended solution containing 1×10^7 pfu of d12.CALP or equal amount of virus buffer was injected into the tumors by using a 30-guage needle, respectively. The same operation was repeated 9 days later. The tumors were measured at the predetermined times after injection, and tumor volumes were calculated by using the formula $[0.53 \times \text{length} \times \text{width}^2]$. In the experiments to test whether d12.CALP can be directed to tumors at the opposite side, SK-LMS-1 xenografts were settled beneath the skin in the flanks of both sides of 6-week-old male nude mice, and the viruses were injected into the tumor at one side.

For histological studies, 1×10^7 pfu/tumor volume of 100 mm³ of d12.CALP was administered once, and then the nude mice with tumors were sacrificed on the predetermied days. The subcutaneous tumors were removed and fixed with 2% paraformaldehyde, 0.5% glutaraldehyde, in PBS containing 1mM MgCl₂ overnight at 4°C. Then, the tumors were placed in a substrate solution, containing X-gal (1mg/ml), 5mM K₃Fe (CN₆), 5mM K₄Fe (CN₆) and 1mM MgCl₂ in PBS for 3 hours at 37°C, and washed with PBS containing 3% DMSO.

To assess the distribution of infected viruses by PCR, DNA was prepared from fresh tissues of infected or non-infected tumors, and brain, lung, liver, kidney, heart, small intestine and uterus or testis. The conditions for PCR amplification were: denaturation at

94°C for 40 seconds, annealing at 60°C for 30 seconds, extension reaction at 72°C for 90 seconds, and the cycle was repeated 30 times. As for ICP6 (ribonucleotide reductase) primer, 5'-gacagccatatacctgagc-3' [forward primer 3 (FP3); Seq. ID No. 9] and 5'-actcacagatcggttgacgaccg-3' [reverse primer 3 (RP-3); Seq. ID No.10], as for primer of glycoprotein E, 5'-gagatgcgaatatacgaat-3' [forward primer 4 (FP4); Seq. ID No.11] and 5'-gtgggtgggctcggccaaat-3' [reverse primer 4 (RP4); Seq. ID No.12], as for a primer of Escherichia coli of LacZ, 5'-gcgttacccaacttaatcg-3' [forward primer 5 (FP5); Seq. ID No.13] and 5'-tgtgagcgagtaacaacc-3' [reverse primer 5 (RP5); Seq. ID No.14]; as for the primer for glyceraldehyde 3-phosphate dehydrogenase: GAPDH), 5'-cccatcaccatcttccagga-3' [forward primer 6 (FP6); Seq. ID No.15] and 5'-ttgtcataccaggaaatggc-3' [reverse primer 6 (RP6); Seq. ID No.16] were used to amplify DNA of 221bp, 320bp, 731bp, respectively (J. Virol.74, 3832-3841, 2000).

A-8 (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 embedded in paraffin. Sections of 4 µm thickness were mounted on a poly-L-lysine coated microslides, treated in xylene, dehydrated through graded alcohol, and immersed in 70% methanol with H₂O₂ to block endogenous peroxidase. Then, antigen retrieval was performed using an autoclave at 121°C for 10 minutes in a 10 mM citrate buffer (pH 7.0). The sections were incubated at room temperature for 1 hour by using 1% (v/v) of goat serum/PBS, washed with PBS, and incubated with a polyclonal antibody against mouse calponin (Genes Cells 3, 685-695, 1998), in 2% (w/v) BSA/PBS overnight at 4°C. The section mentioned above were washed 5 times with 0.005% (v/v) Tween™ 20/PBS, followed by incubation with biotinylated goat

anti-rabbit IgG (TAGO Immunologicals) in 2% (w/v) BSA/PBS for 1 hour at room temperature, then incubated with avidin-biotin-horseradish peroxidase complex (Vector Laboratories) for 30 minutes at room temperature. After being washed in 0.005% (v/v) Tween 20/PBS, the final reaction product was washed with visualized with diaminobenzidine (WAKO Chemicals), and the sections were counterstained with hematoxyline. Tissue specimens treated with goat serum were used as a control to observe the non-specific stain.

A-9 (Statistical analysis)

Statistical differences were determined by using unpaired-Student's t-test. Differences were considered statistically significant with $p < 0.05$.

Example B [Results]

B-1 (Identification of an expression regulatory region of human calponin promoter)

To identify minimal promoter region which regulates the expression of human calponin, the plasmid having various 5' deleted calponin promoter luciferase constructs were transfected into human osteosarcoma cell lines MNNG-HOS and HOS, and mesangium cell line HMC. Mesangium cell line HMC showed a stable growth pattern (hill and valley) characteristic to a smooth muscle-like phenotype and expressed smooth muscle-specific genes such as α -smooth muscle actin and SM22 α . The calponin gene was most highly expressed in HMC among the three cell lines transfected (Figure 1). As described previously (Int. J. Cancer 79, 245-250, 1998), calponin was expressed at the intermediate level in HOS, but was not expressed at all in MNNG-HOS (Figure 1).

