BIO-LRCC-0008
Koropatrick, J.

Modification Form for Permit BIO-LRCC-0008

Permit Holder: James Koropatnick

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Kathleen Calonego

Alayne Brisson

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* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED.*

Classification: 2

Date of last Biohazardous Agents Registry Form: Apr 1, 2008

Signature of Permit Holder: J James Koropatnick

BioSafety Officer(s): Dale Ryder July 30/09

Chair,Biohazards Subcommittee: _____

Modification Form for Permit BIO-LRCC-0008

Permit Holder: James Karopatnick

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. *
Approved Microorganisms	E. coli DH101	
Approved Cells	Human (primary), rodent (primary), Human (established), Rodent (established), Insect (established), Anaplastic astroyctoma SF-268, Breast MCF-7, MDA-MB-435, MDA-MB-231, MDA-MB-468, SK-BR-3, Cervical	HEK293 cells (human embryonic kidney, normal, transformed with Ad 5 DNA); does not generate active virus particles
Approved Use of Human Source Material	blood (whole), blood (fraction) white blood cells, tumour tissue (unpreserved)	
Approved GMO		
Approved use of Animals	CS-1 nu/ni mice	
Approved Toxin(s)		

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Classification: 2

Date of last Biohazardous Agents Registry Form: Apr 1, 2008

Signature of Permit Holder: J James Karopatn

BioSafety Officer(s): Julie Ryder July 30/09

Chair,Biohazards Subcommittee: _____

------- Original Message --------

Subject:RE: Modification Form: Koropatnick

Date: Tue, 22 Sep 2009 11:04:39 -0400

From: James Koropatnick <jkoropat@uwo.ca>

To:'Jennifer Stanley' <jstanle2@uwo.ca>

CC:'Peter J Ferguson' <peter.ferguson@uwo.ca>

References:<4AB3F6A8.3060709@uwo.ca>

Jennifer, the virus used in this study will generated by us in HEK293 cells. We will use commercially-prepared vectors and (from AdMax) that we will transfect into the host cells. The host HEK293 cells will then produce the virus and package it, by a well-understood and standard. The virus that is produced cannot replicate itself in the glioblastoma cells that will be exposed to it.

James Koropatnick

Professor and Director, Cancer Research Laboratory Program (CRLP), London Regional Cancer Program
Assistant Director and Scientist, Lawson Health Research Institute
London Health Sciences Centre, London, Ontario, Canada
Director, CIHR/UWO Strategic Training Program in Cancer Research and Technology Transfer (CaRTT)
Departments of Oncology, Microbiology and Immunology, Physiology and Pharmacology, and Pathology
The University of Western Ontario
790 Commissioners Road East
London, Ontario, Canada N6A 4L6
E-mail: jkoropat@uwo.ca
Telephone: 519-685-8654
Fax: 519-685-8673

Koropatnick

Information for Biohazard Approval and Animal Protocol Modification

The adenovirus that will be used for these studies is made from a commercially available kit called AdMax (www.microbix.com). The company product information sheet and the primary reference research article are attached. Briefly, two vectors are cotransfected into HEK293 cells that generate and package the new adenovirus. One vector codes for a modified version of the type 5 adenovirus and the other codes for the foreign gene of interest. The virus produced is non-replicative.

Infection of cells with the adenovirus will result in protein expression of RGS2 and the RGS2 minigene (a small fragment of full length RGS2). It is not expected that any discernable change in cellular phenotype will occur from the infection, aside from a reduction in their proliferation rates.

The proposed work is an extension of our *in vitro* studies that characterized the anti-proliferative properties of RGS2 and the RGS2 minigene in U87 glioblastoma cells. We have secured funding from WORLDiscoveries (C4 POP) to examine their potential anti-tumour effects in an *in vivo* animal tumor model.

Subject: Re: Viral Vector Policy and Koropatnick project
From: Alayne Brisson <Alayne.Brisson@lhsc.on.ca>
Date: Fri, 04 Sep 2009 16:14:02 -0400
To: Donald J Koropatnick <jkoropat@uwo.ca>, Jennifer Stanley <jstanle2@uwo.ca>

Hi Jennifer,

There is reference to the virus used here in the second paragraph of the manuscript that is attached. It states: "First-generation Ad vectors typically have foreign DNA inserted in place of early region 1 (E1) and/or E3. E1-depleted vectors are replication deficient and are propagated in E1-complementing cells such as the 293 cell line (Graham et al., 1977)."

E-1 depleted vectors are the type that are to be used in the protocol.

Cheers,
Alayne

Jennifer Stanley <jstanle2@uwo.ca> 04/09/2009 3:46 PM >>>

Hi Dr. Koropatnick

Can you provide the information/documentation for question #1?

Thanks,
Jennifer

****

Hi Jennifer, Thanks for the extra info. To answer your questions:

1. Does this gene have oncogenic potential?
NO
2. Are there two viruses? One with full length RGS2 and one for the truncated version?
Are both to be used?
There are 3 viruses that will be used total. One is packaged with full-length RGS2, one is packaged with a truncated segment of RGS2 and the last is the empty adenovirus vector.

Sorry for the confusion, I am not so familiar with this new project myself.

Alayne

Microbix_supplement.pdf Content-Type: application/pdf

Koropatnick. Aus protocol (Viral vector Policy)

Subject: Viral Vector Policy and Koropatnick project
From: Jennifer Stanley <jstanle2@uwo.ca>
Date: Wed, 05 Aug 2009 11:39:06 -0400
To: "Gregory A. Dekaban" <dekaban@robarts.ca>, "Gerald M. Kidder" <avpres@uwo.ca>

Hi Jerry and Greg:

I am looking for some advice on how to apply the Viral Vector Policy (attached) to the following protocol: The information in red below is from the Animal Use Protocol/Researcher. The statements in quotes is directly from the Protocol.

Rodents are being injected with "U87 cells infected with adenovirus. The U87 cells will not shed the adenovirus and therefore the virus will be contained with the tumour in the animal"

Details on adenovirus: adenovirus type 5, replication incompetant

Vector: p?E1sp1A1lox

Source of vector: www.micrbix.com

Gene transfected: RGS2

Details: "Infection of cells with the adenovirus will result in protein expression of RGS2 and the RGS2 minigene (a small fragment of full length RGS2). This should inhibit proliferation."

Based on the viral vector policy, I would classify this as Level 2 (assuming that proliferation is really inhibited). If the researcher provides evidence (that that the virus is not shed or is only shed for a limited time), he could potentially lower the containment (ie Level 1) or limit the duration of the containment required. However, the Researcher plans to use Containment Level 2 at this point.

Any thoughts on this would be appreciated!

Jennifer

Viral_vector_policy_ Final_ June_2009.pdf **Content-Type:** application/pdf
Content-Encoding: base64

Koropatnick

Microbix
Biosystems Inc.

AdMax™ Adenovirus Vector Creation Kits

Clone, cotransfect and GO! Small shuttle plasmids, single cloning step, cotransfections without restriction, 100% reliability. The simplest, most efficient, most flexible system for construction of adenovirus expression vectors.

HOW FAST? How fast can you clone your gene into a small pUC based shuttle plasmid and prepare 100mg plasmid DNA? Add 7 to 10 days to that!

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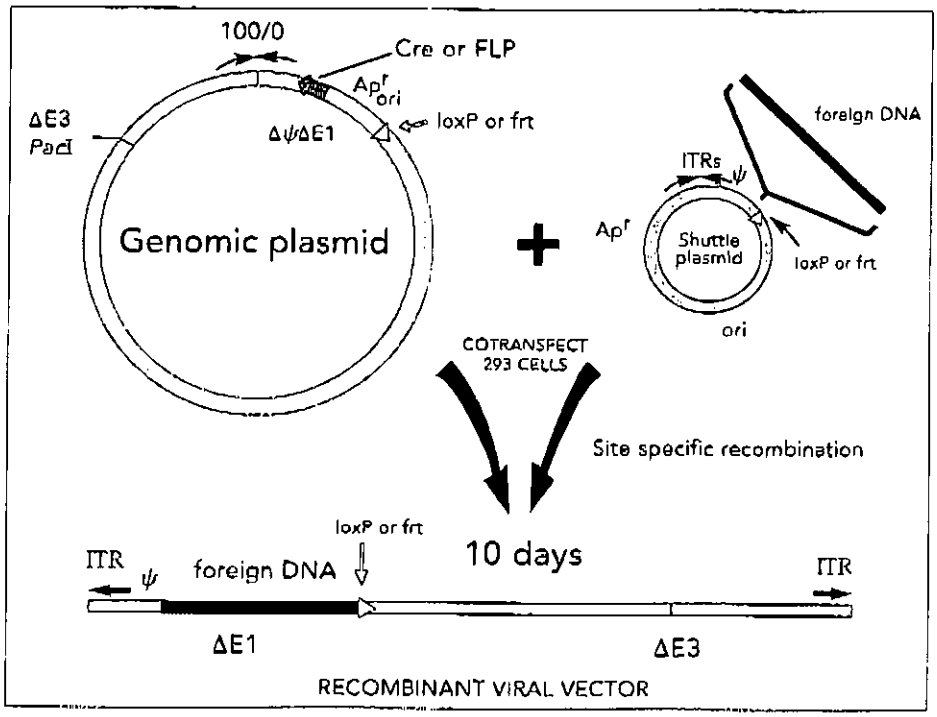
HOW SIMPLE? Only two steps. No homologous recombination in difficult to handle bacterial systems; use your favourite bacterial strain. No transfer of candidate plasmids from one bacterial strain to another. No need for expensive, exotic restriction enzymes or for linearization of plasmid DNA prior to cotransfection of 293 cells. The system does not require lambda packaging or yeast technologies that are not standard procedures in the majority of labs.

HOW FLEXIBLE? Cassettes can be inserted in E1 or E3 or transgenes can be cloned into both regions. For example a transactivator can be inserted in E3 and a regulated expression cassette in E1. Vectors can be designed with an E3 deletion, a wild type E3 region or, if the transgene in E1 is small, a stuffer sequence can be inserted in E3 to prevent formation of RCA. You have a choice of two site specific recombinases: Cre or FLP, with similar high rescue efficiencies.

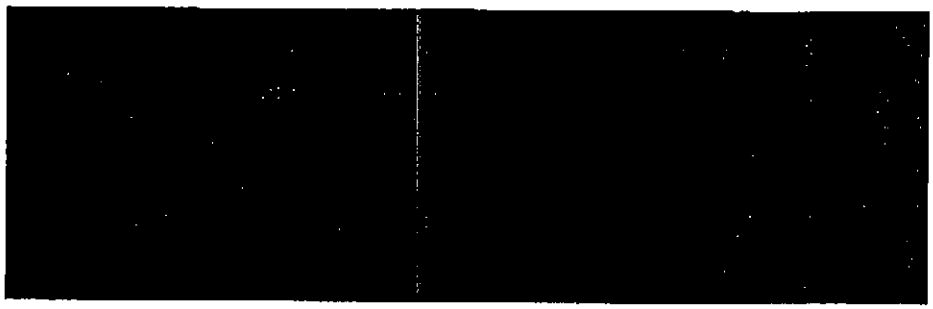
HOW EXPENSIVE? The initial cost of our kits is competitive with other systems, but unlike other kits ours allow for an infinite number of vector rescues. If you can grow plasmid DNA there is no need to purchase our kits more than once.

Figure 1 outlines the principles of the AdMax™ system with Cre-lox as an example. Recombination in cotransfected cells introduces the gene of interest into infectious Ad DNA while simultaneously excising the recombinase gene[1,2] Neither the small shuttle plasmid nor the genomic plasmid need be digested with restriction enzymes prior to cotransfection.

AdMax™ for generation of Adenovirus vectors



Any E1 complementing cell line such as 293 cells, 911 cells or PERC6 cells can be used for cotransfections. Although rescue of viral vectors is highly efficient (over 100 fold greater than with the original two plasmid method of Bett et al.[3]), and 100% of viruses generated by cotransfection should carry the transgene, it is good laboratory practice to build up working stocks of virus from plaque isolates before extensive experimentation. Microbix provides low passage 293 cells that are especially cultured to maintain the strong adherence and plaque forming properties of the original 293 cells. For rapid production of vectors to be used in preliminary experiments, it may be possible to produce recombinant viruses by incubating cell cultures under liquid medium following cotransfections.



Because the only restriction enzymes required with the AdMax™ system are common enzymes used for cloning into the small shuttle plasmids the AdMax™ system is simpler and more economical than methods requiring rare cutters[4,5,6]. Moreover those rescue protocols typically use enzymes such as Pac I or Swa I to linearize plasmid DNA *prior* to transfection. If the transgene contains these sites then these methods are not practical. PacI sites, for example, are found surprisingly often in eukaryotic DNA.

The E3 deleted genomic plasmids contain a unique PacI cloning site in E3. It is possible to insert a reporter gene or a gene for a transactivator in the E3 region to create a modified genomic plasmid that can then be combined with cassettes inserted in the E1 shuttle plasmid. Thus, for example, a series of vectors expressing genes under regulation by tet or by RU486 can be readily constructed using the AdMax™ system.