As a result of transient transfection assay of plasmids p-288Luc and p-260Luc into HOS and HMC cells, luciferase activities for

both increased by 4 times in HOS cell, 6 times in HMC cells than that of when p-1159Luc was transfected. This shows that there is an expression regulatory region between -1159 to -288 of the calponin promoter region. There was a significant correlation between the expression of calponin mRNA and the transcriptional activities of the promoter region from -385 and -260. As the bases were further deleted from -260 to -219, the promoter activity largely decreased in both HOS and HMC cells. Furthermore, when the constructs wherein a wide range of 5' region of calponin gene promoter region are deleted (p-201Luc, p-176Luc and p-153Luc) were transfected, the luciferase activity was the same as when p-219Luc was used. These results indicate that the sequence between -260 to -219 is a positive expression regulatory region of calponin gene transcription in both HOS and HMC cells.

The region between -260 and -219 of the calponin gene promoter mentioned above, includes some sequence motifs similar to consensus binding sequences for Sox (AACAAT) at -258 and GATA-1 (CACAATCAGC) at -250. When a part of -260 to -239 of the calponin gene promoter is deleted from p-260Luc, the transcriptional activity is decreased 50%. To test whether the putative binding site of Sox and GATA-1 and a region downstream to -239 show an expression regulatory function, 3 mutations were prepared by substituting -255/-254 (AA to GG), -246/-244 (A to G at -246, C to T at -244), -232/-231 (CC to TT) of the plasmid p-260Luc, and the plasmids were transfected. In the transfection experiments in HMC cells, p-260 Luc activity for the 3 mutations mentioned above were $73 \pm 0.2\%$, $76 \pm 0.2\%$, $39 \pm 0.1\%$, respectively. These results suggest that all the sequence encompassing -260 to -219 were required for the transcriptional activity of the calponin promoter.

B-2 (Regulation at the transcriptional level of the expression of calponin gene in human soft tissue and bone tumor cells)

To further assess whether there is a correlation between the expression of calponin and the transcriptional activity of 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 (Mol. Cell Biol. 9, 2588-2597, 1987) inserted upstream to p-260Luc (p4F2-260Luc). By RT-PCR analysis, the expression of calponin mRNA was observed in synovial sarcoma cells and SK-LMS-1 leiomyosarcoma cells. By contrast, the expression of calponin in OST osteosarcoma cells was very low (Figure 2). As it is shown in Figure 2, in all the cells examined, the transcriptional activities of p-260 Luc and p4F2-260Luc were correlated with the expression level of transcripts of calponin mRNA. These experimentation results indicate that the expression of calponin gene 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, 4F2 enhancer was inserted upstream of the calponin promoter, and the transcriptional activity of p-260Luc in calponin-positive synovial sarcoma and SK-LMS-1 cells increased from 3 to 5 times. Therefore, in the following experiment, the 4F2 enhancer/-260 calponin promoter sequence was used to regulate expression of the HSV ICP4 gene in human soft tissue and bone tumor cells.

B-3 (Selective replication of a recombinant HSV vector in calponin-positive cells in vitro)

To construct an HSV vector that replicates selectively in calponin-positive cells and proliferating cells, a DNA fragment containing the 4F2 enhancer/-260 calponin promoter/ICP4 (pTKΔ-CALP-

ICP4) was inserted into the TK locus (U_L23) of ICP4-HSV mutant d120 (J. Virol. 56, 558-570, 1985) to prepare d12.CALP. The plasmid pTKΔ-CALP-ICP4 contains 2 chimeric transgenes expressing ICP4 protein and β-galactosidase wherein Escherichia coli-derived LacZ was inserted (Figure 3A). Human cell lines with or without calponin expression were used to assess the selectivity of d12.CALP viral replication (Figure 3B).

The cell lines constructed as mentioned above were infected with d.12CALP or hrR3 at the multiplicity of infection of 0.001 for 48 hours. Plaque formation was assessed to evaluate viral replication (Figure 3C). In calponin-positive synovial sarcoma cells, SK-LMS-1 cells and HOS cells, d12.CALP showed similar cytopathic effects as that of hrR3. In contrast, in calponin-negative SKN cells, OST cells, MNNG-HOS cells and HUVEC cells, no apparent cell lysis by d12.CALP was observed. Desmoid cells, which showed the slowest proliferation speed, expressed mRNA of calponin at a same level as SK-LMS-1 cells, but no apparent formation of plaque by d12.CALP was observed. These results show that the cytopathic effect by d12.CALP depends on the both expression of calponin and the cell proliferation speed.

As it can be seen from Figures 4A and 4B, when d12.CALP is infected to SK-LMS-1 cells and synovial sarcoma cells at low multiplicity of infection (MOI:0.001), a complete ontolysis of the cultures in a 10cm-dish was observed 96 hours after infection. It was also confirmed that the cytolysis of synovial sarcoma cells spread from cell to cell (Figure 4A). Among the SK-LMS-1 cells infected, some were multi-nucleated before lysis (Figure 4B, arrow).

Viral titers were assessed by single step growth assays. d12.CALP replicated in calponin-positive SK-LMS-1 cells but the titers of d12.CALP decreased in calponin-negative OST cells 48 hours after infection from $1/10^6$ to $1/10^7$ compared to SK-LMS-1 cells (Figure 5A).