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CATALOGUE #	PRODUCT
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PD-01-67	Kit F (contains pDC411, pDC412, pDC415, pDC416, pBHG10, pBHGE3 and pFG140)
INDIVIDUAL ADMAX™ PLASMIDS	
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PD-01-30	pDC412
PD-01-31	pDC415
PD-01-32	pDC416

For consultation on AdMax™ or to obtain a quotation please contact:

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Fax: 416.234.1626
customerservice@microbix.com
www.microbix.com

References

[1] Ng, P., Parks, R. J., Cummings, D. T., Evelegh, C. M., Sankar, U., & Graham, F. L. (1999). A high efficiency Cre/loxP based system for construction of adenoviral vectors. Hum. Gene Ther. 10, 2667-2672.
[2] Ng, P., Cummings, D. T., Evelegh, C. M. and Graham, F. L. The yeast recombinase FLP functions effectively in human cells for construction of adenovirus vectors. BioTechniques 29: 524-528, 2000.
[3] Bett, A. J., Haddara, W., Prevec, L. and Graham, F.L. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. Proc. Natl. Acad. Sci. US 91: 8802-8806, 1994.
[4] Chartier, C., Degryse, E., Gantzer, M., Dieterle, A, Pavirani, A., and Mehtali, M. (1996). Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli. J. Virol. 70, 4805-4810.
[5] He, T. C., Zhou, S., Da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998). A simplified system for generating recombinant adenoviruses. Proc. Natl. Acad. Sci. USA 95, 2509-2514.
[6] Mizuguchi, H., and Kay, M. A. (1998). Efficient construction of a recombinant adenovirus vector by an improved *in vitro* ligation method. Hum. Gene Ther. 9, 2577-2583.

Koropatnick

HUMAN GENE THERAPY 10:2667–2672 (November 1, 1999)
Mary Ann Liebert, Inc.

A High-Efficiency Cre/*loxP*-Based System for Construction of Adenoviral Vectors

P. NG,[1] R.J. PARKS,[2] D.T. CUMMINGS,[1] C.M. EVELEGH,[1] U. SANKAR,[1] and F.L. GRAHAM[1,3]

ABSTRACT

Adenovirus (Ad) vectors provide a highly efficient means of mammalian gene transfer and are widely used for high-level protein expression in mammalian cells, as recombinant vaccines and for gene therapy. A commonly used method for constructing Ad vectors relies on *in vivo* homologous recombination between two Ad DNA-containing bacterial plasmids cotransfected into 293 cells. While the utility of this two-plasmid approach is well established, its efficiency is low owing to the inefficiency of homologous recombination. To address this, we have developed an improved method for Ad vector construction based on Cre-mediated site-specific recombination between two bacterial plasmids, each bearing a *loxP* site. Ad vectors are generated as a result of Cre-mediated site-specific recombination between the two plasmids after their cotransfection into 293 cells expressing Cre recombinase. The frequency of Ad vector rescue by Cre-mediated site-specific recombination is significantly higher (~30-fold) than by *in vivo* homologous recombination. The efficiency and reliability of this method should greatly simplify and expedite the construction of recombinant Ad vectors for mammalian gene transfer.

OVERVIEW SUMMARY

Ad vectors are commonly constructed by homologous recombination between two plasmids cotransfected into 293 cells. This method has numerous advantages but results in low numbers of plaques owing to inefficient recombination. We have developed an improved method based on Cre-mediated site-specific recombination, which results in vector rescue at frequencies ~30-fold higher than by homologous recombination. This method should greatly simplify and expedite the construction of recombinant Ad vectors for mammalian gene transfer.

INTRODUCTION

Adenoviruses (Ads) possess several features that make them attractive as mammalian gene transfer vectors. They can efficiently infect a wide variety of quiescent and proliferating cell types from various species to direct high level viral gene expression, their 36-kb double-stranded DNA genome can be manipulated with relative ease by conventional molecular biology techniques, and they can be readily propagated and purified to yield high-titer preparations of stable virus. Consequently, Ads have been extensively used as vectors for recombinant vaccines, for high-level protein production in cultured cells, and for gene therapy (Berkner, 1988; Graham and Prevec, 1992; Hitt *et al.*, 1997, 1999).

First-generation Ad vectors typically have foreign DNA inserted in place of early region 1 (E1) and/or E3. E1-depleted vectors are replication deficient and are propagated in E1-complementing cells such as the 293 cell line (Graham *et al.*, 1977). A number of strategies for Ad vector construction have been developed (Gerard and Meidell, 1995; Graham and Prevec, 1995; Hitt *et al.*, 1995, 1998; Spector and Samaniego, 1995). Typically, foreign DNA is inserted into a small shuttle plasmid containing Ad sequences from the left end of the genome with the E1 region deleted. The foreign DNA can be rescued into virus by direct *in vitro* ligation of the shuttle plasmid with viral DNA digested with appropriate restriction enzymes or by *in vivo* homologous recombination after cotransfection of 293 cells with the shuttle plasmid and restricted viral DNA. How-

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[2]Ottawa General Hospital Research Institute, Ottawa, Ontario, Canada, K1H 8L6.
[3]Department of Pathology, McMaster University, Hamilton, Ontario, Canada, L8S 4K1.

ever, the usefulness of these methods is hampered by the large size of the Ad genome, which limits the number of useful restriction sites available for the *in vitro* ligation method, and by the inefficiency of homologous recombination. Another disadvantage is the requirement for infectious viral DNA as a substrate, which can result in parental virus contamination due to incomplete digestion or self religation.

A popular method for Ad vector construction relies on *in vivo* homologous recombination between the overlapping Ad sequences of the shuttle plasmid and an Ad genomic plasmid after their cotransfection into 293 cells. The Ad genomic plasmid is modified to be noninfectious, thereby virtually eliminating any possibility of generating viruses other than the desired recombinant (Bett *et al.*, 1994). The utility of this method is well proved, but vector rescue is sometimes difficult, perhaps owing to inefficient homologous recombination in cotransfected 293 cells. In an attempt to overcome this limitation methods have been developed that make use of homologous recombination between two plasmids in *Escherichia coli* (Chartier *et al.*, 1996; Crouzet *et al.*, 1997; He *et al.*, 1998) and yeast (Ketner *et al.*, 1994). However, these approaches are more complex, time consuming, and technically demanding, the former requiring transformation of an unconventional *E. coli* strain followed by transfer of candidate plasmids to a second *E. coli* strain for large-scale plasmid DNA preparation. The latter method requires yeast cell culture and manipulation, which is not routine in most laboratories. A more recently described method involves transfection of 293 cells after *in vitro* ligation of foreign DNA into an Ad genomic plasmid (Mizuguchi and Kay, 1998). However, the recombinant plasmid must be digested with at least two restriction enzymes prior to transfection into 293 cells, thus rendering this method unsuitable for foreign DNAs that contain recognition sequences for these enzymes.