By conducting immunoblot analysis of cell extracts 22 hours after infection, it was found that ICP4 protein was expressed in SK-LMS-1 cells but that ICP4 protein was not expressed in OST cells. This was consistent with the result of viral replication assay (Figure 5B). In contrast, d120 viral vector did not show generation of viral progenies at all in cultures of SK-LMS-1 and OST.

B-4 (Treatment of human leiomyosarcoma xenografts with a recombinant HSV vector)

To evaluate the effect of d12.CALP in a treatment in vivo, SK-LMS-1 leiomyosarcoma xenografts were settled to nude mice, and 1×10^7 pfu d12.CALP per tumor volume of 100mm^3 was administered twice. As a control, the virus buffer alone was administered to the tumor sites. Before the treatment, there was no significant difference between the tumors treated with d12.CALP and the control tumors, in tumor volume (138 ± 20 and $139 \pm 28 \text{ mm}^3$, respectively, $n=5$), or in the expression levels of immuno-reactive calponin. The infection of d12.CALP showed an association with the growth suppression of SK-LMS-1 tumors, but there were no association with the growth suppression of calponin-negative OST tumors (Figure 6A). On the contrary, in the treatment of SK-LMS-1 xenografts with a virus buffer alone, the growth of progressive tumor and the death of all the animals ($n=5$) were confirmed by 89 days after the treatment, and it was found that it was associated with the progressive tumor growth and death of animals (Figure 6B). By 5 weeks after the initial d12.CALP infection, it was confirmed that the tumors were completely regressed in 4 out of 5 mice (Figure 7). In one mouse, the tumor was regrown. Therefore, the recurrent tumor of said mouse was treated again with d12.CALP, and the tumor growth was suppressed in a stable manner.

In histochemical staining with X-gal, the expression of β -

galactosidase was observed by introducing LacZ to TK locus in SK-LMS-1 tumor cells treated with d12.CALP (Figures 8A and 8B), but was not observed in control tumor cells. By this result, the region wherein d12.CALP viruses spread in vivo was identified. At day 8, necrosis began to be noticeable, and the expression of LacZ was lacking in this region (Figure 8A, arrow). At higher magnification, as it was observed in cytopathic assays in vitro, it was found that some of the cells among the tumor cells stained blue were multi-nucleated (Figure 8C, arrow), and these were losing their typical morphological appearance of SK-LMS-1 cells. However, as it is shown in Figure 8D, the expression of LacZ was negative for smooth muscle cells surrounding normal blood vessels in virus-infected mice. Furthermore, by PCR analysis, LacZ sequence specific to d12.CALP could not be confirmed in DNA prepared from brain, lung, liver, kidney, heart, small intestine or uterus prepared 8 days after administration of d12.CALP into the tumors (Figure 8E). In organs including aortic or gastrointestinal smooth muscles, viral replication and expression of LacZ were not observed histologically.

B-5 (Spread of recombinant HSV vector in tumor)

In order to assess whether d12.CALP which was injected into SK-LMS-1 xenograft and replicated could target tumor cells which are located in a distant place through blood vessels, d12.CALP was inoculated in tumors in SK-LMS-1 xenograft in the right flank, and the virus distribution at SK-LMS-1 xenograft in the left flank was examined. As it is shown in Figure 9A, the expression of β -galactosidase in tumor cells in the left flank was confirmed at day 20, as well as in the site of inoculation. Histologically, tumor necrosis were observed in a wide range, in both tumors at the right flank and tumors at the left flank but as it is shown in Figure 9B, no

effect by d12.CALP was observed in calponin-positive smooth muscle cells surrounding normal blood vessels.

By performing PCR analysis using primers to ribonucleotide reductase (ICP6), glycoprotein E or Escherichia coli-derived LacZ, which were inserted in TK locus, it was found that virus DNA derived from d12.CALP spread in tumor tissues in both flanks but did not spread in tumor tissues in brain or testis (Figure 9C).

B-6 (Selective injury activity to tumor neovascular smooth muscle cells)

Human leiomyosarcoma cells were xenotransplanted beneath the skin of both dorsal parts of a nude mouse, and d12.CALP was injected into one side (right side). At day 8 after injection, bleeding occurred to the xenograft of the opposite side (left side), and the tumors diminished (n=4) (Figure 10). As for the leiomyosarcoma used in this experiment, it was confirmed that d12.CALP do not injure the tumor cells itself in an infection experiment in vitro, the retraction of the tumors was thought to be the result of d12.CALP that has attained hematogenously the tumor at the opposite side and has injured the blood vessel in the tumors. Actually, by investigating histologically the tumors 2 days after bleeding, an image of a wide hemorrhagic necrosis of the tumor cells and a disruption image of the blood vessels were observed. Moreover, by an immunohistochemical analysis, the expression of LacZ and ICP 4 protein were observed in the cells surrounding blood vessels. On the other hand, in the smooth muscle cells of normal blood vessels contacting the tumors, cell injury was not observed.