Considering the importance of the Ad vector as a tool for mammalian gene transfer, especially in the emerging field of human gene therapy, development of improved systems for their efficient and reliable construction is clearly important. This study describes a simple and efficient method of constructing Ad vectors based on Cre-mediated site-specific recombination between two bacterial plasmids after their cotransfection into 293 cells expressing Cre. The flexibility, efficiency, and reliability of this method promise to greatly simplify and expedite construction of Ad vectors for mammalian gene transfer.

MATERIALS AND METHODS

Construction of plasmids

Plasmids were constructed by standard protocols, prepared by the alkaline lysis method (Birnboim and Doly, 1978), and purified by CsCl density-gradient centrifugation (Sambrook *et al.*, 1989). The plasmid pCA36lox was constructed by inserting a synthetic *loxP* site (5′ GATCCAATAACTTCGTATAGCATACATTATACGAAGTTATAAGTACTGAATTCG 3′ and 5′ GATCCGAATTCAGTACTTATAACTTCGTATAATGTATGCTATACGAAGTTATTG 3′) into the *Bgl*II site of pCA36 (Addison *et al.*, 1997). The plasmid pCA36loxΔ was constructed by digestion of pCA36lox with *Nru*I and partial digestion with *Sca*I, followed by self-ligation to delete all Ad sequences downstream of the *loxP* site. The plasmid pBHGloxΔE1,3 was constructed by replacing the 4604-bp *Bst*I 107I fragment of pBHG10 (Bett *et al.*, 1994) with the 2326-bp *Eco*RV/*Bst*I 107I fragment from pΔE1sp1Alox. The plasmid pΔE1sp1Alox was constructed by inserting the synthetic *loxP* site (see above) into the *Bgl*II site of pΔE1sp1A (Bett *et al.*, 1994).

Cells and viruses

Propagation of 293 (Graham *et al.*, 1977) and 293Cre4 (Chen *et al.*, 1996) cells was performed as described (Hitt *et al.*, 1998). Cotransfection of 293 and 293Cre4 with the appropriate plasmids was used to generate Ad vectors that formed plaques on the cell monolayer (Hitt *et al.*, 1998) and the infectious Ad genomic plasmid pFG140 (Graham, 1984) was included to assess the transfection efficiency. Plaques were isolated and expanded, and viral DNA was extracted and analyzed by restriction enzyme digestion as described (Hitt *et al.*, 1998). β-Galactosidase expression from virus-infected or mock-infected cells was determined by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining as follows: X-Gal was added, to a final concentration of 0.4 mg/ml, to the culture supernatant of mock-infected or virus-infected cells after complete cytopathic effect was observed, and the cells were incubated at 37°C for 1 hr.

RESULTS AND DISCUSSION

We have previously developed a method for constructing Ad vectors based on *in vivo* homologous recombination between two bacterial plasmids cotransfected into 293 cells (Bett *et al.*, 1994). In this method, the foreign DNA is inserted into a small shuttle plasmid that contains a portion of the left end of the Ad genome including the inverted terminal repeat (ITR), the packaging signal, and a multiple cloning region for insertion of foreign DNA in place of E1. The second plasmid used in this system contains essentially the entire Ad genome but is modified to be noninfectious. The foreign DNA is rescued into recombinant viruses by *in vivo* homologous recombination between the overlapping Ad sequences of the two plasmids after their cotransfection into 293 cells (Fig. 1A).

While this method is widely used and well proved, the efficiency of vector rescue is low. One possible explanation is that homologous recombination is a rate-limiting step in the generation of infectious virus. Consistent with this hypothesis is the observation that the number of plaques generated by transfection of 293 cells with infectious Ad genomic plasmids, such as pFG140 (Graham, 1984), is ~100-fold higher than that generated by a typical cotransfection for vector rescue (F.L. Graham, unpublished observations). It might therefore be possible to increase the efficiency of vector rescue by using a more efficient recombination system such as that mediated by Cre, a recombinase, encoded by bacteriophage P1, that efficiently and specifically catalyzes recombination between 34-bp target *loxP* sites (Hoess *et al.*, 1982). To test this hypothesis, we have developed a Cre-mediated site-specific recombination system for the construction of Ad vectors and