Industrial Applicability

A malignant tumor derived from mesenchymal cells, that is a

sarcoma, is resistant to chemotherapy or radiotherapy and continues to relapse even after surgical resection, and by eventually spreading to lung, liver, peritonuem and the like, lead the patient to death. The number of cases in Japan is up to 5000 to 10000 annually, including mainly sarcoma to bone and soft tissue in the field of orthopedic surgery, and including leiomyosarcoma in the field of gynecological, stroma sarcoma in the field of digestive surgery, malignant mesothelioma in the field of chest/digestive surgery, fibrosarcoma, malignant meningioma, neurilemmoma in the field of neurosurgery and the like. Although it represents only about 1% of carcinoma, as it generates frequently also to young people, and as there is no effective treatment, the development of a new treatment method is strongly required socially. The present invention can respond to such requirements, and the present invention can provide a cell-specific replication-competent vector which does not act to a normal cell, which expresses specifically in particular cells such as malignant tumor cells and the like, and by using said cell-specific replication-competent vector, the first gene therapy in the world being selective to sarcoma cells can be possible. Especially, as calponin gene is expressed in 31% of gastrointestinal stromal tumor (GIST), 91% of leiomyosarcoma, 38% of stroma tumor in the field of digestive surgery, 60% of osteosarcoma, and 32% of soft part sarcoma other than leiomyosarcoma, the therapeutic drug of the present invention can be used as an effective treatment of cancer to all solid cancers and among them, can be used effectively in a new gene therapy that selectively disrupts tumor vessels.

SEQUENCE LISTING

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20

Claims:

1. An isolated DNA consisting of the nucleotide sequence of Seq. ID No. 1 or the complementary sequence thereof.
2. An isolated DNA consisting of the nucleotide sequence of Seq. ID No. 2 or the complementary sequence thereof.
3. An isolated DNA consisting of the nucleotide sequence of Seq. ID No. 3 or the complementary sequence thereof.
4. An isolated DNA consisting of the nucleotide sequence of Seq. ID No. 1, Seq. ID No. 2 or Seq. ID No. 3 and an enhancer sequence operably linked upstream of said nucleotide sequence.
5. The isolated DNA according to claim 4, wherein the enhancer is a 4F2 enhancer.

Application number / numéro de demande: JP02/04334

Figures: 4/5/7/8/9/10

Pages: _____

Unscannable items
received with this application
(Request original documents in File Prep. Section on the 10th floor)

Documents reçu avec cette demande ne pouvant être balayés
(Commander les documents originaux dans la section de préparation des dossiers au
10ème étage)