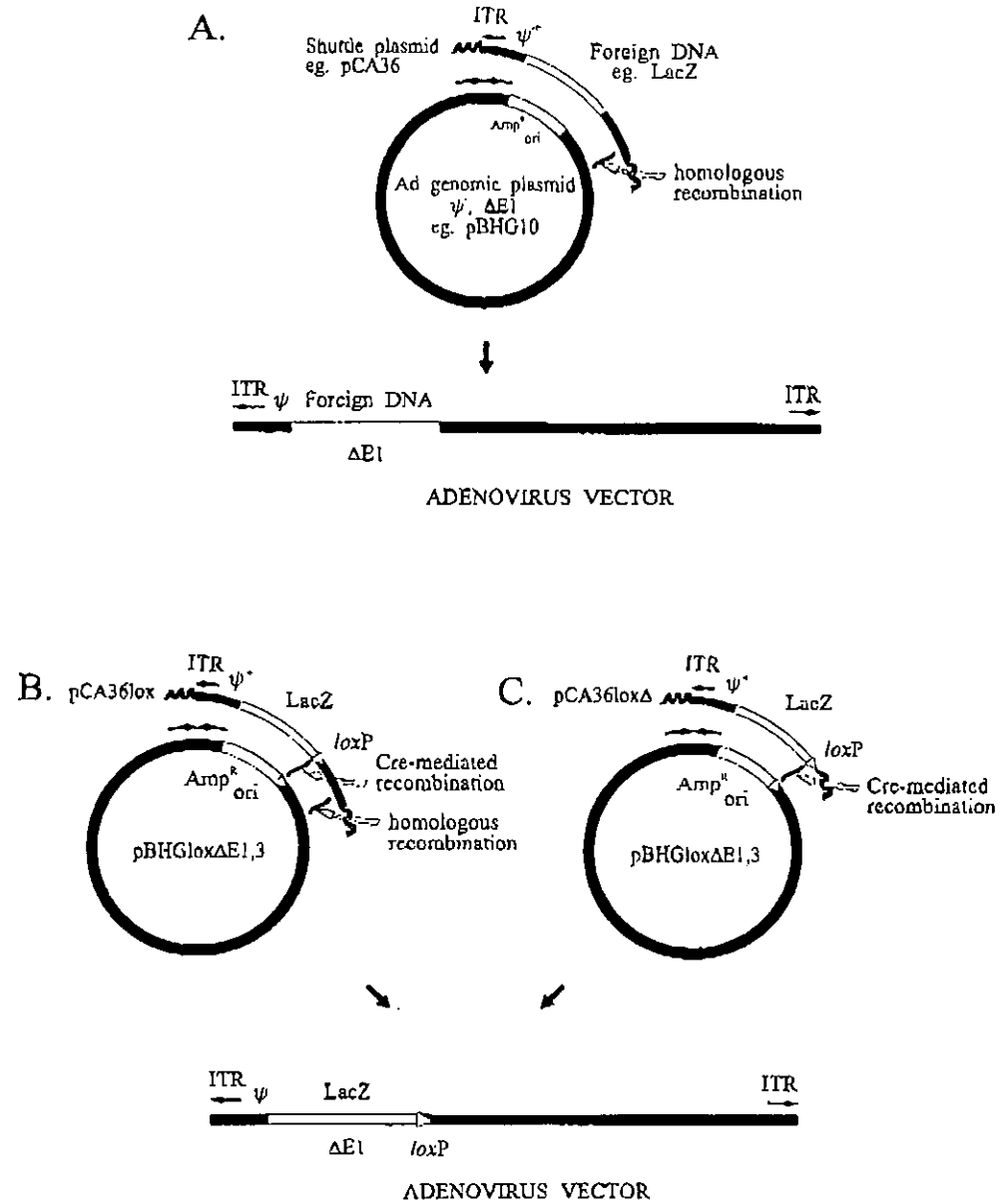

FIG. 1. Construction of Ad vectors by (A) *in vivo* homologous recombination after cotransfection of 293 cells with a shuttle plasmid (e.g., pCA36) and an Ad genomic plasmid (e.g., pBHG10), (*B*) *in vivo* homologous recombination or Cre-mediated site-specific recombination after cotransfection of 293Cre4 cells with pCA36lox and pBHGloxΔE1,3, and (C) Cre-mediated site-specific recombination after cotransfection of 293Cre4 cells with pCA36loxΔ and pBHGloxΔE1,3. Ad sequences are shown in black and the position and orientation of the *loxP* site is represented by a white triangle. Only the relevant portions of the shuttle plasmids are shown.

have evaluated this method by rescuing a recombinant vector expressing β-galactosidase.

The plasmids used were derived from pCA36 (Addison *et al.*, 1997) and pBHG10 (Bett *et al.*, 1994) (Fig. 2). The shuttle plasmid pCA36 contains the left end of the Ad genome including the ITR, a packaging signal, and a *lacZ* expression cassette substituting for the E1 region. The plasmid pBHG10 contains essentially the entire Ad genome but with an E3 deletion and deletion of E1A sequences including the packaging signal. Neither plasmid alone is capable of producing infectious virus in transfections of 293 cells; however, when cotransfected, homologous recombination between their overlapping regions of Ad homology results in a recombinant vector bearing the *lacZ* expression cassette (Fig. 1A). To modify this system to utilize Cre-mediated recombination, a *loxP* site was introduced into pCA36, immediately downstream of the *lacZ* expression cassette, to generate pCA36lox (Fig. 2) and into pBHG10, immediately upstream of the pIX gene, to generate pBHGloxΔE1,3 (Fig. 2). Cre-mediated recombination between the *loxP* sites in pCA36lox and pBHGloxΔE1,3 was accomplished by cotrans-

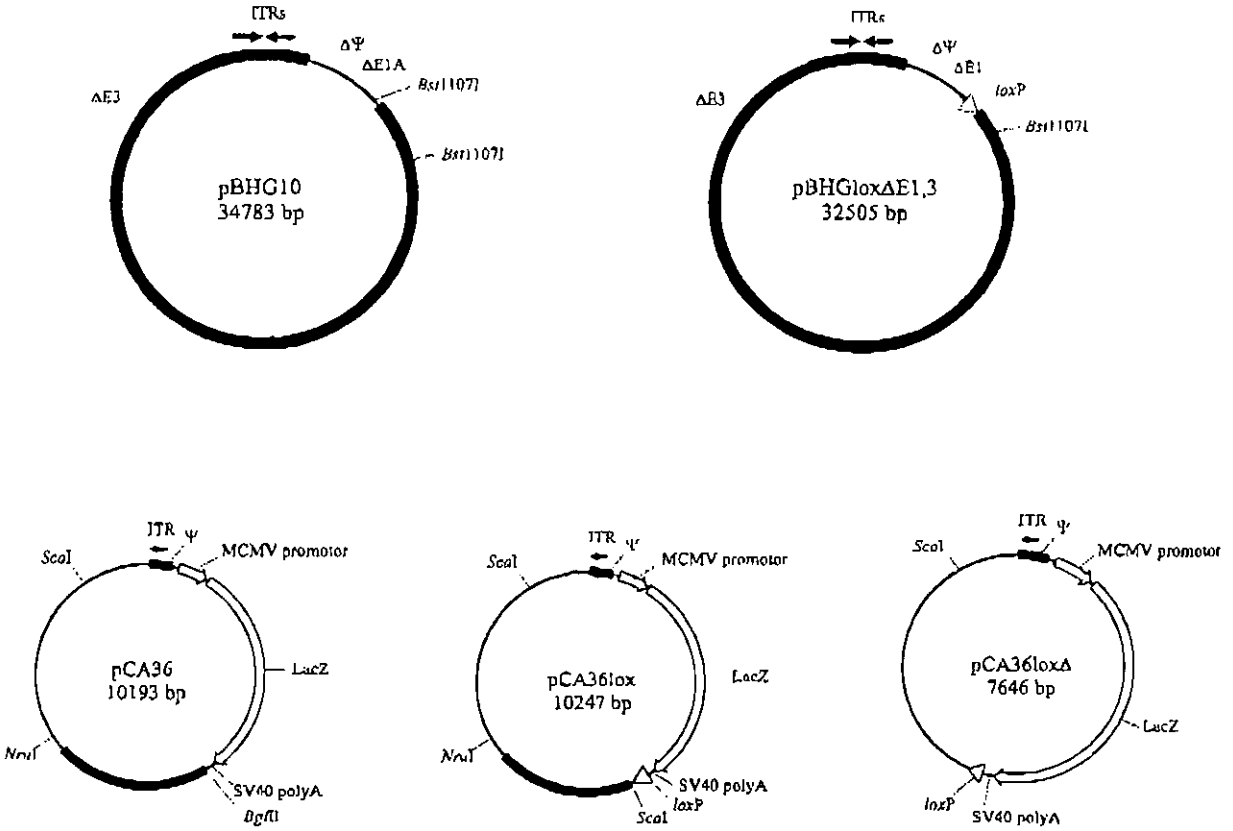

FIG. 2. Plasmids used to rescue recombinant Ad vectors expressing β-galactosidase. The plasmid pBHG10 contains essentially the entire Ad serotype 5 genome except for deletions encompassing E1A and the packaging signal, and the E3 regions (Bett *et al.*, 1994). The plasmid pBHGloxΔE1,3 was derived from pBHG10 by insertion of a *loxP* site as described in Materials and Methods. The plasmid pCA36 contains a portion of the left end of the Ad genome into which the *lacZ* expression casette has been inserted in place of the E1 region (Addison *et al.*, 1997). A *loxP* site was inserted into pCA36 to generate pCA36lox and Ad sequences downstream of the *loxP* site in pCA36lox were deleted to generate pCA36loxΔ as described in Materials and Methods. Thick lines represent Ad sequences, thin lines represent bacterial plasmid sequences, and the position and orientation of the *loxP* site are represented by a white triangle. Plasmids are not drawn to scale.

fection into 293 cells expressing Cre (293Cre4) (Chen *et al.*, 1996). The vector DNA structure generated by Cre-mediated recombination is expected to be identical to that generated by homologous recombination (Fig. 1A) except for a 54-bp insertion containing a *loxP* site (Fig. 1B).

To compare the efficiency of vector rescue by homologous recombination versus Cre-mediated recombination, 293 and 293Cre4 cells were cotransfected with various combinations of shuttle plasmids and Ad genomic plasmids, in various amounts, and the numbers of plaques generated were counted. The results of a typical experiment are presented in Table 1. The number of plaques generated by cotransfection of 293 cells with pCA36 and pBHG10 provides a measure of the efficiency of vector rescue by homologous recombination. A similar efficiency was obtained by cotransfection of 293Cre4 cells with this plasmid combination, indicating that homologous recombination activity responsible for vector rescue was comparable for both cell lines. A similar efficiency was also obtained when only one of the two plasmids used to cotransfect 293 or 293Cre4 cells contained a *loxP* site (pCA36 + pBHGloxΔE1,3 or pCA36lox + pBHG10) or when 293 cells were cotransfected with pCA36lox and pBHGloxΔE1,3. This was expected since vectors could be rescued only by homologous recombination because the requirements for Cre-mediated recombination were not met in these cotransfections. No plaques were obtained by cotransfection of 293Cre4 cells with pCA36 and pBHGloxΔE1,3 in the experiment presented in Table 1, whereas in other identical cotransfections a few plaques were obtained (Table 2). These results illustrate that while vector rescue can be achieved by homologous recombination, its low efficiency can occasionally result in failure.

In contrast to the low number of plaques generated by the cotransfections described above, when 293Cre4 cells were cotransfected with pCA36lox and pBHGloxΔE1,3 the number of plaques generated was ~30-fold higher, suggesting that vector rescue by Cre-mediated recombination was significantly more efficient than by homologous recombination (Table 1). Twenty-six plaques from this cotransfection were analyzed and all were positive for β-galactosidase expression and had the expected DNA structure from Cre-mediated recombination (data not shown).

To confirm that the enhanced efficiency of vector rescue by

TABLE 1. EFFICIENCY OF Ad VECTOR RESCUE BY COTRANSFECTION

Plasmid	DNA/dish[a]	Plaques/dish (average/dish)	
		293 cells	293Cre4 cells
pCA36:pBHG10	5:5	0, 0, 0, 0 (0)	0, 1, 2, 0 (0.75)
	5:10	0, 0, 0, 1 (0.25)	1, 0, 0, 0 (0.25)
	10:10	2, 0, 1, 1 (1)	1, 2, 0, 0 (0.25)
pCA36:pBHGloxΔE1,3	5:5	0, 0, 0, 1 (0.25)	0, 0, 0, 0 (0)
	5:10	0, 0, 0, 1 (0.25)	0, 0, 0, 0 (0)
	10:10	0, 0, 2, 1 (0.75)	0, 0, 0, 0 (0)
pCA36lox:pBHG10	5:5	1, 3, 1, 0 (1.25)	0, 1, 0, 1 (0.5)
	5:10	0, 1, 0, 0 (0.25)	0, 0, 1, 2 (0.75)
	10:10	0, 0, 0, 0 (0)	0, 1, 1, 0 (0.5)
pCA36lox:pBHGloxΔE1,3	5:5	1, 0, 0, 1 (0.5)	5, 14, 20, 20 (17.25)
	5:10	0, 0, 0, 0 (0)	11, 15, 12, 16 (13.5)
	10:10	0, 0, 1, 1, (0.5)	18, 9, 10, 8 (11.25)
pFG140	0.5	99, 100 (99.5)	49, 50 (49.5)

[a]Micrograms of shuttle plasmid:Ad genomic plasmid cotransfected per 60 mm dish of the indicated cell line.

cotransfection of 293Cre4 cells with pCA36lox and pBHGloxΔE1,3 was mediated by Cre, a derivative of pCA36lox, called pCA36loxΔ, was constructed from which Ad sequences to the right of the *loxP* site were deleted, thus virtually precluding vector rescue by homologous recombination. 293 and 293Cre4 cells were cotransfected with pBHGloxΔE1,3 and either pCA36, pCA36lox, or pCA36loxΔ and vector rescue efficiencies were compared (Table 2). Consistent with the preceding results, cotransfections of 293 cells with pBHGloxΔE1,3 and pCA36 or pCA36lox and cotransfection of 293Cre4 cells with pBHG10 and pCA36lox resulted in low numbers of plaques, while cotransfection of 293Cre4 cells with pCA36lox and pBHGloxΔE1,3 resulted in a ~20-fold higher number of plaques. For pCA36loxΔ, cotransfection of 293 cells with pBHGloxΔE1,3 gave no plaques, as expected since virtually all overlapping sequence homology required for vector rescue had been removed from pCA36loxΔ. However, cotransfection of 293Cre4 cells with this plasmid combination resulted in a large number of pluques, comparable to that obtained by cotransfection of 293Cre4 cells with pCA36lox and pBHGloxΔE1,3, indicating that increased vector rescue efficiency was mediated by Cre. Nine plaques generated by cotransfection of 293Cre4 cells with pCA36loxΔ and pBHGloxΔE1,3 were analyzed, and all were positive for β-galactosidase expression and had a viral DNA structure expected from Cre-mediated recombination (data not shown). In subsequent experiments 39 additional plaques obtained from Cre-mediated recombination have been analyzed and all were shown to express β-galactosidase (data not shown).