FIG 1

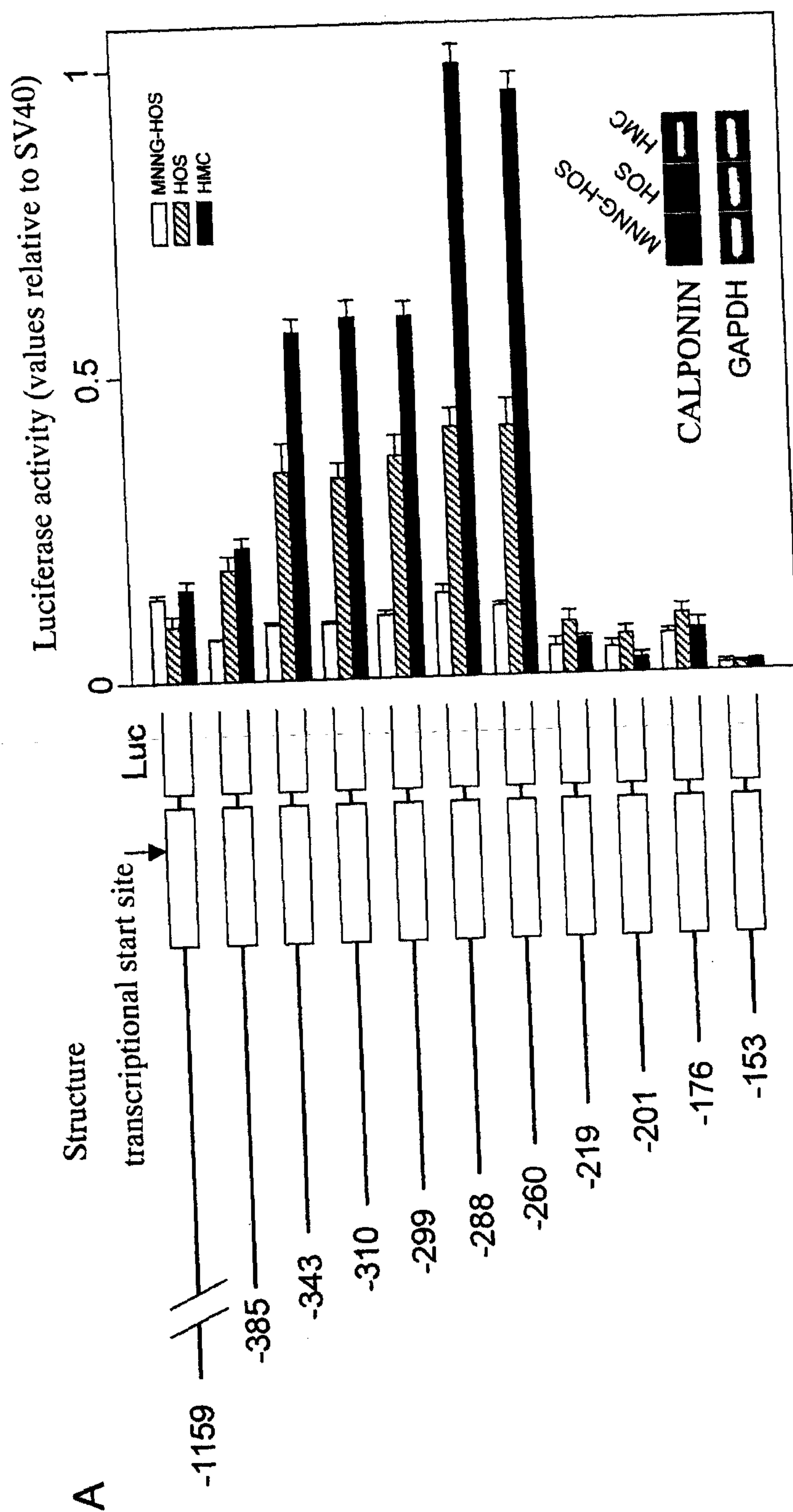


FIG 2

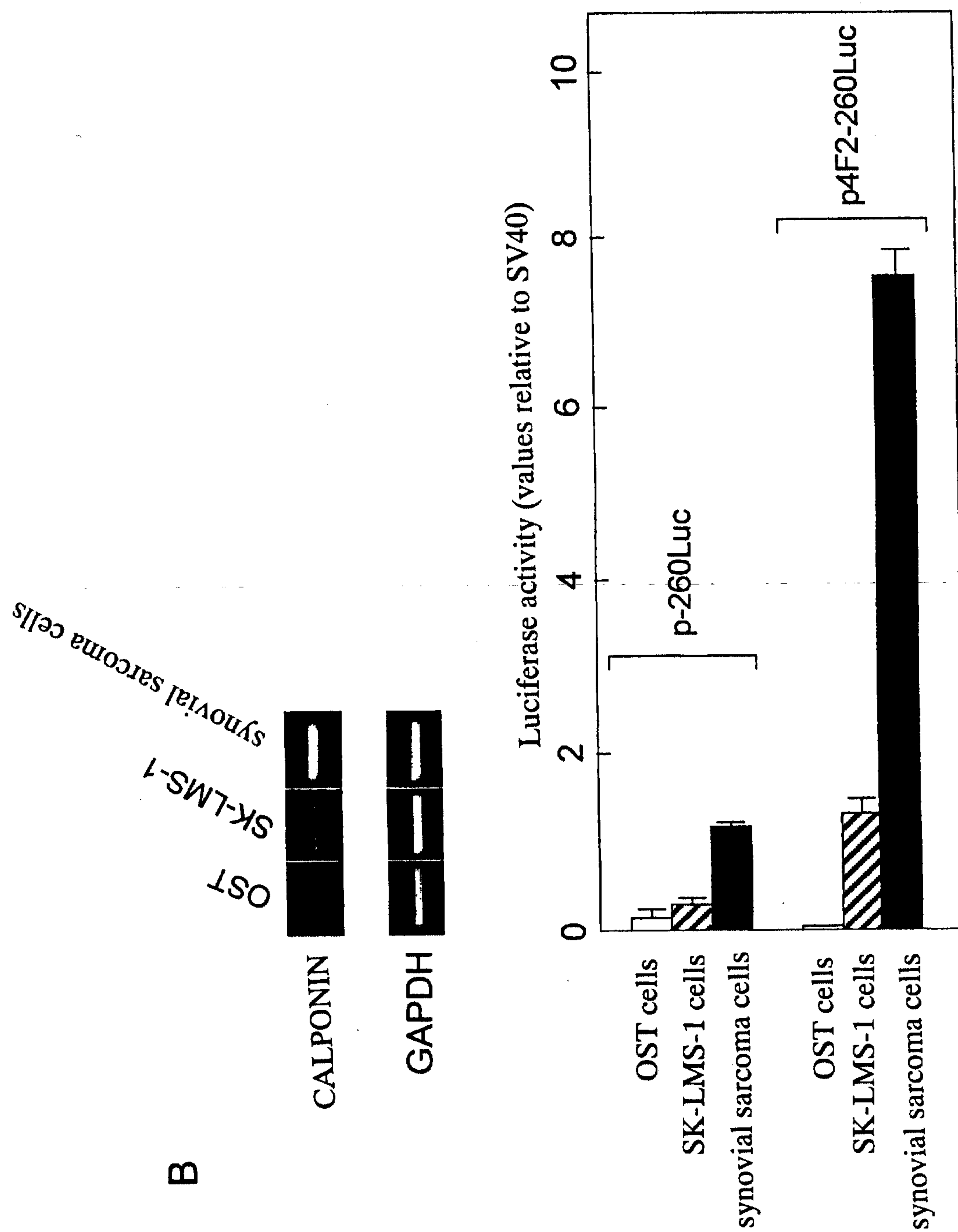


FIG 3

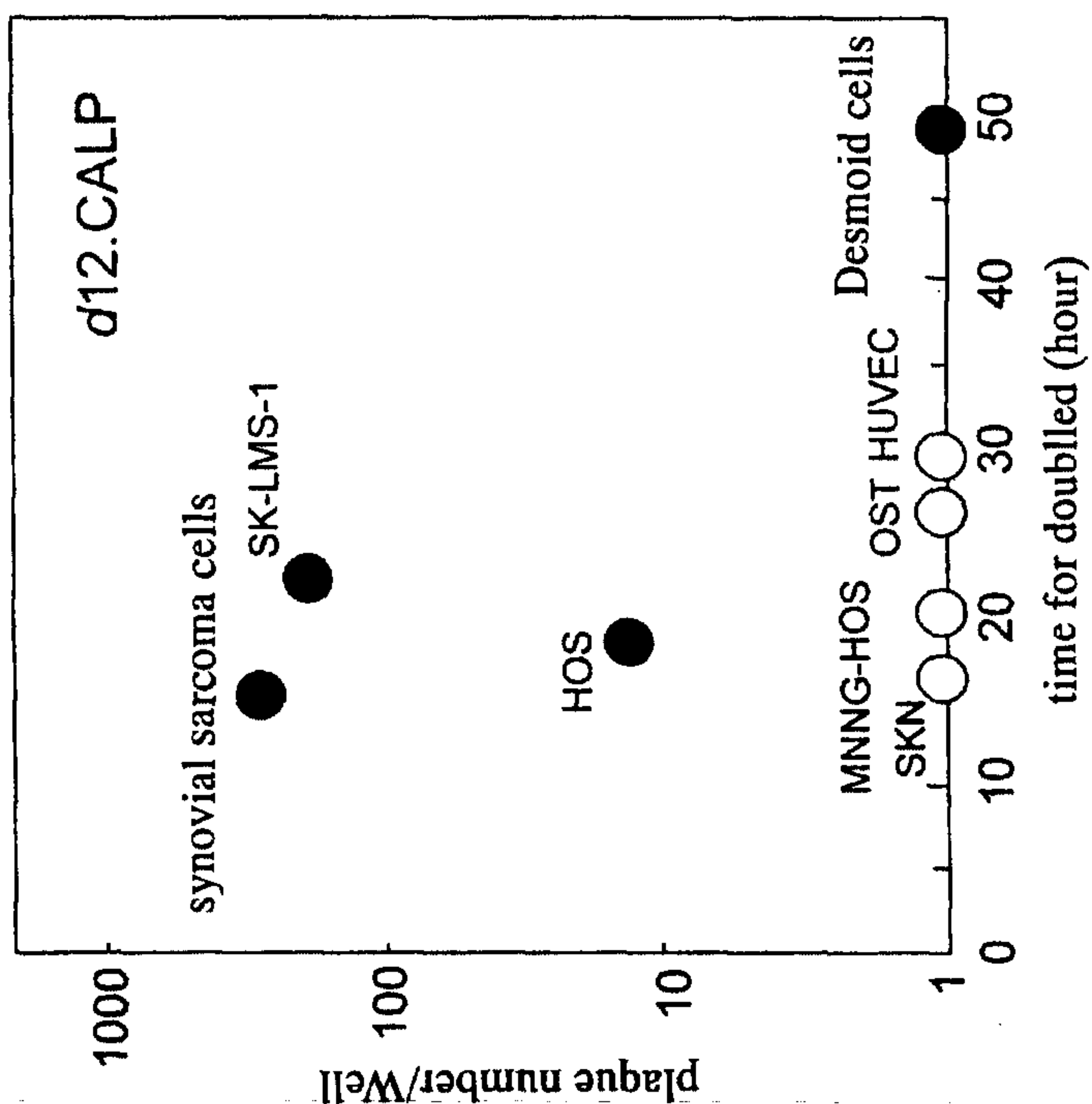
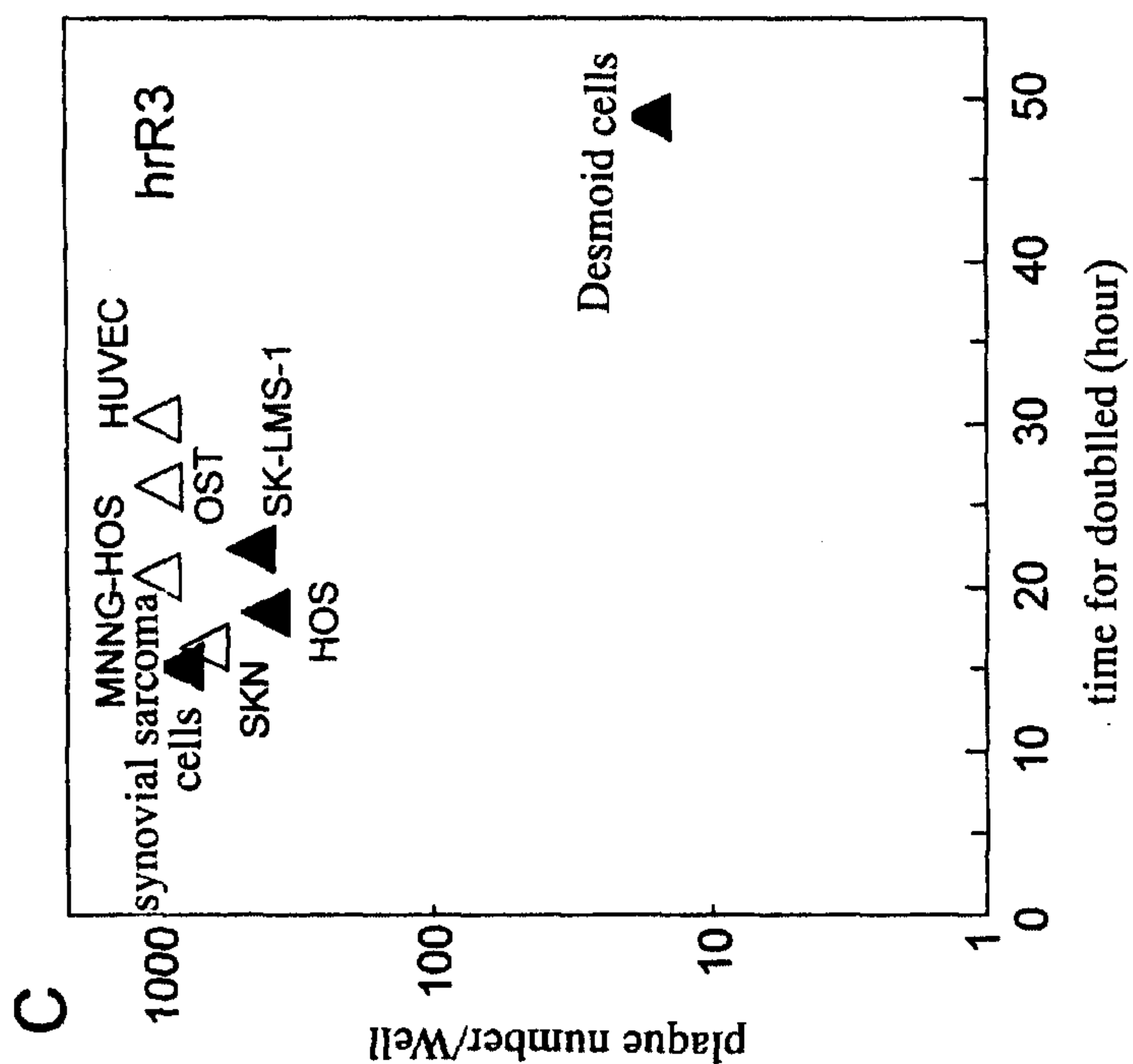
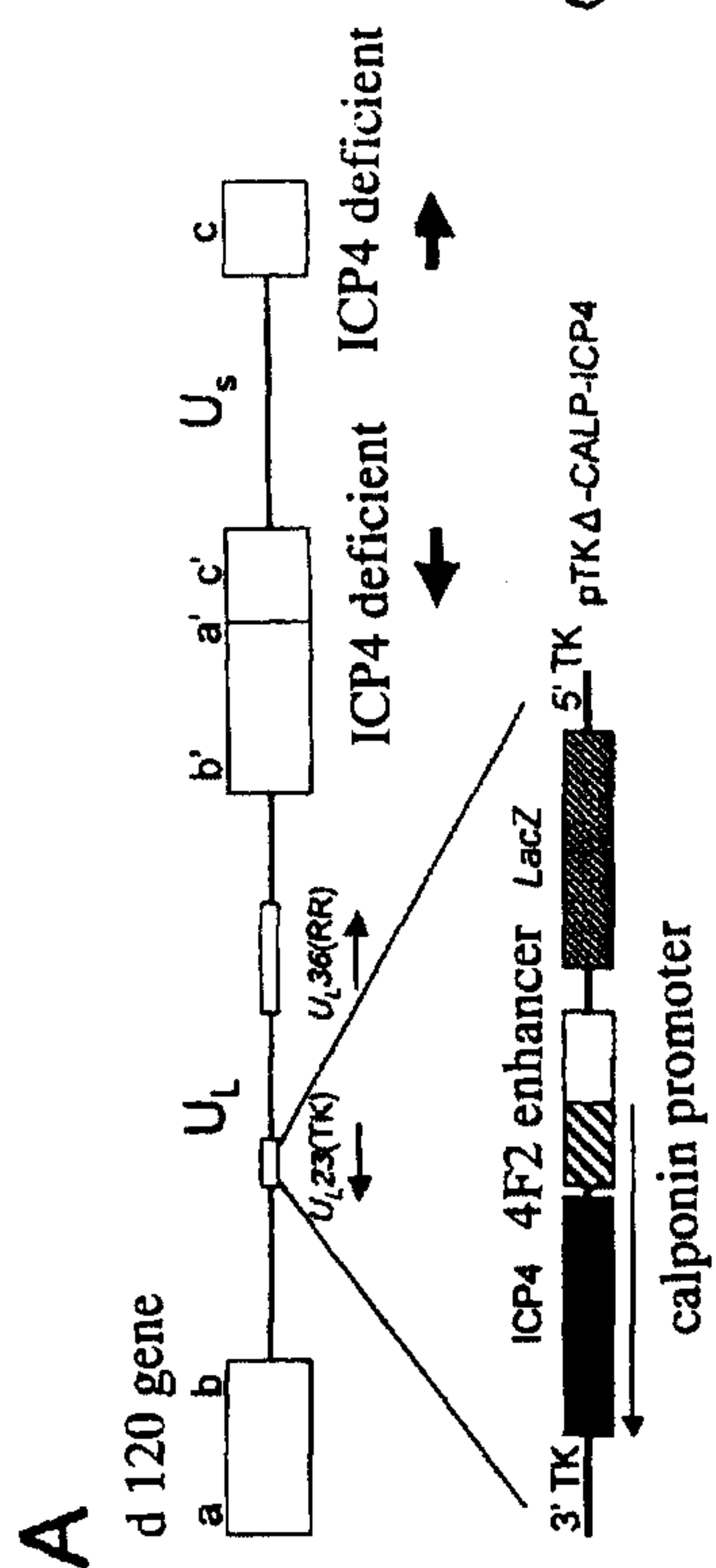
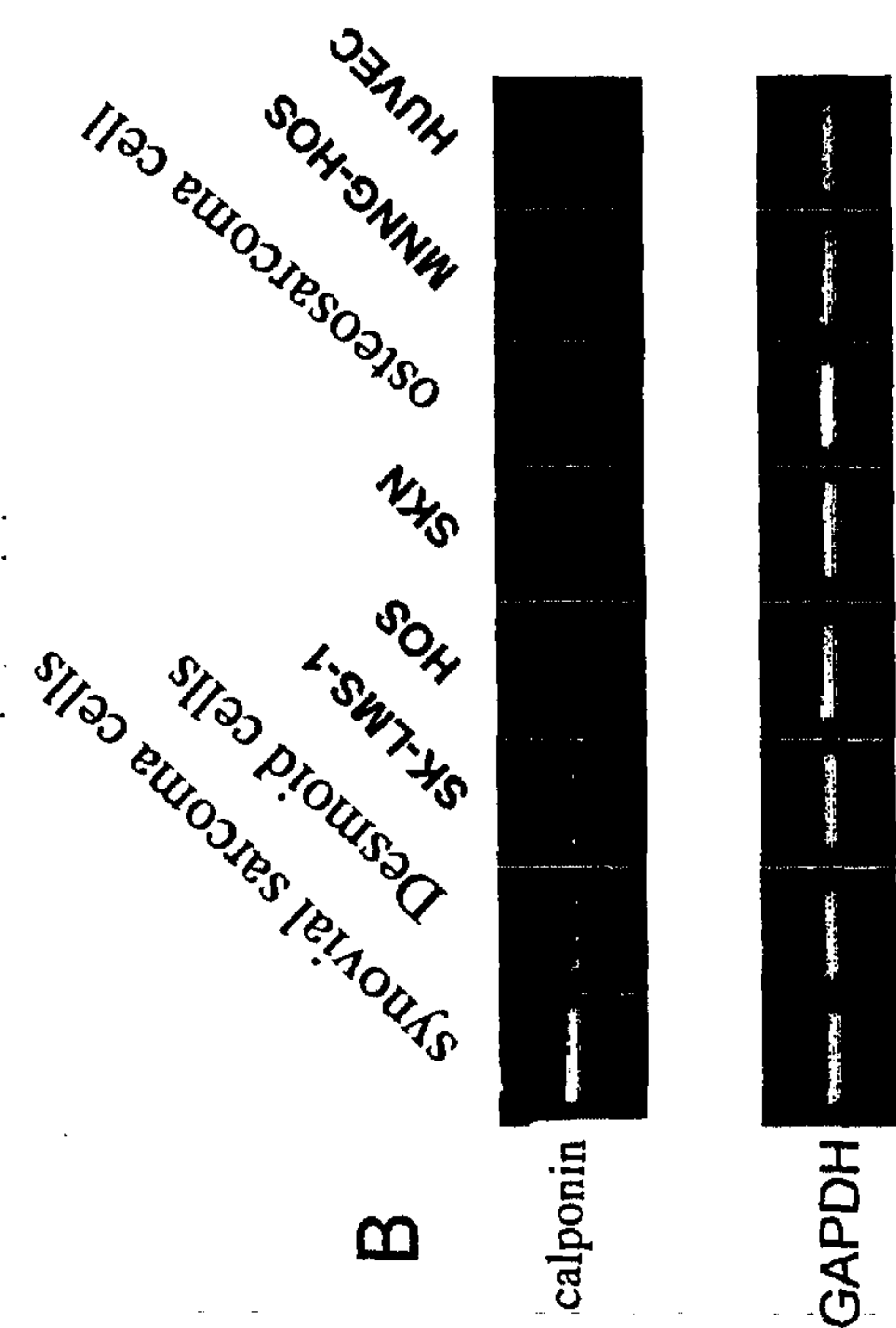


FIG 6