A method has been described that uses Cre-mediated recombination for the construction of Ad vectors; it involves transfection of 293 cells expressing Cre recombinase (CRE8) with a shuttle plasmid bearing a *loxP* site and infection with a donor virus or transfection with purified donor viral DNA containing a packaging signal flanked by *loxP* sites (Hardy *et al.*, 1997). Cre-mediated excision of the donor virus packaging signal followed by site-specific recombination with the shuttle plasmid produces the recombinant vector. However, since Cre is not 100% efficient, the vector preparations are contaminated with the donor virus, which must be reduced by passage in CRE8 cells (Hardy *et al.*, 1997). This is in contrast to our method, which utilizes only noninfectious components so that all infectious viruses generated are the desired recombinant.

We have now constructed a variety of shuttle plasmids containing a *loxP* site to permit cloning of virtually any expression cassette for rescue into Ad vectors and several Ad genomic plas-

TABLE 2. EFFICIENCY OF Ad VECTOR RESCUE BY COTRANSFECTION

Plasmid	Plaques/dish (average/dish)	
	293 cells	293Cre4 cells
pCA36[a]	1, 1, 2, 6, 2, 3 (2.5)	1, 1, 2, 1, 2, 3 (1.7)
pCA36lox[a]	1, 2, 2, 2, 2, 1 (1.7)	41, 44, 41, 41, 44, 31 (40.3)
pCA36loxΔ[a]	0, 0, 0, 0, 0, 0 (0)	41, 36, 55, 34, 24, 40 (38.3)
pFG140[b]	72, 72 (72)	150, 115 (132.5)

[a]All cotransfections performed with 5 μg of the indicated shuttle plasmid and 5 μg of pBHGloxΔE1,3 per 60 mm dish of the indicated cell line.
[b]One microgram was transfected per dish of the indicated cell line.

mids containing a *loxP* site with either a wild-type, or variously deleted, E3 region. This system offers several advantages over other methods: (1) foreign DNA is cloned into a small, easily manipulated shuttle plasmid of ~3.4–4.4 kb; (2) the method requires, as substrate for vector construction, only plasmid DNA that can be propagated in any *E. coli* strain routinely used for recombinant DNA manipulation; (3) the plasmids do not require restriction endonuclease digestion prior to cotransfection; (4) cotransfection of 293Cre4 cells results in a large number of plaques, so that successful rescue of the desired vector is practically guaranteed (unless the expression cassette encodes a toxic product); and (5) all plaques generated contain the foreign DNA and have the expected DNA structure. The simplicity, reliability, and efficiency of our system should greatly expedite the construction of Ad vectors for mammalian gene transfer.

ACKNOWLEDGMENTS

This work was supported by grants from the Natural Sciences and Engineering Research Council (NSERC), the Medical Research Council (MRC), and the National Cancer Institute of Canada (NCIC), and by Merck Research Laboratories. R.J.P. was an MRC Postdoctoral Fellow and F.L.G. was a Terry Fox Research Scientists of the NCIC.

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BIO - LRCC - 0008

THE UNIVERSITY OF WESTERN ONTARIO
BIOHAZARDOUS AGENTS REGISTRY FORM
Revised Biohazards Subcommittee: January, 2007

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario where the use of biohazardous infectious agents are described in the experimental work proposed. The form must also be completed if animal work is proposed involving the use of biohazardous agents or animal carrying zoonotic agents infectious to humans. Containment Levels will be required in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Health Canada (HC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Completed forms are to be returned to Occupational Health and Safety (Stevenson-Lawson Building, Room 60) for forward to the Biohazard Subcommittee. For questions regarding this form, please contact the Biosafety Coordinator at extension 81135. If there are changes to the information on this form (excluding grant title and funding agencies) modifications must be completed and sent to Occupational Health and Safety. See website: www.uwo.ca/humanresources

PRINCIPAL INVESTIGATOR
SIGNATURE J James Koropatnick
DEPARTMENT Oncology
ADDRESS LHSC, Victoria Campus, Victoria Research Building, Room A4-114, A4-116
PHONE NUMBER extension 58654
EMAIL jkoropat@uwo.ca

Location of experimental work to be carried out: Building(s) Victoria Research Building Room(s) A4
*For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to it being sent to Occupational Health and Safety (See Section 12.0, Approvals). For research being done at Lawson Health Research Institute, London Regional Cancer Centre, Child and Parent Research Institute or Robarts Research Institute, University Biosafety Committee members can also sign as the Safety Officer.

TITLE OF GRANT(S):

CIHR MOP-49441, Metallothionein in monocyte function, hormone responsiveness, and signal transduction **(Attachment 1)**

CIHR MOP-62836, Antisense downregulation of thymidylate synthase as an anticancer therapy **(Attachment 2)**

NIH RO1 ES11288-01, Mercury and monocyte activation **(Attachment 3)**

PLEASE ATTACH A BRIEF DESCRIPTION OF YOUR WORK, SUCH A THE RESEARCH GRANT SUMMARY(S) THAT EXPLAINS THE BIOHAZARDS USED. PROJECTS SUBMITTED WITHOUT A SUMMARY WILL NOT BE REVIEWED.

FUNDING AGENCY/AGENCIES CIHR, NIH

Names of all personnel working under Principal Investigators supervision in this location:
i) Dr. Peter Ferguson
ii) Ms. Alayne Brisson
iii) Ms. Kathleen Calonego

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

iv) Dr. Julio Masabanda
v) Dr. Reza Mazaheri
vi) Mr. Mark Niglas
vii) Ms. Kristen Reipas
viii) Mr. Alexander Sykelyk

1.0 Microorganisms

1.1 Does your work involve the use of microorganisms or biological agents of plant or animal origin (including but not limited to viruses, prions, parasites, bacteria)? __ YES __ NO
If no, please proceed to Section 2.0

1.2 Please complete the table below:

Name of Biological agent(s)	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time?
E. Coli DH101	__ Yes X No	__ Yes X No	__ Yes X No	4 litres
	__ Yes __ No	__ Yes __ No	__ Yes __ No	
	__ Yes __ No	__ Yes __ No	__ Yes __ No	
	__ Yes __ No	__ Yes __ No	__ Yes __ No	

1.3 For above named organism(s) or biological agent(s) circle HC or CFIA Containment Level required. 2

1.4 Source of microrganism(s) or biological agent(s)? ATCC Bacteriology Collection

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? X YES __NO

If no, please proceed to Section 3.0

2.2 Please indicate the type of primary cells (ie. derived from fresh tissue) that will be grown in culture in the table below

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue
Human	X Yes __ No	Cancer patients (LRCP), ethically obtained through the Clinical Cancer Research Unit
Rodent	X Yes __ No	Mice, VRL Vivarium or UWO Vivarium
Non-human primate	__ Yes X No	
Other (specify)		

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

2.3 Please indicate the type of established cells that will be grown in culture in the table below.

Cell Type	Is this cell type used in your work?	Specific cell line(s)	Supplier / Source
Human	X Yes __ No	See list below	ATCC
Rodent	X Yes __ No	See list below	ATCC
Non-human primate	__ Yes X No		
Other (specify)	X Yes __ No	Insect: see list below	Collaborators in London

Cell Culture - Koropatnick Laboratory - London Regional Cancer Program, Cancer Research Laboratories - VRL

Cell Lines Used:

Human:
Anaplastic astrocytoma SF-268
Breast MCF-7, MDA-MB-435, MDA-MB-231, MDA-MB-468, SK-BR-3
Cervical epithelial HeLa
Colon tumour HT-29, CaCo-2
Epidermoid carcinoma A431
Erythroleukemia K562
Foreskin fibroblast NIH3T3
Gastric adenocarcinoma AGS, Hs746T, N87
Glioma U87, A172, SF-295, SNB-19, U373MG
Hepatoma Hep-G2
Kidney tumour 293T (T-antigen-expressing)
Leukemia, promyelocytic HL-60
Lung fibroblast WI-38
Lymphoblastoid W1-L2
Mammary epithelial line 1001-8 (ATCC)
Melanoma SK-MEL-5
Muscle tumour BC_3H1
Non-small cell lung carcinoma A549, H520
Ovarian carcinoma OV-90
Pancreatic carcinoma PANC-1, Panc 02.03, Panc 03.27, Panc 10.05
Prostate carcinoma DU145, LNCaP
Small cell lung carcinoma DMS114, DMS153, H69, SHP-77
Squamous cell carcinoma HN-5a
Testicular Leydig cell tumour line MA10
Umbilical vein epithelial cell (HUVEC)
~~Mesothelioma~~ NCI-H28, NCI-H2052, COLO-587, MSTO-211H

Al. per e-mail attached

Rodent:
Mouse kidney primary
Mouse embryonic fibroblast (MEF)
Mouse mammary tumour 2305
Mouse melanoma B16 F10
Chinese hamster ovary

Insect:
SF9

* DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED*

2.4 For above named cell types(s) circle HC or CFIA containment level required 1 2 3
All the above cells lines will be handled at containment level 2

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? X YES __NO
If no, please proceed to Section 4.0

3.2 Indicate if the following will be used in the laboratory

- Human blood (whole) or other bodily fluids X YES __ NO Specify: blood
- Human blood (fraction) or other bodily fluids X YES __ NO Specify: white blood cells
- Human organs (unpreserved) __ YES X NO Specify
- Human tissues (unpreserved) X YES __ NO Specify: tumour tissue

3.3 Is human source known to be infected with and infectious agent __ YES X NO
If YES , please name infectious agent _____

3.4 For above named materials circle HC or CFIA containment level required. 2

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents or cells described in Sections 1.0 and 2.0 ? X YES __ NO
If no, please proceed to Section 5.0

4.2 Will genetic sequences from the following be involved:

- HIV __ YES X NO
if YES specify _____
- HTLV 1 or 2 or genes from any CDC class 1 pathogens __ YES X NO
if YES specify _____
- Other human or animal pathogen and or their toxins __ YES X NO
if YES specify _____

4.3 Will intact genetic sequences be used from

- SV 40 Large T antigen __ YES X NO___ If YES specify
- Known oncogenes __ YES X NO___ If YES specify

4.4 Will a live vector(s) (viral or bacterial) be used for gene transduction __ YES X NO
If YES name virus

4.5 List specific vector(s) to be used: _____

4.6 Will virus be replication defective __ YES __ NO

4.7 Will virus be infectious to humans or animals __ YES __ NO

4.8 Will this be expected to increase the Containment Level required __ YES __ NO

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

5.0 Human Gene Therapy Trials

5.1 Will human clinical trials using the viral vector in 4.0 be conducted? __ YES X NO
If no, please proceed to Section 6.0
If YES attach a full description of the make-up of the virus.

5.2 Will virus be able to replicate in the host? __ YES __ NO

5.3 How will the virus be administered? _____

5.4 Please give the Health Care Facility where the clinical trial will be conducted: _____

5.5 Has human ethics approval been obtained? __ YES __ NO

6.0 Animal Experiments

6.1 Will any of the agents listed be used in live animals? ✓ YES X NO
If no, please proceed to section 7.0

6.2 Name of animal species to be used CD-1 nu/nu mice

6.3 AUS protocol # 2004-040-04

6.4 If using murine cell lines, have they been tested for murine pathogens? X YES __ NO

7.0 Use of Animal species with Zoonotic Hazards

7.1 Will any of the following animals or their organs, tissues, lavages or other bodily fluids including blood be used:

- Pound source dogs __ YES X NO
- Pound source cats __ YES X NO
- Sheep or goats __ YES X NO
- Non- Human Primates __ YES X NO If YES specify species _____
- Wild caught animals __ YES X NO If YES specify species _____
  colony # _____

8.0 Biological Toxins

8.1 Will toxins of biological origin be used? __ YES X NO
If no, please proceed to Section 9.0

8.2 If YES, please name the toxin _____

8.3 What is the LD_{50} (specify species) of the toxin _____

9.0 Import Requirements

9.1 Will the agent be imported? X YES __ NO
If no, please proceed to Section 10.0
If yes, country of origin USA (primarily from the American Type Culture Collection)

9.2 Has an Import Permit been obtained from HC for human pathogens? X YES __ NO

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

9.3 Has an import permit been obtained from CFIA for animal pathogens? __ YES __ NO

9.4 Has the import permit been sent to OHS? X YES __ NO
If yes, Permit # BIO-LRCC-0008

10.0 Training Requirements for Personnel named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biohazardous agents in Sections 1.0 to 9.0 have been trained.

SIGNATURE _____

11.0 Containment Levels

11.1 For the work described in sections 1.0 to 9.0, please underline the highest HC or CFIA Containment Level required. 1 2 3

11.2 Has the facility been certified by OHS for this level of containment? X YES __ NO

11.3 If yes, please give the date and permit number: MARCH 12, 2008
Dave Ryder

12.0 Approvals

UWO Biohazard Subcommittee

Signature _____

Date 1 Apr. 2008

Safety Officer for Institution where experiments will take place

Signature _____

Date 15/4/08

Only approved if area is assessed and approved as level 2 biosafety level.
Completed MAR 12, 2008
Dave Ryder

Safety Officer for University of Western Ontario (if different than above)

Signature _____

Date April 1, 2008

* DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED*