development of wild-type or naturally attenuated oncolytic viruses, intentional genetic modification is not introduced into the virus genome, the tumor selective targeting, tumor-selective replication and pathogenicity of these oncolytic viruses are dependent on the method of selecting the strains. During the characterization of wild-type and attenuated oncolytic viruses, it is extremely important to analyze the molecular mechanisms of the tumor-selectivity and tumor-specific replication, as well as the genetic stability, etc.

There are several strategies used to design and construct the tumor selectivity of genetically engineered oncolytic viruses [10, 11, 23, 24]. One strategy is to engineer viruses through the deletion of virus genes critical for viral replication in normal cells but non-essential in tumor cells. For example, adenovirus E1B55K is responsible for binding and inactivating p53. E1B55K-deleted adenovirus has acquired the ability to propagate and induce cell death selectively in tumor cells, and then to spread to surrounding cells and tissues [25, 26]. Another strategy is transcriptional targeting, in which tumor- or tissue-specific promoters that are active in tumor cells are inserted into the viral genome to regulate the expression of essential viral genes and to restrict viral replication in tumor cells. The promoters used are categorized into different types; telomerase reverse transcriptase (TERT), S-phase of cell cycle promoter (E2F) and hypoxia promoter (HIF-1) are utilized as targeting promoters to all tumor cells; and prostate-specific antigen (PSA) promoter, α-fetoprotein (hepatoma) promoter and albumin promoter for hepatoma are used as tissue-specific promoters. The third strategy is the receptor-mediated targeting of replication-competent viruses to tumor cells [23, 27]. In this approach, the natural virus tropism of a replication-competent virus is adapted to the tumor cells through genetic modification of the virus coat or envelopes. This approach requires the ablation of the natural virus infection pathway and the introduction of new ligands into the virus surface without disrupting viral integrity. To improve the selectivity of oncolytic viruses to tumor cells and to improve safety, constructs with multiple modifications to tumor selectivity are developed. In addition, to improve efficacy, oncolytic viruses carrying a transgene (armed oncolytic viruses) have recently been developed [14, 28, 29].

In the endorsement of clinical trials or the approval of oncolytic virus products, the scientific rational of the design of the oncolytic virus construct must be thoroughly justified. Furthermore, non-clinical studies should be designed in each case to verify predictions of efficacy and safety. In this context, it is recommended that animal models be developed to provide valuable evidence concerning the non-clinical safety of these products and to assess their proof of concept.

MANUFACTURING AND CHARACTERIZATION OF ONCOLYTIC VIRUSES

There are to date no specific regulatory guidelines related to the manufacture and characterization of oncolytic viruses for clinical use. However, guidelines concerning the manufacturing and characterization of gene therapy products have been issued by the Food and Drug Administration (FDA), the European Medicines Agency (EMEA) and the government of Japan [30-33]. While there are some differences in the format of these guidelines, the underlying scientific principles are not fundamentally different, and the scientific principles covered by the above guidelines for gene therapy products may be applicable to the evaluation of the manufacturing and characterization of oncolytic viruses.

The guidelines should require that the rational behind the selection of the virus, helper virus and cells used in the production of the virus be described, including the genetic construct of the oncolytic virus, and of the helper virus if applicable. In cases in which the manufacturing method of the oncolytic virus in question has a specific feature, a justification of the feature must be included. The DNA or RNA sequence of the oncolytic virus must be clarified as much as possible, with particular attention to any regions of the virus genome that have been modified. Sequence analysis should be performed by a validated method which must also be described. In the case of genetically modified oncolytic viruses, a full explanation must be provided of the origin and detailed derivation of all constitutive components, such as promoters, enhancers, duplication units, selection markers and other base sequence parts from other constructs of oncolytic virus DNA or RNA. When a transgene is inserted into an oncolytic virus sequence, the construing procedure, amplification method, purification method and any flanking area that may have an important effect on the transcription, translation or stability of the translation sequence must be described in detail.

Cell and Virus Bank System

It is important to establish a cell and virus banking system in order to maintain consistency in the production of oncolytic viruses. A cell banking system for manufacturing oncolytic viruses should be designed and fully characterized; in general, a cell banking system includes a Master Cell Bank (MCB) and Working Cell Bank (WCB) for producing and packaging cell lines ("International conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH)" guideline Q5D [34]). The concept of a two-tiered cell bank, in which the MCB is used to generate WCBs, is generally considered to be the most practical approach to providing a supply of cell substrates for the continued manufacture of oncolytic virus products. The strategy for providing a continued supply of cells from their cell bank(s) must be described, including the anticipated utilization rate of the cell bank(s) for production, the expected intervals between the generations of new cell bank(s), and the criteria for qualification of cell bank(s). Generally, the MCB is created first, usually directly from an initial clone or from a preliminary cell bank derived from an initial clone. A WCB is derived from one or more containers of the MCB. It is the WCB which is typically used to directly provide cells for the manufacturing process.

The characterization and testing of banked cells is critical for the control of oncolytic viruses. The MCB and WCB must be subject to extensive quality control, and the established guidelines will be applicable to evaluate these banks (ICH Q5D or regional guidelines). Characterization of the MCB allows the sponsor to assess the source with regard to the presence of cells from other lines, adventitious agents, endogenous agents and molecular contaminants (e.g., toxins or antibiotics from the host organism). A characterization of the criteria for cell banks may include appearance, identity, cell count and viability for cell banks, as well as the sterility, mycoplasma, purity, absence of adventitious viruses and absence of specific human viruses. The objective of this testing is to confirm the identity, purity

and suitability of the cell substrates for manufacturing oncolytic viruses.

Another dimension of cell characterization is the appropriateness for their intended use in oncolytic virus production. There are two concerns for cell substrate stability: the consistent production of the oncolytic virus and the retention of production capacity during storage under defined conditions.

A two-tiered virus banking system, a Master Virus Bank (MVB) and a Working Virus Bank (WVB), is generally constructed for the production of oncolytic virus products. The MVB and WVB should also be characterized and should be subject to extensive quality control; the established guidelines may be applicable to evaluate these banks (ICH Q5D or regional guidelines). A characterization of the criteria for virus banks may include particle number and infectious titer, sterility, mycoplasma, purity, absence of adventitious viruses, replication-competent viruses and molecular variants, and absence of specific human viruses. A MVB is produced from an initial seed virus, and a WVB is derived from one or more containers of the MVB. The MVB and WVB should be produced under optimized culture conditions for viral growth and harvest, and be thoroughly defined, giving an efficient and reproducible downstream purification process. The quality, safety and efficacy of the final formulation of the oncolytic virus in which the virus will be stable for long periods in storage is guaranteed by the establishment of a well-defined virus banking system.

Sponsors are also encouraged to employ state-of-the-art methods and technological improvements in oncolytic virus characterization and testing as they become available, as long as the specificity, sensitivity and precision of the newer methods are at least equivalent to those of existing methods. Since oncolytic virus therapy has been developed only very recently, technologies for the characterization of oncolytic viruses remain to be fully elucidated. There remain a number of technical challenges concerning oncolytic virus testing and product characterization.

Manufacturing of Oncolytic Viruses

The manufacturing method for oncolytic viruses (vectors) must be fully described, including a description of the cells used for the production of the oncolytic viruses, and all relevant data on the name, manufacturing method, pathogenicity, propagation, growth factor dependence, phenotype, tumorigenecity, stability, etc. Changes in the character of the original cells must be clarified and the cultivation method of the cells described, including the medium, serum, antibiotics or other growth factors used. When a packaging cell is used, the manufacturing procedure, selection, identification method and isolation purification method to produce a seed cell strain must be established and characterized and the genetic stability of any sequence inserted into a packaging cell should also be described. The purification method of oncolytic viruses should be described in detail. When scaling up for manufacturing, suitable validation data to describe the contents should be made available. Additionally, descriptions must be included of the preparation and storage method of the MCB and WCB, as well as of the controlling and renewal methods. Finally, tests should be performed to confirm that the cell phenotype between the lots has not changed during the cultivation period. The test period, method and results of any safety tests necessary for quality control should be justified.

Genetic Stability, Replication-Competent Viruses (RCVs) and Molecular Variants

The biological and manufacturing consistency of oncolytic viruses depends primarily on the genetic stability of virus genomes as well as on the nature of the producer cells. A well-defined cell banking system partially ensures the genetic stability of oncolytic viruses during the manufacturing process. Relevant concerns include the generation of replication-competent viruses (RCVs) and molecular variants during manufacturing. RCVs in products can be evaluated by bioamplification assay [35]. Semiquantitative bioamplification systems are used to detect recombination that may occur during manufacturing. These assays are able both to detect contaminating wild-type viruses and to evaluate the genomic stability of an engineered virus; the oncolytic virus product tested in such assays requires multiple passages. Wild-type viruses that contaminate a preparation of engineered oncolytic virus are also typically detected using quantitative polymerase chain reaction (PCR) [36]. When the molecular variants are predicted by recombination, a preparation of engineered oncolytic virus should be tested for molecular variants using quantitative PCR [35].

The selection of the cell substrate is another strategy to minimize the appearance of recombinant RCVs. In the case of adenovirus production, the amount of replication-competent adenovirus (RCA) detected is higher in batches produced in conventional cell lines (e.g., 293 cells [37]) compared to that found in batches produced in recently engineered cell lines (e.g., PER.C6 cells [38]) because of the sequence homology between the engineered adenoviruses and the integrated sequences in the 293 cells. PER.C6 cells are reported to have produced no RCAs in large-scale adenovirus product [39]. A novel cell line, C139 derived from A549 human lung cancer cells, it has been reported that the E1a and E1b coding regions were reduced to their minimal sequences and that native promoters were deleted [40]. Additionally, it has been reported that neither RCAs nor cytopathic effect (CPE)-inducing replication-deficient recombinants are generated during the production of adenoviral vector using C139.

Adventitious Agent Testing

For more information on adventitious agent testing, ICH guidance Q5A: "Guidance on Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin" [41] should be referred if applicable. *In vitro* viral testing should be performed on the MCB, WCB, MVB, WVB, CAL (cells at the limit of *in vitro* cell age used for production) and oncolytic virus products. In such testing, the test sample (for example, MCB or MVB) is inoculated onto various susceptible indicator cell lines such as the human or primate cell line. The choice of cells used would depend on the species of origin of the oncolytic virus and the cell substrate used. In addition, the test would include a measure of both cytopathic and hemadsorbing viruses.

In vivo viral assays should be carried out by inoculating the test sample (MCB, MVB, etc.) into animals such as adult and

suckling mice, and embryonated hen's eggs. Additional testing of guinea pigs, rabbits or monkeys should also be considered. An assay for species-specific viruses should be performed and rodent cell lines used during production should be tested for rodent-specific viruses. If human cell lines are used in the therapeutic product, testing for human pathogens, including cytomegalovirus (CMV), human immunodeficiency virus (HIV) -1 and 2, human T-cell lymphotropic virus (HTLV) 1 and 2, Epstein-Barr virus (EBV), hepatitis B virus (HBV), hepatitis C virus (HCV), B19, and other human viral agents should be performed if appropriate. Human viral agents may be tested using a PCR-based test system. Retroviral contamination in MCB and MVB must be analyzed using reverse transcriptase (RT) assays and electron microscopic analysis.

Adventitious agent testing may be particularly challenging for oncolytic virus products. One strategy is based on the neutralization of the oncolytic virus with a specific antibody prior to testing for adventitious agents both for *in vitro* and *in vivo* assays. This is done to prevent the product from generating false positive results.

Batch Release

Typical release criteria for oncolytic viruses may be based on appearance, identity, virus titer, virus particles, potency, purity (including product-related and process-related impurity), safety (sterility, mycoplasma, endotoxins, adventitious viruses), and characterization.

In general, a standard potency assessment for oncolytic virus products is carried out based on the ratio of virus particle numbers to infectious titers in the final products. For replication-defective adenoviral vectors, the ratio of adenovirus vector particles to infectious titers must be less than 30:1 to satisfy FDA guidelines [31]. At present, however, no specific guidelines exist concerning the acceptable ratio of physical and infectious titers for oncolytic viruses. In addition to measuring tumor cell line killing in an *in vitro* assay, biological characteristics such as viral infectious titer and particles to infectious unit ratio are very useful to ensure batch-to-batch consistency.

Furthermore, it will be necessary to develop standardized testing procedures that will allow the evaluation and comparison of the selectivity, potency and toxicity of oncolytic viruses. If applicable, a wild-type strain may be useful as a positive control in order to allow normalization of infectivity and viral replication capability between different cell types. In the case of adenovirus products, the Adenovirus Type 5 Reference Material established by FDA and Adenovirus Reference Material Working Group can be used to standardize and normalize quantification methods, particle numbers and infectious titers [42].

The following numerical estimation of expressing the selectivity of an oncolytic virus effect in tumor cells compared to a normal cell line has been proposed [43]:

$$\text{Selectivity} = \frac{[\text{effect of oncolytic virus in tumor cell/effect of wt in tumor cell}]}{[\text{effect of oncolytic virus in normal cell/ effect of wt in normal cell}]}$$

"Effect" can be measured in terms of viral yield (or burst size, CPE (IC50 values), viral late protein expression or viral DNA replication level. Progeny production is considered the most relevant for the desired analytical effect [9].

NON-CLINICAL STUDIES

Non-clinical studies of oncolytic virus products are crucial to establish the safety and proof of concept in advance of clinical trials. Since oncolytic viruses have very unique safety issues, such as the emergence of genetic variants and the risk of germline transmission, international harmonized guidelines such as ICH S6 or other documents do not seem to be applicable. The design of non-clinical studies for oncolytic virus products will depend on the type and nature of the specific oncolytic virus product.

In each case, the objective and design of the animal studies, including the type of animal and the reason for selecting it, must be explained. Non-clinical studies should be designed to obtain data that demonstrate the proof-of-principle of oncolytic virus products and that provide biosafety features: target organs for toxicity, risk of shedding, etc. In general, animal models are valuable for testing non-clinical safety and assessing proof of concept, however, they have certain limitations. Some viruses have species-specific susceptibility to viral infection and replication, there may be differential tropism in tumor-xenograft models, and it is impossible to model all aspects of the immune response. Differences in the tissue architecture between animal models and humans are also an important factor, especially with respect to the role of the connective tissue and intermixed normal cells. Nevertheless, animal models are useful to address specific questions such as the choice of a route of administration, biodistribution, safety/toxicity, dose selection and dose regimen. When possible, the selectivity of virus replication has also been studied using *in vivo* models.

Non-clinical safety studies should initially include single-dose toxicity studies, repeated-dose toxicity studies if appropriate, and biodistribution studies, which can incorporate pharmacodynamic-like endpoints. The type and duration of repeated-dose non-clinical safety studies should be considered dependent on the type of oncolytic virus and potential concerns about insertional mutagenesis, for instance.

A quantitative nucleic acid amplification test (NAT) may be used to investigate tissue distribution and the persistence of the oncolytic virus sequence in biodistribution studies. If the administered oncolytic virus sequence is detected in unintended tissues or organs by a NAT assay, this may assist in determining mRNA for the gene product by RT-PCR. Additionally, RT-NAT immunological-based assays may be used to in verify the duration and level of expression of the gene product to detect functional protein.

According to the potential risk of inadvertent germline integration of oncolytic viruses based on the vector type, route of administration and patient population, it may also be necessary to determine whether or not the nucleic acid of the oncolytic virus is incorporated into gonads. The key element in the assessment of inadvertent germline integration is a well-conducted biodistribution study in animal models.

CLINICAL STUDIES AND SAFETY EVALUATION

Due to the complexity of oncolytic virus products and the limited usefulness of animal models, many concerns, including safety issues, remain to be addressed in early-phase clinical studies. These studies must focus on safety and definitions of dose and clinical strategy.

Clinical Pharmacokinetics

With respect to the pharmacokinetics of oncolytic viruses, both quantitative PCR and infectivity assays may be used to monitor patients. In some cases, quantitative monitoring of administered oncolytic virus genomes may provide data supporting viral replication in permissive tissues.

DOSE SELECTION AND DOSE REGIMEN

The dose selection and dose regimen of oncolytic viruses in clinical use should be carefully assessed to ensure their safety and evaluate their toxicity for humans. In one case, a patient who was enrolled in a phase-one clinical trial using replication-deficient adenovirus vector died due to the injection of high-dose vector [44]. The use of replication-competent viruses poses special concerns since the replication of the virus in the patient may lead to an enhanced level of and prolonged exposure to the virus, and thus might increase the risk of virus-induced toxicity. Replication competence of the oncolytic virus does not eliminate the need to perform dose ranging studies to determine an effective dose level.

Viral Shedding and Risks of Contact Person

Since data on viral shedding are limited with respect to oncolytic viruses [29, 45-49], precautions to reduce the risk of exposure of healthcare providers, family members and other patient contacts should be taken. The possibility of virus shedding and the site of shedding may depend on the site and route of administration, dose and replication efficacy of the virus in question. During clinical trials, risk must be monitored not only in the patient but also in the general population. The monitoring of viral shedding and mobilization/recombination with wild-type strains is recommended, and the need to establish long-term follow-up programs must be evaluated. However, all of these measures should take into account the special aspects of oncolytic viruses, such as the disease spectrum and pathogenicity of wild-type strains versus modified oncolytic viruses, the level of pre-existing immunity in the general population, and the ability of the virus to evade the immune system. The onset of tropism-modified versions of some oncolytic viruses requires additional control since the tropism can be narrowed or expanded, and previous clinical experience with non-modified strains is not necessarily relevant.

Schedule for Patient Follow-Up

It is important to establish observation and follow-up schedules for patients, including investigation of the *in vivo* distribution of the administered oncolytic virus, survival and functional expression terms of the gene of interest, symptoms caused by replication-competent viruses or molecular variants, etc. If the oncolytic virus is found to be transiently distributed to the gonads in animal studies, assaying patient semen for the presence of vector may be considered. However, if the patient population is sterile, or if the patient has a severe disease condition with short life expectancy, monitoring of semen samples may not be necessary.

ABBREVIATIONS

CMV = Cytomegalovirus
CPE = Cytopathic effect
E2F = S-phase of cell cycle promoter
EBV = Epstein-Barr virus
EMEA = European Medicines Agency
EOP cells = End of production cells
FDA = Food and Drug Administration
HBV = Hepatitis B virus
HCV = Hepatitis C virus
HIF-1 = Hypoxia-inducible factor-1
HIV = Human immunodeficiency virus
HSV = Herpes simplex virus
HTLV = Human T-cell lymphotropic virus
ICH = International conference on harmonization of technical requirements for registration of pharmaceuticals for human use
MCB = Master cell bank
MVB = Master virus bank
NAT = Nucleic acid amplification test
NDV = Newcastle disease virus
PCR = Polymerase chain reaction
PSA = Prostate-specific antigen
RCA = Replication-competent adenovirus
RCV = Replication-competent virus
RT = Reverse transcriptase
TERT = Telomerase reverse transcriptase
VSV = Vesicular stomatitis virus
WCB = Working cell bank
WVB = Working virus bank

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Received: July 05, 2006 **Revised: September 03, 2006** **Accepted: September 10, 2006**

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Journal of Virological Methods 143 (2007) 95–103

Journal of Virological Methods

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Optimization of the virus concentration method using polyethyleneimine-conjugated magnetic beads and its application to the detection of human hepatitis A, B and C viruses

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Received 25 September 2006; received in revised form 22 February 2007; accepted 26 February 2007
Available online 12 April 2007

Abstract

To enhance the sensitivity of virus detection by polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR), a novel virus concentration method using polyethyleneimine (PEI)-conjugated magnetic beads was developed in our previous study. However, several viruses could not be concentrated by this method. In this paper, the conditions of virus concentration were optimized to concentrate a wide range of viruses more efficiently. The PEI beads adsorbed viruses more efficiently than other cationic polymers, and the optimum virus concentration was obtained under weak acidic conditions. Mass spectrometric analysis revealed that several serum proteins, such as complement type 3, complement type 4 and immunoglobulin M (IgM), were co-adsorbed by the PEI beads, suggesting that the beads may adsorb viruses not only by direct adsorption, but also via immune complex formation. This hypothesis was confirmed by the result that poliovirus, which PEI beads could not adsorb directly, could be concentrated by the beads via immune complex formation. On the other hand, hepatitis A (HAV) and hepatitis C (HCV) viruses were adsorbed directly by PEI beads almost completely. Like poliovirus, hepatitis B virus (HBV) was concentrated efficiently by the addition of anti-HBV IgM. In conclusion, virus concentration using PEI beads is a useful method to concentrate a wide range of viruses and can be used to enhance the sensitivity of detection of HAV, HBV and HCV.
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Keywords: Polyethyleneimine; Virus concentration; Immune complex; HAV; HBV; HCV

1. Introduction

Many useful biological/biotechnological medicinal products are produced from biological materials and by cell culture techniques. Recent progress in gene therapy and cell therapy products has provided new hope for the treatment of grave genetic diseases and lethal disorders. These innovative medicinal products, however, involve some risk in terms of the spread of transmissible agents and virus-mediated infectious diseases. To ensure the viral safety of biological/biotechnological products, it is important to confirm that the starting materials, intermediate products and final products are free from virus contamination. This is especially important for cell therapy products, since it is difficult to inactivate and/or remove contaminated viruses from these products.

Polymerase chain reaction (PCR) is a highly sensitive method for the detection of virus genomes (Saiki et al., 1988). Several nucleic acid amplification test (NAT) methods other than PCR have also been developed (Alter et al., 1995; Kamisango et al., 1999; Kern et al., 1996; Notomi et al., 2000; Sarrazin et al., 2000). These tests are reported to be able to detect only some copies of virus genomes. Therefore, in many countries, NAT methods have been employed to detect specific viruses in the virus screening of blood-derived products (Willkommen et al.,

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doi:10.1016/j.jviromet.2007.02.014

1999). NAT methods are also thought to be useful in testing biotechnology products derived from cell lines and cell therapy products. However, since all NAT methods used for the detection of viruses have a detection limit, it is impossible to deny virus contamination completely. In order to reduce the virus risk of both biological/biotechnological products and cell therapy products, it is essential to develop more sensitive methods of virus detection. One way to improve the sensitivity of virus detection is to concentrate the target viruses before NAT testing.

Recently, a novel viral concentration method using polyethyleneimine (PEI)-conjugated magnetic beads was developed (Satoh et al., 2003). It was shown that PEI beads efficiently adsorbed many model viruses, such as simian virus 40 (SV-40), herpes simplex 1 virus (HSV-1), Sindbis virus and vesicular stomatitis virus (VSV), and that the method improved the sensitivity of NAT for the detection of virus genomes about 10 to 100 times. It has also been reported that PEI beads efficiently adsorb amphotropic murine leukemia virus, and that the virus concentration method provided sensitive detection of replication-competent retrovirus in retrovirus vector products (Uchida et al., 2004). However, several small non-enveloped viruses such as poliovirus could not be concentrated or were only partially concentrated by PEI beads (Satoh et al., 2003). In addition, the mechanism of virus adsorption by PEI beads remains to be elucidated.

In the present study, the viral concentration method using PEI beads was optimized in order to allow the efficient concentration of several viruses. It was demonstrated that poliovirus can be concentrated by PEI beads via the formation of immune complexes. In addition, it was shown that the virus concentration method using PEI beads is applicable to human infectious viruses such as the hepatitis A(HAV), hepatitis B (HBV) and hepatitis C (HCV) viruses, which are important viruses to test for in order to ensure the viral safety of biological products and cell therapy products.

2. Materials and methods

2.1. Viruses

SV-40 virus, HSV-1 (stain F), porcine parvovirus (PPV; strain 90HS) and poliovirus (strain Sabin 1) were obtained and amplified as described previously (Satoh et al., 2003). Briefly, the supernatants of Vero cells infected with HSV-1 or poliovirus were used as virus samples. CV-1 cells were infected with SV-40 virus, and 5 days after infection, the supernatant was saved as the SV-40 sample. The supernatant of ESK cells infected with PPV was used as the PPV sample. In order to remove cell debris from the collected virus suspension, each virus suspension was centrifuged at 3000 rpm for 10 min. After removing cell debris, the resulting stock viruses (SV40: 4×10^7 copies/ml; PPV: 1×10^6 copies/ml; HSV-1: 1×10^8 copies/ml; poliovirus: 2×10^7 copies/ml) were aliquoted and stored at −80 °C until use. Human adenovirus type 5 reference material (ATCC VR-1516; 5.8×10^{11} particles/ml) was obtained from the American type culture collection (ATCC) and used without amplification. HAV was obtained from ATCC (strain HM175/18f), infected into FRhK-4 cells, and the supernatant of the cell was saved 9–11 days later as the HAV sample (1×10^8 PFU/ml). The first Japanese national standard for HBV DNA (Genotype C; potency: 4.4×10^5 IU/ml) and the first Japanese national standard for HCV RNA (Mizusawa et al., 2005); genotype HCV-1b; potency: 100,000 IU/ml) were directly used as the HBV sample and HCV sample, respectively.

2.2. Preparation of PEI beads

PEI beads were prepared by coupling PEI (MW 70,000; Wako Pure Chemical Industries, Ltd., Tokyo, Japan) with magnetic beads (IMMUTEX-MAG™; mean diameter: 0.8 μm; JSR Inc., Tokyo, Japan) by the 1-ethylene-3-(3-dimethylaminopropyl) carbodiimide coupling method, as described previously (Satoh et al., 2003). The final concentration of the PEI beads was 50 mg/ml. Different molecular weights of PEI beads were prepared as described above but including the coupling of PEI with a molecular weight (MW) of 1800 or PEI (MW 10,000) to magnetic beads, instead of PEI (MW 70,000). Polyarylamine (PAA)-conjugated magnetic beads and poly-L-lysine (PLL)-conjugated magnetic beads were prepared in the same way as PEI beads, using PAA (MW 150,000) or PLL (MW > 300,000) instead of PEI, respectively.

2.3. Virus concentration using PEI beads

The essential adsorption procedure for each virus was as follows. Virus samples were diluted with virus dilution medium (Dulbecco's modified Eagle's medium (DMEM) or DMEM supplemented with 2% fetal calf serum (FCS)). The exact concentration of the virus suspension used for each experiment is described in the corresponding figure legends. Next, 1 ml of each virus suspension was incubated with 100 μl of PEI beads for 10 min at room temperature. The complexes of virus and PEI beads were trapped by a magnetic field (Magnetic Trapper™; Toyobo Co., Tokyo, Japan) for 5 min and separated from the supernatant fraction. The virus DNA or RNA was extracted from virus-bead complex or from untreated virus suspensions (100 μl) with an SMI-TEST EX R&D Kit (Medical & Biological Laboratories Co., Nagano, Japan) following the manufacturer's instructions. Extracted DNA or RNA was dissolved in 50 μl of TE buffer (10 mM Tris–HCl (pH 7.4)/0.1 mM EDTA), and 10 μl of the solution was used for real-time PCR or RT-PCR reaction.

2.4. Effect of pH on virus concentration by PEI beads

Good's buffers with pH 4–9 (1 M MES, pH 4.0; 1 M MES, pH 5.0; 1 M MES, pH 6.0; 1 M HEPES, pH 7.0; 1 M HEPES, pH 8.0; 1 M Tricine, pH 9.0) were prepared and added to the virus dilution medium at a final concentration of 20 mM. Virus samples were then diluted with the virus dilution media at different pH values, and concentrated with PEI beads as described. The exact concentration of the virus suspension used for each experiment is described in the corresponding figure legends.

2.5. Real-time PCR and RT-PCR

Real-time PCR and reverse transcription PCR (RT-PCR) were carried out in a 50-μl reaction mixture containing 10 μl of extracted DNA or RNA, 0.5 μM of each primer set with a fluorescence probe, 25 μl of PCR master mix and, in the case of RT-PCR, a reverse transcriptase mix prepared according to the kit manual. The following real-time PCR and RT-PCR master mix kits were used: a QuantiTect Probe PCR kit (Qiagen, Hilden, Germany) for HSV-1, SV-40, adenovirus and PPV; a QuantiTect Probe RT-PCR kit (Qiagen) for poliovirus, HAV and HCV; and a Platinum Quantitative PCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, CA, USA) for HBV. The 5′-primers, 3′-primers and fluorescence probes used for the real-time PCR and RT-PCR detection of viruses are shown in Table 1. The real-time PCR and RT-PCR were performed on an ABI PRISM 7000 Sequence-Detection System (Applied Biosystems, Foster City, CA, USA).

2.6. SDS-PAGE analysis of serum proteins adsorbed on PEI beads

The virus suspension (HSV-1) diluted with DMEM supplemented with 5% FCS was incubated with PEI beads for 10 min. The fraction of serum proteins adsorbed on the beads and the untreated virus suspension were then boiled with sodium dodecly sulfate (SDS) sample buffer and applied to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out on a slab gel ($T = 7.5\%$) with a BE-120 system from Biocraft (Tokyo, Japan). Protein bands were visualized by Coomassie Brilliant Blue staining.

2.7. In-gel digestion

Protein bands of interest were excised from the SDS-PAGE gel, destained three times in 50% acetonitrile and 25 mM ammonium bicarbonate for 10 min each time, and dehydrated in acetonitrile. The gel pieces were dried in a vacuum centrifugal concentrator and incubated with 10 mM dithiothreitol (DTT) in 25 mM ammonium bicarbonate at 56 °C for 60 min. After cooling to room temperature, the DTT solution was replaced with roughly the same volume of 55 mM iodoacetamide in 25 mM ammonium bicarbonate. After incubation for 45 min at room temperature in the dark, the gel pieces were washed in 25 mM ammonium bicarbonate for 5 min and dehydrated by the addition of 50% acetonitrile and 25 mM ammonium bicarbonate for 5 min. After this procedure was repeated twice, the gel pieces were dried in a centrifugal concentrator. The gel pieces were allowed to swell in 2 μl of a digestion buffer containing 25 mM ammonium bicarbonate, 0.1% octyl glucoside, and 25 ng/μl trypsin (sequence grade; Promega, Madison, WI, USA) in ice for 5 min, and then 15 μl of a digestion buffer without trypsin was added. After 30 min, the supernatant was discarded, and the gel pieces were incu-

Table 1
Primer and probe sets used for the real-time PCR and RT-PCR

Virus	Primer and probe set
HSV-1	Forward primer: 5′-GCGTCATGGTACTGGCAAG-3′ Reverse primer: 5′-TTGACTCTACGGAGCTGGCC-3′ Probe: 5′-FAM-TGGAGCTGATGCCGTAGTCGG-TAMRA-3′
SV-40	Forward primer: 5′-GACATTCCTAGGCTCACCTCACA-3′ Reverse primer: 5′-ACCTTGCCAAACTGTCCCTTAAA-3′ Probe: 5′-FAM-CTTGAAAGAAGAACCCAAAGA-TAMRA-3′
PPV	Forward primer: AACAACTACGCAGCAACTCCAATA-3′ Reverse primer: ACGGCTCCAAGGCTAAAGC-3′ Probe: 5′-FAM-AGGAGGACCTGGATTT-MGB-3′
Adenovirus*1	Forward primer: TCCGGTCCTTCTAACACACCTC-3′ Reverse primer: ACGGCAACTGGTTTAATGGG-3′ Probe: 5′-FAM-TGAGATACACCCGGTGGTCCCGC-TAMRA-3′
Poliovirus	Forward primer: 5′-CCCGAGAAATGGGACGACTA-3′ Reverse primer: 5′-TGGAGCTGTTCCGTAGGTGTAA-3′ Probe: 5′- FAM-ACATGGCAAACCTCATCAAATCCATCAATC-MGB-3′
HAV*2	Forward primer: 5′-GGTAGGCTACGGGTGAAAC-3′ Reverse primer: 5′-AACAACTCACCAATATCCGC-3′ Probe: 5′-FAM-CTTAGGCTAATACTTCTATGAAGAGATGC-TAMRA-3′
HBV*3	Forward primer: 5′-GGACCCCTGCTCGTGTTACA-3′ Reverse primer: 5′-GAGAGAAGTCCACCMCGAGTCTAGA-3′ Probe: 5′-FAM-TGTTGACAARAATCCTCACCATACCRCAGA-TAMRA-3′
HCV*4	Forward primer: 5′-TGCGGAACCGGTGAGTACA-3′ Reverse primer: 5′-CTTAAGGTTTAGGATTCGTGCTCAT-3′ probe: 5′-FAM-CACCCTATCAGGCAGTACCACAAGGCC-TAMRA-3′

Each primer set was prepared according to the original papers described below (*1 to *4) or designed using Primer Express software (Applied Biosystems). *1 Adenovirus (Ishii-Watabe et al., 2003), *2 HAV (Jothikumar et al., 2005), *3 HBV (Pas et al., 2000), *4 HCV (Martell et al., 1999).

bated overnight at 37 °C. To extract tryptic fragments, the gel pieces were shaken in 50% acetonitrile and 5% trifluoroacetic acid (TFA) for 30 min. After this procedure was repeated twice, the extraction solutions were pooled, dried in a centrifugal evaporator, and dissolved in 20 μl of 0.1% TFA. The samples were then absorbed onto reverse-phase ZipTipC18 (Millipore, Bedford, MA, USA). The resin was washed with 0.1% TFA and the peptides were eluted with 3 μl of 75% acetonitrile/0.1% TFA. The eluate was analyzed by mass spectrometry (MS) as described below.

2.8. MS and database searching

The peptide mixture (0.5 μl volume) elution was deposited onto a matrix assisted laser desorption/ionization (MALDI) target plate, and this was closely followed by the deposition of 0.5 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% TFA. MS and tandem MS (MS/MS) analysis of the peptide mixtures was performed using a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA). Peptide mass fingerprinting and MS/MS ion searches were performed for protein identification by a Mascot search based on the MSDB protein database.

2.9. Preparation of anti-mouse IgG-rabbit IgM antibody

Anti-mouse immunogloblulin G (IgG) rabbit antiserum was obtained from rabbits immunized with highly purified mouse IgG (11 mg/ml; Jackson ImmunoResearch, West Grove, PA, USA) at 11 days after immunization, when IgM titer was increased. The antiserum (3 ml) was then diluted with an equal volume of phosphate buffered saline (PBS) (−), and applied to a mouse-IgG agarose affinity column (Invitrogen). After washing with 10 ml of PBS (−), the bound fraction was eluted with 0.1 M glycine–HCl (pH 3.0) and neutralized with 1 M Tris–HCl (pH 8.0). A PEI-sepharose-6MB column was prepared by coupling PEI to CNBr-activated sepharose-6MB (GE Healthcare Bioscience, Piscataway, NJ, USA). Anti-mouse IgG rabbit antiserum purified with a mouse IgG-agarose column was applied to a PEI-sepharose-6MB column and washed with PBS (−), and the bound fraction was eluted with 1.4 M NaCl/50 mM HEPES (pH 7.6). The eluted fraction was concentrated and used as anti-mouse IgG rabbit IgM antibody (final concentration, 4 μg/ml).

2.10. Poliovirus concentration via immune complexes

When poliovirus suspension was concentrated by PEI beads via immune complex formation, anti-poliovirus 1 mouse monoclonal antibody (IgG1: 5 μl; Chemicon International, Temecula, CA, USA) and purified anti-mouse IgG rabbit IgM antibody (20 μl), or anti-poliovirus 1 mouse monoclonal antibody and human complement C1 (5 μl; Merck Biosciences/Calbiochem, Darmstadt, Germany) and C4 (3 μl; Calbiochem) were added to the virus suspension before incubation with PEI beads.

2.11. Preparation of anti-HBV IgM antibody

Anti-hepatitis B surface antigen (HBsAg) IgM antibody was prepared as follows. Rabbits were immunized with a mixture of the adw and adr subtypes of recombinant HBsAg (Advanced ImmunoChemical, Long Beach, CA, USA). Anti-HBsAg rabbit antiserum was obtained at 10 days after immunization, when IgM titer was increased. The antiserum (3 ml) was diluted with an equal volume of PBS (−), applied to a PEI-sepharose-6MB column, washed with 20 ml PBS (−), and eluted with 1.4 M NaCl/100 mM HEPES (pH 7.0). PEI-sepharose-6MB-bound fractions were pooled, desalted with a PD-10 column equilibrated with 1.2 M NaCl/50 mM HEPES buffer (pH 7.4), and purified with an ImmunoPure IgM purification kit (Pierce Biotechnology, Rockford, IL, USA). IgM fractions were concentrated and used as anti-HBsAg IgM antibody.

3. Results

3.1. Optimization of the virus concentration method using PEI beads

In order to optimize the virus concentration method using PEI beads, the relationship between the MW of PEI coupled with magnetic beads and the efficiency of the virus concentration was examined. When PEIs with average molecular masses of 1800, 10,000 and 70,000 Da were compared, the PEI of MW 70,000 Da efficiently concentrated HSV-1, while magnetic beads with the PEI of MWs 1800 and 10,000 Da could not adsorb HSV-1 (Fig. 1). Therefore, the PEI beads with MW 70,000 Da were used in the following experiments.

Next, the virus adsorption ability of PEI was compared to that of other cationic polymers. As shown in Fig. 2, PEI beads exhibited a markedly higher virus adsorption ability than PAA- or PLL-conjugated magnetic beads for all model viruses tested.

The effect of pH on the efficiency of virus concentration was then examined. HSV-1 and SV-40 virus suspensions at different

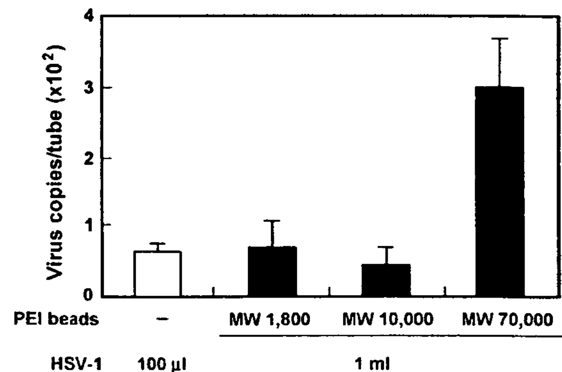


Fig. 1. Comparison of virus concentrations by magnetic beads coupled with PEIs of three different molecular weights. HSV-1 suspension (1×10^3 copies/ml, 1 ml/tube) was incubated with PEI beads whose PEI had a molecular weight of 1800, 10,000 or 70,000 Da. Viral genome DNA was extracted from the PEI bead fraction and from untreated HSV-1 suspension (100 μl). Virus copy numbers were determined by real-time PCR. Data are expressed as the mean ± S.D. ($n = 3$).

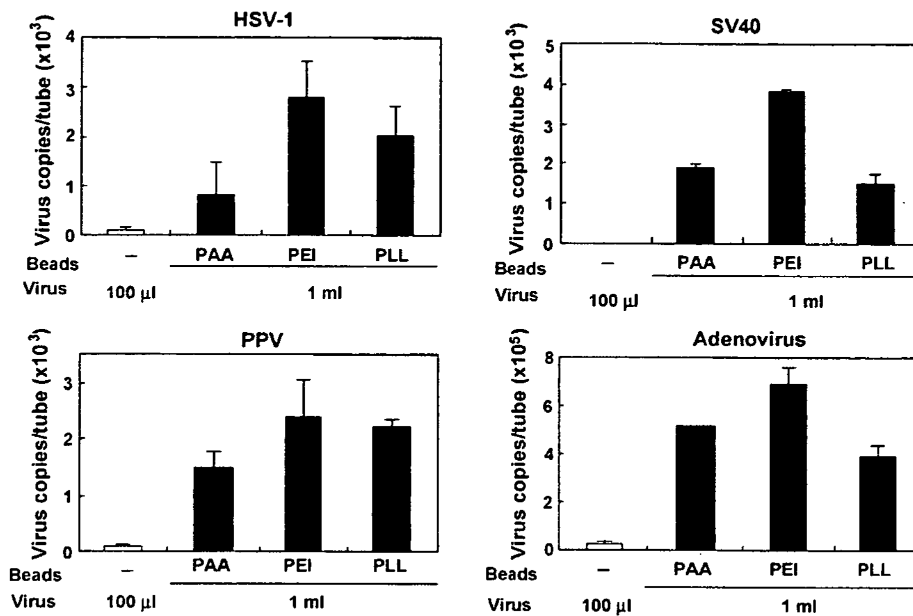


Fig. 2. Comparison of virus concentration by magnetic beads coupled with three different cationic polymers. HSV-1 (5×10^3 copies/ml), SV-40 (5×10^3 copies/ml), PPV (5×10^3 copies/ml) and adenovirus suspensions (1×10^6 copies/ml) (1 ml each) were incubated with PAA-, PEI- or PLL-conjugated magnetic beads. Viral genome DNA was extracted from each magnetic bead fraction and from untreated virus suspensions (100 μl each). Virus copy numbers were determined by real-time PCR. Data are expressed as the mean ± S.D. ($n = 3$).

pH levels (pH 5–9) were concentrated by PEI beads following the standard method. A pH levels of 6 was found to be optimal for the concentration of these viruses (Fig. 3).

3.2. Analysis of serum proteins adsorbed on PEI beads

To improve the virus concentration method using PEI beads, the serum components co-adsorbed by the beads during virus concentration were analyzed. When a virus suspension containing 5% FCS was concentrated by PEI beads and analyzed by SDS-PAGE, several proteins were specifically adsorbed by the beads (Fig. 4). Using MS and MS/MS analyses of these protein bands, complement type 3, complement type 4 and IgM heavy chain were identified as serum components concentrated by PEI beads. Since complement components and IgM were adsorbed by the beads, it is hypothesized that PEI beads may adsorb viruses not only by direct adsorption, but also via the formation of immune complexes that involve IgM antibody and/or complements.

3.3. Concentration of poliovirus by PEI beads via immune complexes

To confirm this hypothesis, concentrations of poliovirus, which PEI beads could not adsorb directly, via the formation of immune complexes were examined. Instead of anti-poliovirus IgM antibody, anti-poliovirus mouse monoclonal antibody (IgG) was used in combination with anti-mouse IgG rabbit IgM anti-

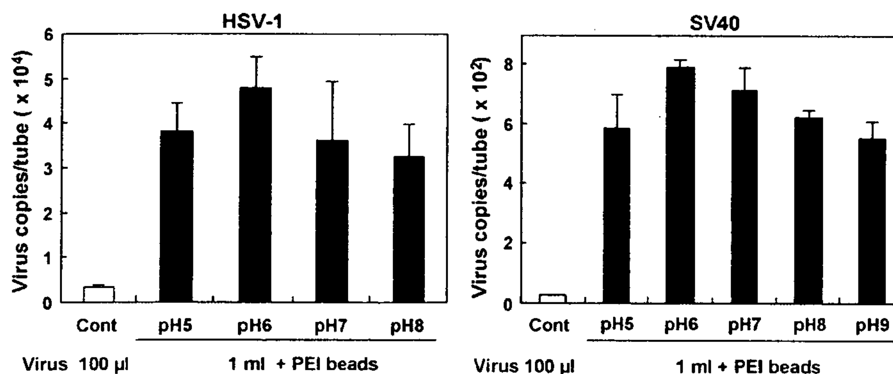


Fig. 3. Effect of pH on the efficiency of virus concentration by PEI beads. HSV-1 (5×10^4 copies/ml) and SV-40 (1×10^3 copies/ml) suspensions diluted with virus dilution medium at different pH levels (HSV-1: pH 5, 6, 7 and 8; SV-40: pH 5, 6, 7, 8 and 9) (1 ml each) were incubated with PEI beads. Viral genome DNA was then extracted from PEI bead fraction and from untreated virus suspensions (100 μl each). Virus copy numbers were determined by real-time PCR. Data are expressed as the mean ± S.D. ($n = 3$).

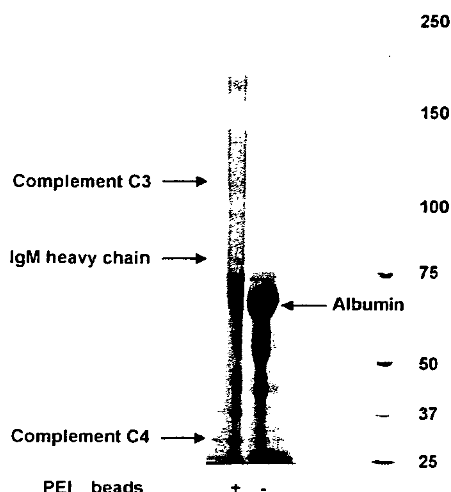
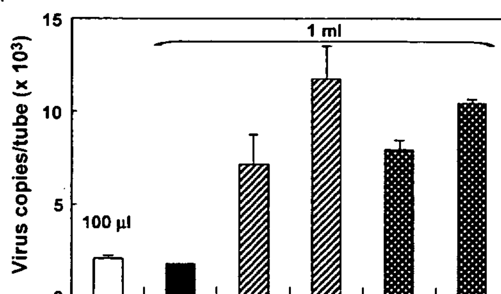


Fig. 4. Serum proteins adsorbed on PEI beads during virus concentration. HSV-1 suspension diluted with DMEM supplemented with 5% FCS was incubated with PEI beads. PEI bead fraction (+) and untreated virus suspension (−) were then boiled with SDS sample buffer and applied to SDS-PAGE. Serum protein bands concentrated by PEI beads were identified by MS/MS analysis, as shown in Fig. 5.



PEI beads	-	+	+	+	+	+
Anti-poliovirus mouse IgG MoAb	-	-	+	+	+	+
Anti-mouse IgG-rabbit IgM	-	-	-	+	-	-
C1 + C4	-	-	-	-	+ (r.t.)	+ (37°C)

Fig. 6. Concentration of poliovirus by PEI beads via the formation of immune complexes. Poliovirus suspension (2×10^4 copies/ml, 1 ml each) was incubated with PEI beads at room temperature or 37 C in the absence or presence of anti-poliovirus mouse IgG monoclonal antibody, anti-mouse IgG-rabbit IgM, or a combination of complements C1 and C4. Viral genome RNA was extracted from the PEI bead fraction and from the untreated virus suspension (100 µl). Virus copy numbers were determined by real-time RT-PCR. Data are expressed as the mean ± S.D. ($n = 3$).

body to induce the formation of immune complexes. Anti-mouse IgG rabbit IgM antibody was prepared from rabbit anti-mouse IgG antiserum and purified by a mouse-IgG affinity column followed by a PEI-sepharose-6MB column. Since the PEI-sepharose-6MB column adsorbed IgM (Fig. 5) but not IgG (data not shown), the PEI-sepharose-6MB adsorbed fraction was used as the anti-mouse IgG rabbit IgM antibody. When poliovirus alone was incubated with the PEI beads, it was not adsorbed, but poliovirus was adsorbed when coincubated with anti-poliovirus IgG antibody, and a further significant improvement in the efficiency of virus concentration was achieved by the addition of anti-mouse IgG rabbit IgM along with the anti-poliovirus IgG (Fig. 6). The addition of the combination of complement C1, complement C4 and anti-poliovirus IgG to the reaction mixture of virus and PEI beads also increased the efficiency of virus concentration by PEI beads, but only when the complement system was activated by [incubation at] 37 °C (Fig. 6).

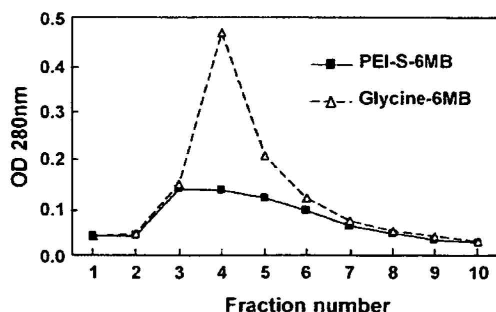


Fig. 5. Adsorption of IgM to a PEI-sepharose column. One ml of human IgM solution (1 mg/ml) was applied to a PEI-sepharose 6MB (PEI-S-6MB) column or to a control column without PEI (Glycine-6 MB) and washed with PBS (−). The eluates were fractionated into ten 1 ml fractions, and the OD280 of each fraction was determined using a spectrophotometer.

3.4. Application of the virus concentration method using PEI beads to human hepatitis viruses

The virus concentration method using PEI beads was applied to human HAV, HBV and HCV. Fig. 7 shows the effect of pH on the virus concentration efficiency. HAV was efficiently adsorbed by the PEI beads (Fig. 7A). The number of viral copies obtained in the PEI bead fraction when using 1 ml of virus suspension was about 10-fold the number extracted from untreated virus suspension (100 µl), suggesting that the concentration of HAV almost reached the predicted level. Neither the presence or absence of serum nor the pH condition affected the efficiency of the HAV concentration. HCV was also efficiently adsorbed by PEI beads, even in the presence of 2% FCS, and the optimum pH was found to be 5 (Fig. 7C). On the other hand, the efficiency of HBV concentration by PEI beads was lower than the efficiencies of HAV and HCV concentrations. The number of viral copies obtained in the PEI bead fraction under the optimum condition of pH 5 without serum was about six-fold the number extracted from untreated virus suspension (Fig. 7B). The presence of FCS significantly reduced the adsorption of HBV by PEI beads.

In order to improve the concentration of HBV obtained by PEI beads, anti-HBV IgM antibody was prepared and the concentration of HBV via immune complex formation was examined. As shown in Fig. 8, the concentration of HBV by PEI beads was improved by the addition of anti-HBV IgM antibody. Under the optimum condition, the number of viral copies obtained in the PEI bead fraction was more than seven-fold the number extracted from the untreated virus suspension even in the

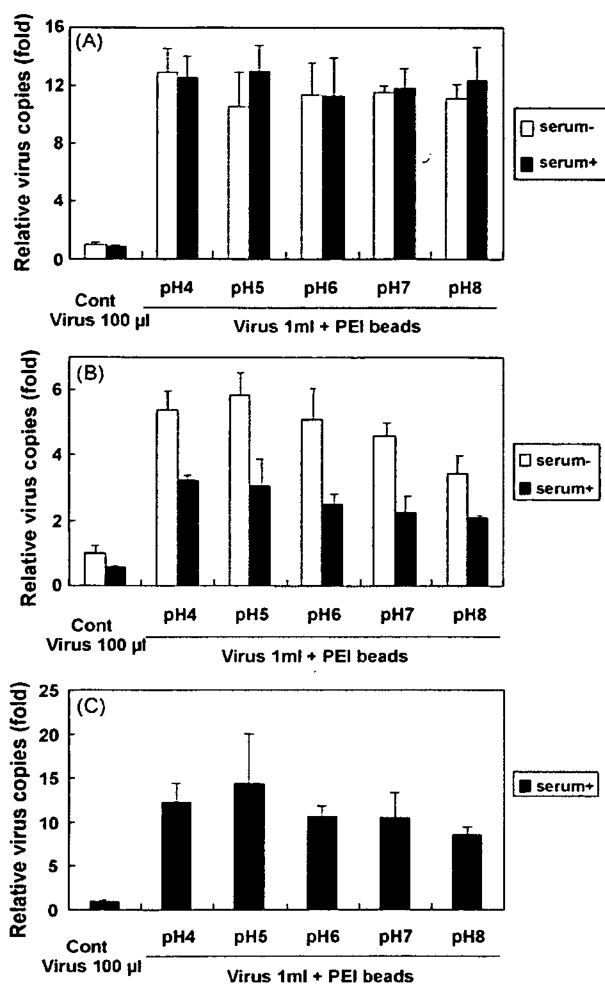


Fig. 7. Effect of pH on the concentration of HAV, HBV and HCV by PEI beads. HAV (A), HBV (B), and HCV (C) were diluted with virus dilution media of different pH levels supplemented with or without 2% FCS. Virus suspensions (HAV: 5×10^4 PFU/ml; HBV: 8.8×10^3 IU/ml; HCV: 1×10^3 IU/ml; 1 ml/tube) with different pH levels were incubated with PEI beads. Viral genome DNA and RNA were then extracted from PEI bead fraction and analyzed by real-time PCR and RT-PCR. Data are expressed as the mean ± S.D. ($n = 3$).

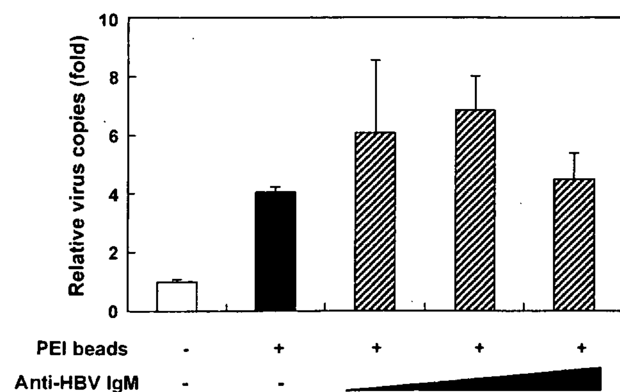


Fig. 8. Effect of anti-HBV IgM antibody for HBV concentration by PEI-beads. HBV suspensions (8.8×10^3 IU/ml; 1 ml/tube) were incubated with PEI beads in the absence or presence of 5, 15 or 50 μl of anti-HBV IgM antibody. Viral genome DNA was then extracted from the PEI bead fraction and analyzed by real-time PCR. Data are expressed as the mean ± S.D. ($n = 3$).

presence of serum. Therefore, the virus concentration achieved by PEI beads was shown to be enhanced by the formation of immune complexes.

Table 2 shows a summary of virus concentrations by PEI beads for all of the viruses examined. A wide range of viruses, including small non-enveloped viruses and human hepatitis viruses (HAV, HBV and HCV), were efficiently concentrated by PEI beads under the optimum condition, either directly or by the formation of immune complexes.

4. Discussion

In the present study, the virus concentration method using PEI beads (Satoh et al., 2003) was optimized, and was applied to human hepatitis A, B and C viruses.

First, the effects of various cationic polymers, PEI molecular weights, and pH values were examined in order to determine the optimal conditions for virus concentration. Among PEI beads with three different molecular weights (1800, 10,000 and 70,000 Da), only the PEI whose MW was 70,000 Da was able to adsorb viruses (Fig. 1). With respect to the cationic polymers, PEI magnetic beads showed a higher virus adsorption ability than PAA- or PLL-conjugated magnetic beads (Fig. 2). The optimum pH for the concentration of model viruses by PEI beads was subacidic (Fig. 3). The virus adsorption mechanism of PEI beads remains unclear. However, it is hypothesized that the positively charged field of the PEI molecule may interact tightly with the negative charge of surface lipids or the negatively charged surface proteins on viruses (Satoh et al., 2003). PEI is a polycationic polyamine with the highest cationic charge density among existing polymers (Futami et al., 2005). PEI has a branched backbone containing primary, secondary and tertiary amine groups. In contrast, PAA is a linear polycation having only primary amine groups, and PLL is a linear polycation with primary and secondary amine groups. Therefore, it is suggested that the high-density cationic charge of PEI and its branched structure on the surface of the magnetic beads may be important for efficient virus adsorption. According to the analysis of Owada et al. (1999), the interaction between PEI-coated membranes and human immunodeficiency virus type 1 (HIV-1) or plasma protein may be dependent on the surface area of each particle, and this fundamental principle was consistent with their observation that PEIs with higher MWs bound more intensely to HIV-1. This is also consistent with the data that PEI with a MW of 70,000 Da was able to adsorb viruses more efficiently than PEIs of 1800 Da or 10,000 Da.

In order to improve the efficiency of virus concentration by PEI beads, the serum components co-adsorbed by the beads were analyzed. MS analysis revealed that several proteins, including complement type 3, complement type 4 and IgM, were specifically co-adsorbed by PEI beads during virus concentration (Fig. 4), suggesting that the beads were able to adsorb immune complexes that involved IgM antibody and/or complements. Therefore, it is hypothesized that in addition to direct adsorption, PEI beads may adsorb viruses via the formation of immune complexes. This hypothesis was confirmed by the fact that PEI beads were able to adsorb poliovirus under con-

Table 2
Summary of concentration of viruses by PEI beads

Viruses	Natural host	Virus genome	Envelope	Size (nm)	PEI beads concentration
Model viruses cytomegalovirus (CMV)	Simian	DNA	+	180–200	+
Herpes symplex virus type 1 (HSV-1)	Human	DNA	+	150–200	+
Vesicular stomatitis virus (VSV)	Bovine	RNA	+	70–150	+
Amphotropic murine leukemia virus	Murine	RNA	+	80–110	+
Sindbis virus	Human	RNA	+	60–70	+
Adenovirus type 5	Human	DNA	−	70–90	+
Simian virus 40 (SV40)	Simian	DNA	−	40–50	+
Porcine parvovirus (PPV)	Porcine	DNA	−	18–24	+
Poliovirus sabin 1	Human	RNA	−	25–30	+[a]
Human hepatitis viruses hepatitis B virus (HBV)	Human	DNA	+	40–45	+[a]
Hepatitis C virus (HCV)	Human	RNA	+	40–50	+
Hepatitis A virus (HAV)	Human	RNA	−	25–30	+

[a] Concentrated by the addition of antibodies.

ditions which fostered immune complex formation, such as the addition of anti-poliovirus mouse IgG antibody with anti-mouse IgG rabbit IgM, or the addition of anti-poliovirus IgG antibody with activated complements (Fig. 6). Poliovirus is a very small (25–30 nm) non-enveloped virus, and could not be concentrated by PEI beads in our previous study (Satoh et al., 2003). Another possible explanation is that the increase in the surface area of virus particles due to the formation of immune complexes enhances the interaction between the poliovirus and the PEI beads, as hypothesized by Owada et al. (1999).

The results obtained from model viruses suggest that the virus concentration method using PEI beads may be applicable to a wide range of viruses. Therefore, this method was applied to human hepatitis viruses. A recent study reported that in some HAV patients, the duration of the viremic phase persisted for more than 1 year with low viral load levels (10^3–10^4 HAV genome equivalents/ml) (Normann et al., 2004). In the case of HBV, the presence of occult HBV infection (HBV DNA positivity in the setting of negative serum hepatitis B surface antigen) has been documented, and the majority of these infections were associated with low viral loads ($<10^5$ copies/ml) (Minuk et al., 2004). Several studies have demonstrated high rates of transmission of HCV through transfusions with extremely low viral loads (Operskalski et al., 2003). HCV is particularly infectious during the early window period, with levels as low as 1 viral copy in 20 ml plasma able to transmit infection by transfusion (Busch et al., 2003), though intermittent low-level HCV viremia can occur as long as 2 months before the periods of exponential increase in viral load (Glynn et al., 2005). Therefore, it is extremely important to develop a highly sensitive detection method for these viruses. In the present study, it was possible to concentrate HAV and HCV by PEI-beads to almost the predicted levels (Fig. 7). In contrast, HBV was not fully concentrated even under optimum conditions around pH 5. Therefore, the concentration of HBV via the formation of immune complexes was tested. As expected, the concentration of HBV was improved by the addition of anti-HBV IgM antibody (Fig. 8), indicating that the virus concentration method using PEI beads is applicable for the concentration and sensitive detection of HAV, HBV and HCV by PCR and RT-PCR reaction.

To enhance/establish the utility of this virus concentration method using PEI meads for viral safety of biological products and cell therapy products, examination using actual patient sera and different genotypes/subtypes of each virus may be required. In a preliminary experiment, it is confirmed that this PEI beads method can be used for hepatitis virus samples spiked in human plasma. Applicability to different genotypes will be examined using a Japanese genotype panel of HBV and HCV, which will be available soon.

PEI beads may be applicable not only for virus concentration but also for the efficient infection of viruses. Scherer et al. (2002) report that superparamagnetic nanoparticles coated with PEI enhanced the infection of adenovirus and retrovirus vectors under a magnetic field. This infection method (magnetofection) also enhanced the infection of measles virus (Kadota et al., 2005). In a preliminary experiment, the PEI beads used in the present study also enhanced the infectivity of several viruses under a magnetic field. Therefore, it is suggested that PEI beads may be useful for the sensitive detection of both virus genomes and virus infectivity.

In conclusion, the present study demonstrates that the virus concentration method using PEI beads is effective for the concentration and sensitive detection of a wide range of viruses, including HAV, HBV and HCV.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Health and Labor Science Research (H17-SAISEI-021) from the Japanese Ministry of Health, Labor and Welfare, as well as by a Grant-in-Aid for Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation. The authors are grateful to Ms. Momoko Komesu for her technical assistance.

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HUMAN GENE THERAPY 18:74–80 (January 2007)
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DOI: 10.1089/hum.2006.129

Technical Report

Rapid Construction of Small Interfering RNA-Expressing Adenoviral Vectors on the Basis of Direct Cloning of Short Hairpin RNA-Coding DNAs

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ABSTRACT

In the conventional method for constructing an adenoviral (Ad) vector expressing small interfering RNA (siRNA), short hairpin RNA (shRNA)-coding oligonucleotides are introduced downstream of a polymerase III (or polymerase II)-based promoter cloned into a shuttle plasmid. An siRNA expression cassette, which is cloned into the shuttle plasmid, is then introduced into the E1 deletion region of the Ad vector plasmid by *in vitro* ligation or homologous recombination in *Escherichia coli*, and the linearized plasmid is transfected into 293 cells, generating an Ad vector expressing siRNA. Therefore, two-step plasmid manipulation is required. In this study, we developed a method by which shRNA-coding oligonucleotides can be introduced directly into the Ad vector plasmid. To do this, we constructed a new vector plasmid into which the human U6 promoter sequence was cloned in advance. Unique restriction enzyme sites were introduced at the transcription start site of the U6 promoter sequence in the vector plasmid. Luciferase and p53 genes were efficiently knocked down by Ad vectors generated by the new method and expressing siRNA against the target gene. This method should be useful for RNA interference-based experiments, and should make it easy to construct an siRNA-expressing Ad vector library for functional screening.

INTRODUCTION

RNA INTERFERENCE (RNAi), which mediates the sequence-specific suppression of gene expression in a wide variety of eukaryotes by double-stranded RNA homologies to the target gene (Scherer and Rossi, 2003), is a powerful tool for the knockdown of gene expression. Transduction of synthetic small interfering RNA (siRNA; 19 to 29 nucleotides of RNA) or the promoter-based expression of siRNA in the cells results in sequence-dependent degradation of target mRNA and subsequent reduction of target gene expression. Most promoter-based RNAi systems express short hairpin RNA (shRNA), which is then trimmed by Dicer, generating functional siRNA. Polymerase III-based promoters, such as the small nuclear RNA U6 promoter or the human RNase P RNA H1 promoter, are widely used for the expression of shRNA (siRNA) (Scherer and Rossi, 2003), although polymerase II-based promoters are also used (Xia *et al.*, 2002; Shinagawa and Ishii, 2003). The promoter-based method has an advantage in that viral vectors as well as nonviral vectors can be used for delivery of the siRNA expression unit, whereas only nonviral vectors are used for delivery of synthetic siRNA.

Recombinant adenoviral (Ad) vectors have been used extensively to deliver foreign genes to a variety of cell types and tissues both *in vitro* and *in vivo* (McConnell and Imperiale, 2004; Volpers and Kochanek, 2004). They can be easily grown to high titers and can efficiently transfer genes into both dividing and nondividing cells. Therefore, Ad vector-mediated

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delivery of an siRNA expression unit, in which a promoter-based shRNA expression cassette is delivered into the cell by the Ad vector, provides a valuable tool for both gene function studies and therapeutic applications.

Construction of Ad vectors used to be a time-consuming and labor-intensive procedure, but several improved methods to facilitate the construction of Ad vectors have been developed (reviewed in Mizuguchi *et al.*, 2001). The homologous recombination method in E1-complementing cell lines (i.e., 293 cells) has been the most widely used method for generating recombinant Ad vectors, and it has greatly contributed to the widespread use of Ad vectors (Bett *et al.*, 1994). The major limitations of this approach are the low frequency of the recombination event and the tedious and time-consuming plaque purification procedure required to select the recombinant virus of interest, because a relatively high percentage of the virus produced is wild type (in most cases, 20–70%), due to recombination with the Ad sequence integrated into the chromosomes of 293 cells. The improved *in vitro* ligation method (Mizuguchi and Kay, 1998, 1999) and the homologous recombination method in *Escherichia coli* (He *et al.*, 1998), which are commercially available from Clontech (Palo Alto, CA) and Invitrogen (Carlsbad, CA), respectively, have now become widely used, because these systems overcome the limitations of the homologous recombination method in 293 cells. To construct an Ad vector expressing siRNA by these two methods, shRNA-coding oligonucleotides are introduced downstream of the polymerase III (or polymerase II)-based promoter cloned in a shuttle plasmid. An shRNA (siRNA) expression cassette, which is cloned in the shuttle plasmid, is then introduced into the E1 deletion region of the Ad vector plasmid, which clones a full Ad genome, by simple *in vitro* ligation or homologous recombination in *E. coli*. The resulting plasmid is then linearized and transfected into 293 cells, generating an Ad vector expressing siRNA. Therefore, two-step *E. coli* transformation and plasmid manipulation is required for the improved *in vitro* ligation method, whereas three-step *E. coli* transformation and plasmid manipulation is required in the homologous recombination method in *E. coli* (because a special *E. coli* strain is used in the latter method, retransformation into a normal stain of *E. coli* is required) (reviewed in Mizuguchi *et al.*, 2001).

In the present study, we developed a simple method for generating an Ad vector expressing siRNA, in which shRNA-coding oligonucleotides could be directly introduced into an Ad vector plasmid containing the human U6 (hU6) promoter sequence. Unique restriction enzyme sites were introduced at the transcription start site of the hU6 promoter sequence cloned into the Ad vector plasmid. Two types of modified hU6 promoter sequence were constructed to develop this method. Using this method, only one-step *E. coli* transformation is required to generate an Ad vector plasmid containing an siRNA expression cassette.

MATERIALS AND METHODS

Cells

A549 and 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). A549-Luc cells, which are stable transformants with luciferase expression, were cultured in DMEM supplemented with 10% FCS. For construction of A549-Luc cells, A549 cells were transfected with luciferase-expressing plasmid pGL3-Control-RSVneo, which contains the simian virus 40 (SV40) promoter/enhancer-luciferase cDNA-SV40 p(A) sequence and the neomycin expression cassette, using SuperFect transfection reagent (Qiagen, Valencia, CA). pGL3-Control-RSVneo was constructed by insertion of the Rous sarcoma virus (RSV) promoter-driven neomycin expression cassette into pGL3-Control (Promega, Madison, WI). Monoclonal A549 cells stably expressing luciferase (A549-Luc) were obtained by geneticin (G418) selection.

Plasmid and virus

The hU6 promoter sequence was amplified from human genomic DNA (Clontech), using the following primers: hU6-S1, hU6-AS1, and hU6-AS2 (Table 1). The hU6a and hU6b promoter sequences were amplified with hU6-S1/hU6-AS1 and hU6-S1/hU6-AS2 primer sets, respectively (see Fig. 2). These promoter sequences were introduced into pHM5 (Mizuguchi and Kay, 1999), and were then transferred into the E1 deletion region of the vector plasmid pAdHM4.1, a derivative of pAdHM4 (Mizuguchi and Kay, 1998) (the *Xba*I site outside the Ad genome of pAdHM4 was deleted), by an *in vitro* ligation method using the I-*Ceu*I and PI-*Sce*I sites (Mizuguchi and Kay, 1998, 1999), resulting in pAdHM4-hU6a and pAdHM4-hU6b, respectively (Fig. 1A). To construct a vector plasmid containing an shRNA-coding sequence against luciferase, oligonucleotides 1/2 and 3/4 were synthesized (Table 1), annealed, and cloned into the *Cla*I and *Xba*I sites of pAdHM4-hU6a or the *Swa*I and *Xba*I sites of pAdHM4-hU6b, generating pAdHM4-hU6a-Lu and pAdHM4-hU6b-Lu, respectively. The target sequence for siRNA is bp 158 to 176 of luciferase cDNA. For the construction of vector plasmid containing shRNA-coding sequence against p53 (Brummelkamp *et al.*, 2002), oligonucleotides 5/6 and 7/8 were used for cloning into the *Cla*I and *Xba*I sites of pAdHM4-hU6a or the *Swa*I and *Xba*I sites of pAdHM4-hU6b, generating pAdHM4-hU6a-p53 and pAdHM4-hU6b-p53, respectively. The target sequence for siRNA is bp 775 to 793 of human p53 cDNA.

The original intact hU6 promoter sequence, derived from an *Eco*RI/*Sal*I fragment of piGene hU6 (iGENE Therapeutics, Tsukuba, Japan), was also introduced into the *Sph*I and *Sal*I sites of pHM5 (Mizuguchi and Kay, 1999), resulting in pHM5-ihU6. pHM5-ihU6 was then digested with *Sal*I and *Xba*I, and ligated with oligonucleotides 9 and 10, resulting in pHM5-hU6. In this case, oligonucleotides 11/12 and 13/14 (for the shRNA-coding sequence against luciferase and p53, respectively) were introduced into the *Bsp*MI site of pHM5-hU6 according to the report of Miyagishi *et al.* (2004) and the manufacturer's instructions (iGENE Therapeutics); and then an siRNA expression cassette was inserted into the E1-deletion region of pAdHM4 (Mizuguchi and Kay, 1998), using the I-*Ceu*I and PI-*Sce*I sites, resulting in pAdHM4-hU6-Lu and pAdHM4-hU6-p53, respectively. The sequence was verified with a DNA sequencer (ABI PRISM 310: Applied Biosystems, Foster City, CA).

Viruses (Ad-hU6-Lu, Ad-hU6a-Lu, Ad-hU6b-Lu, Ad-hU6-p53, Ad-hU6a-p53, and Ad-hU6b-p53) were prepared by the

TABLE 1. OLIGONUCLEOTIDES USED IN THE PRESENT STUDY

Oligonucleotide	*Sequence of oligonucleotide (5′–3′)*
hU6-S1 primer	aaggtcgggcaggaagagggccta
hU6-AS1 primer	ggtctagaagtatcgatttcgtctttccacaagatatat (*Xba*I and *Cla*I recognition sequences are underlined and italicized, respectively)
hU6-AS2 primer	ggtctagaagtatttaaatcgtcctttccacaagatatataa (*Xba*I and *Swa*I recognition sequences are underlined and italicized, respectively)
Oligonucleotide 1	cgacgctgagtacttcgaaatttcaagagaatttcgaagtactcagcgttttttggaaat (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 2	ctagatttccaaaaaacgctgagtacttcgaaattctcttgaaatttcgaagtactcagcgt (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 3	ccacgctgagtacttcgaaatttcaagagaatttcgaagtactcagcgttttttggaaat (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 4	ctagatttccaaaaaacgctgagtacttcgaaattctcttgaaatttcgaagtactcagcgtgg (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 5	cggactccagtggtaatctacttcaagagagtagattaccactggagtctttttggaaat (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 6	ctagatttccaaaaagactccagtggtaatctactctcttgaagtagattaccactggagtc (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 7	ccgactccagtggtaatctacttcaagagagtagattaccactggagtctttttggaaat (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 8	ctagatttccaaaaagactccagtggtaatctactctcttgaagtagattaccactggagtcgg (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 9	tcgacctgcaggcatgcaagcttc (BspMI recognition sequences are underlined)
Oligonucleotide 10	ctaggaagcttgcatgcctgcagg (BspMI recognition sequences are underlined)
Oligonucleotide 11	caccacgctgagtacttcgaaatttcaagagaatttcgaagtactcagcgtttttt (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 12	gcataaaaaacgctgagtacttcgaaattctcttgaaatttcgaagtactcagcgt (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 13	caccgactccagtggtaatctacttcaagagagtagattaccactggagtcttttt (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)
Oligonucleotide 14	gcataaaaagactccagtggtaatctactctcttgaagtagattaccactggagtc (loop sequences and siRNA-coding sequence are underlined and italicized, respectively)

transfection of a *Pac*I-linearized vector plasmid (pAdHM4-hU6-Lu, pAdHM4-hU6a-Lu, pAdHM4-hU6b-Lu, pAdHM4-hU6-p53, pAdHM4-hU6a-p53, and pAdHM4-hU6b-p53, respectively) into 293 cells as described previously (Mizuguchi and Kay, 1998). Ad vectors containing only the original intact hU6 promoter sequence (without a target sequence; Ad-hU6) were similarly constructed with pHM5-hU6 and pAdHM4. The virus was purified by $CsCl_2$ gradient centrifugation; dialyzed with a solution containing 10 m*M* Tris (pH 7.5), 1 m*M* $MgCl_2$, and 10% glycerol; and stored in aliquots at −70°C. Determination of virus particle (VP) titers and infectious titers was accomplished spectrophotometrically by the method of Maizel *et al.* (1968) and with an Adeno-X rapid titer kit (Clontech), respectively. The infectious titer-to-particle ratio was 1:36 for Ad-hU6, 1:31 for Ad-hU6-Lu, 1:28 for Ad-hU6a-Lu, 1:24 for Ad-hU6b-Lu, 1:22 for Ad-hU6-p53, 1:12 for Ad-hU6a-p53, and 1:15 for Ad-hU6b-p53.

Adenovirus-mediated gene transduction and luciferase assay

A549 cells (2×10^5 cells) were seeded into a 12-well dish. The next day, they were transduced with the Ad vectors for 1.5 hr. Determination of luciferase production in the cells and extraction of cellular protein for Western blotting were performed after a 72-hr culture period. Luciferase production in the cells was measured with a luciferase assay system (PicaGene LT 2.0; produced by Toyo Ink [Tokyo, Japan] for Wako [Kyoto, Japan])

Western blotting for p53

Cell extracts were prepared in lysis buffer (25 m*M* Tris [pH 7.5], 1% Triton X-100, 0.5% sodium deoxycholate, 5 m*M* EDTA, 150 m*M* NaCl) containing a cocktail of protease inhibitors (Sigma, St. Louis, MO). The protein content was measured

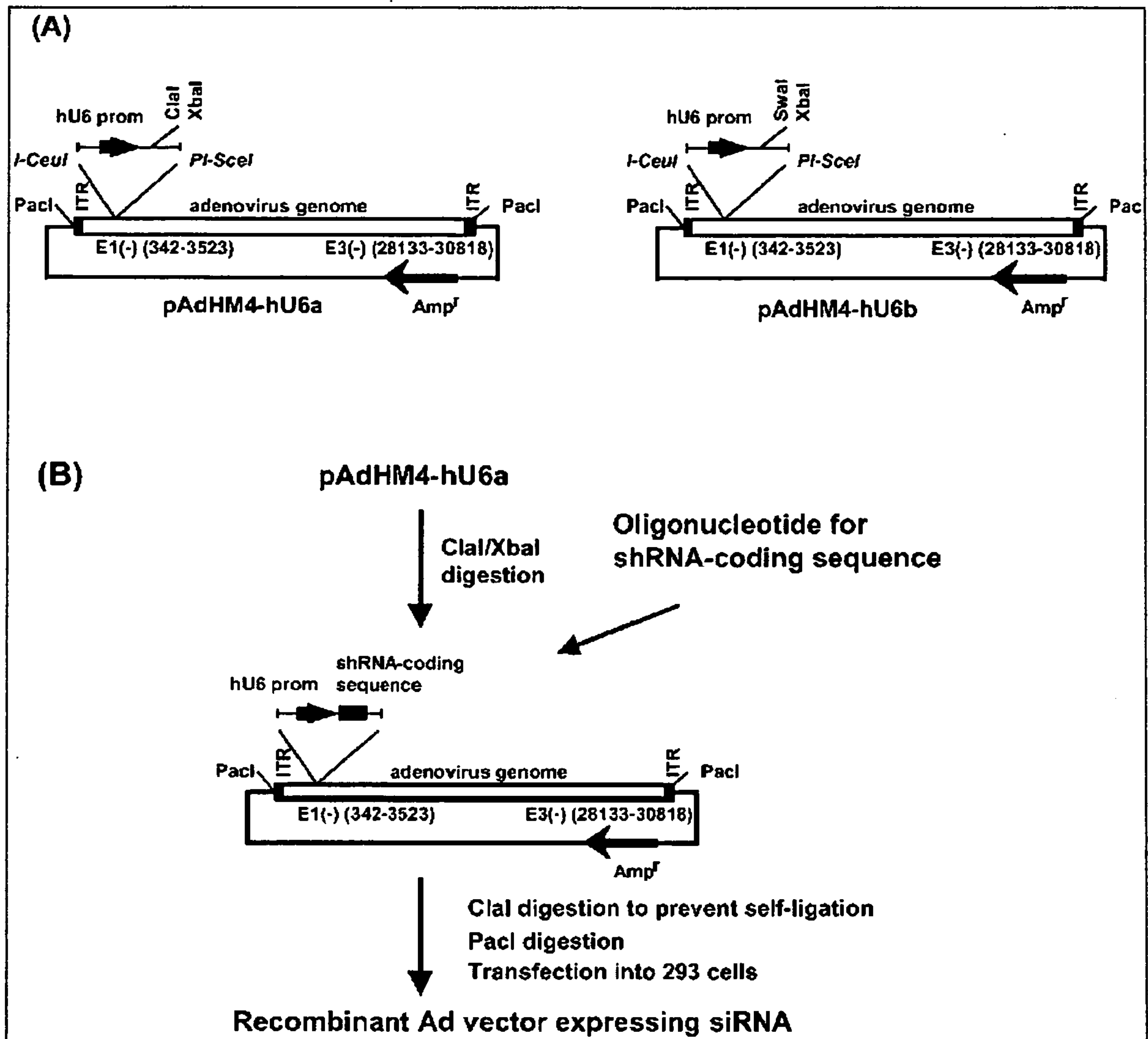

FIG. 1. Vector plasmids and the construction strategy for Ad vectors expressing siRNA. (**A**) Vector plasmids pAdHM4-hU6a and pAdHM4-hU6b. pAdHM4-hU6a contains a unique *Cla*I site at the transcription start site of the hU6 promoter sequence and an *Xba*I site downstream from the promoter sequence. pAdHM4-hU6b contains a unique *Swa*I site at the transcription start site of the hU6 promoter sequence and an *Xba*I site downstream from the promoter sequence. (**B**) Construction strategy for the Ad vector expressing siRNA. pAdHM4-hU6a was digested with *Cla*I/*Xba*I and ligated with oligonucleotides for the shRNA-coding sequence. Ligation products were then digested with *Cla*I to prevent the generation of nonrecombinant parental plasmid. The resulting plasmid was linearized by digestion with *Pac*I and transfected into 293 cells, generating recombinant Ad vectors expressing siRNA. pAdHM4-hU6b was similarly used.

with a Bio-Rad assay kit (Bio-Rad, Hercules, CA), using bovine serum albumin as the standard. Protein samples (10 μg) were electrophoresed on sodium dodecyl sulfate (SDS)– 12.5% polyacrylamide gels under reducing conditions, followed by electrotransfer to Immobilon-P membranes (Millipore, Bedford, MA). After blocking in nonfat dry milk, the filters were incubated with antibodies against p53 (Santa Cruz Biotechnology, Santa Cruz, CA) and actin (Oncogene Research Products/EMD Biosciences, San Diego, CA), followed by incubation in the presence of peroxidase-labeled goat anti-mouse IgG antibody (American Qualex Antibodies, San Clemente, CA) or peroxidase-labeled goat anti-mouse IgM antibody (Oncogene Research Products/EMD Biosciences). The filters were developed by chemiluminescence (ECL Western blotting detection system; GE Healthcare, Piscataway, NJ). The signals were read with an LAS-3000 (Fujifilm, Tokyo, Japan), and quantified with Image Gauge software (Fujifilm).

RESULTS AND DISCUSSION

Rapid and efficient construction of Ad vectors expressing siRNA offers the promise of using RNAi in the context of both gene function analysis and therapeutic applications. In the present study, we developed a simple method for constructing Ad vectors expressing siRNA, based on only one-step *in vitro* ligation. To do this, we first constructed an Ad vector plasmid containing the E1- and E3-deleted Ad genome and the hU6 pro-

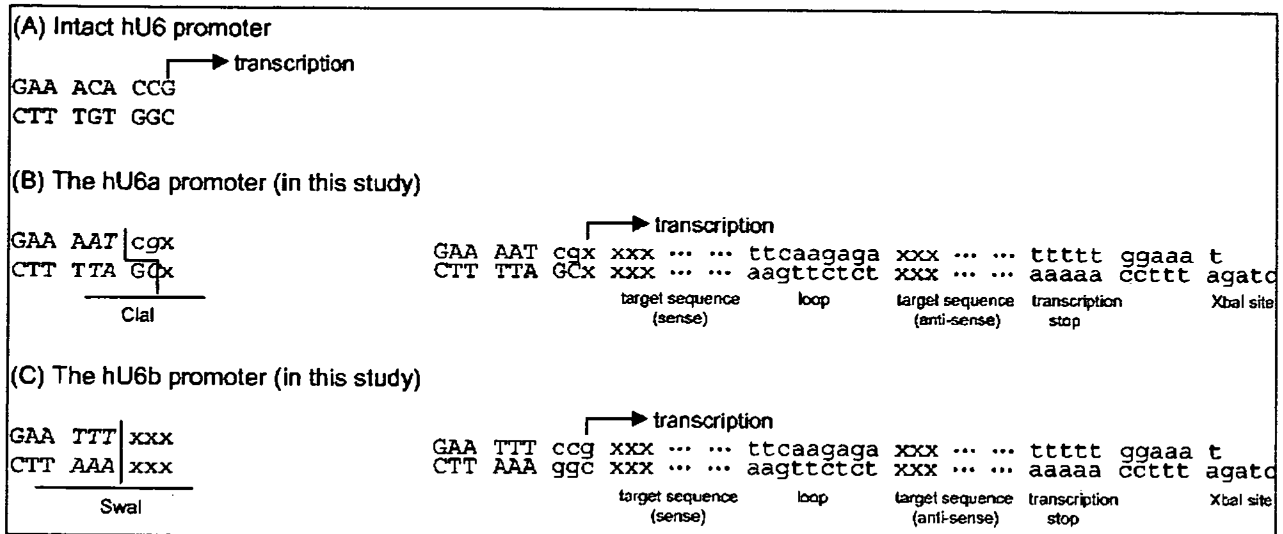

FIG. 2. Sequences at the transcription start site of the new hU6 promoter. (**A**) Intact hU6 promoter sequence. (**B**) hU6a promoter sequence. In this promoter, the *Cla*I site is placed at the transcription start site. (**C**) hU6b promoter sequence. In this promoter, a *Swa*I site is placed at the transcription start site. shRNA-coding oligonucleotides to be synthesized for each promoter are shown as lower-case letters on the right-hand side.

moter sequence in the E1 deletion region, pAdHM4-hU6a and pAdHM4-hU6b (Fig. 1A). By introducing the hU6 promoter sequence into the vector plasmid in advance, the cloning step of the gene of interest from the shuttle plasmid to the vector plasmid, which is an essential step in the conventional method for constructing Ad vectors (namely, the improved *in vitro* ligation method [Mizuguchi and Kay, 1998, 1999] and homologous recombination method in *E. coli* [He *et al.*, 1998]), can be skipped. To make it possible to directly clone the shRNA-coding oligonucleotides downstream of the hU6 promoter sequence, hU6 promoters containing unique restriction enzyme sites at the transcription start site have been developed. The new hU6 promoter sequences contain a *Cla*I or *Swa*I site around the transcription start site and an *Xba*I site downstream from the promoter (Figs. 1 and 2). These enzyme sites were selected because they do not cut the E1- and E3-deleted Ad genome. Because the transcription of shRNA might be influenced by the mutated sequences around the transcription start site, two types of hU6 promoters, differing by only a few nucleotides, were constructed. The hU6a promoter sequence contains a *Cla*I site, whereas the hU6b promoter sequence contains a *Swa*I site. *Cla*I, *Swa*I, and *Xba*I sites are unique in the vector plasmids pAdHM4-hU6a and pAdHM4-hU6b. To generate a recombinant vector plasmid for Ad vectors expressing siRNA, oligonucleotides for shRNA against the target gene were synthesized, annealed, and ligated with *Cla*I/*Xba*I-digested pAdHM4-hU6a or *Swa*I/*Xba*I-digested pAdHM4-hU6b. Oligonucleotides were designed so that recombinant vector plasmid containing the shRNA-coding sequence is redigested with *Xba*I, but not with *Cla*I or *Swa*I. By designing oligonucleotides like the one described above, the generation of self-ligated plasmid can be avoided by digestion of the ligation products with *Cla*I or *Swa*I. On the right side of Fig. 2, DNA sequences, including the shRNA-coding sequence around the transcription start site of the hU6 promoter, are shown. Oligonucleotides that must be synthesized for the shRNA-coding sequence are shown as lower-case letters. By using the method developed in the present study, we could easily generate Ad vectors expressing siRNAs against luciferase and human p53. More than 90% of the recombinant Ad vector plasmids contained the correct insert. Because the *Cla*I- (or *Swa*I-) and *Xba*I-digested pAdHM4-hU6a and pAdHM4-hU6b can be stored at −20°C, only the ligation-based introduction of oligonucleotides into these sites of the vector plasmid would be required for the construction of an appropriate vector.

To examine the function of Ad vectors expressing siRNA against luciferase (Ad-hU6a-Lu and Ad-hU6b-Lu), the efficiency of knockdown of luciferase expression in A549-Luc cells, which stably express luciferase, was examined by treatment with Ad-hU6a-Lu or Ad-hU6b-Lu (Fig. 3). Ad-hU6-Lu, in which the hU6 promoter contains the original intact sequence even after introduction of an shRNA-coding sequence, was used as a positive control. To generate Ad-hU6-Lu, the shRNA-coding sequence was first introduced downstream from the hU6 promoter sequence cloned into the shuttle plasmid, according to the report of Miyagishi *et al.* (2004) and the manufacturer's instructions (iGENE Therapeutics); the shRNA expression cassette was then introduced into the E1 deletion region of the Ad vector plasmid pAdHM4 (Mizuguchi and Kay, 1998). Transfection of a *Pac*I-digested vector plasmid into 293 cells generated Ad-hU6-Lu. Ad-hU6, which contains the intact hU6 promoter without the shRNA-coding sequence, and Ad-hU6-p53, which contains the intact hU6 promoter with the shRNA-coding sequence against human p53, were similarly constructed and used as negative controls. Data showed that Ad-hU6a-Lu and Ad-hU6b-Lu suppressed luciferase expression in A549-Luc cells as efficiently as Ad-hU6-Lu, in a dose-dependent manner (Fig. 3). Ad-hU6 and Ad-hU6-p53 showed no effects on luciferase expression. Ad-hU6a-p53 and Ad-hU6b-p53 (these Ad vectors are used in Fig. 4) also had no influence on luciferase expression (data not shown). The RNAi effect of luciferase expression was relatively weak compared with that of p53 (de-

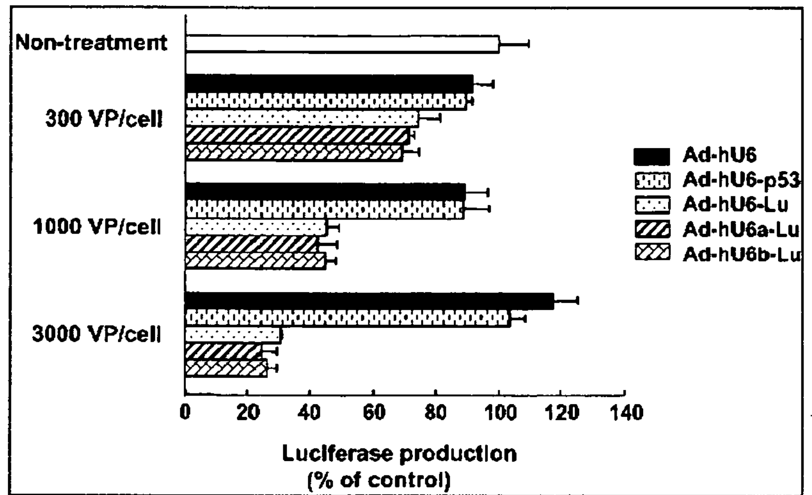

FIG. 3. Suppression of luciferase expression by Ad vector expressing siRNA. A549-Luc cells, which stably express luciferase, were transduced for 1.5 hr with Ad-hU6, Ad-hU6-p53, Ad-hU6-Lu, Ad-hU6a-Lu, or Ad-hU6b-Lu at 300, 1000, or 3000 VP/cell. After culturing for 72 hr, luciferase production in the cells was measured by luminescence assay. Data are expressed as means and SD (n = 4).

scribed below). This difference probably occurred because the A549-Luc cells were expressing luciferase from a strong viral promoter (SV40 promoter and enhancer) and because the levels of luciferase expression were higher than those of endogenous p53 expression.

We next examined the RNAi effect of the siRNA-expressing Ad vector generated in the present study on the endogenous gene. As a model, we silenced p53 expression in A549 cells (Fig. 4). Ad-hU6a-p53 and Ad-hU6b-p53 were generated, and Ad-hU6, Ad-hU6-Lu, and Ad-hU6-p53 were also used. Ad-hU6-p53 contains the intact hU6 promoter sequence, including the transcription start site, even after introduction of the shRNA-coding sequence. A549 cells were transduced with a 300- or 1000-VP/cell of each Ad vector, and cultured for 3 days. Levels of p53 expression were examined by Western blotting. Expression of actin was also measured as an internal control. Expression of p53 in A549 cells was efficiently decreased by treatment with Ad-hU6a-p53 and Ad-hU6b-p53 as well as with Ad-hU6-p53. Levels of p53 expression in cells treated with Ad-hU6-p53, Ad-hU6a-p53, or Ad-hU6b-p53 at 1000 VP/cell were decreased to 7, 2, and 5%, respectively, relative to cells treated with Ad-hU6, according to Image Gauge software (Fujifilm) (in the case of 300 VP/cell, they were decreased to 53, 24, and 30%, respectively). The efficiency of p53 silencing by treatment with Ad-hU6-p53 was slightly lower than that with Ad-hU6a-p53 or Ad-hU6b-p53. This reduced efficiency is likely due to the approximately 1.5 to 2 times lower infectious titer-to-particle titer ratio of Ad-hU6-p53 in comparison with those of Ad-hU6a-p53 and Ad-hU6b-p53. Ad-hU6 and Ad-hU6-Lu did not decrease the level of p53 expression (Fig. 4). These results indicate that new hU6 promoters containing *Cla*I or *Swa*I sites at the transcription start site should transcribe as efficiently as the original hU6 promoter, and that Ad vectors containing the new hU6 promoters efficiently silence target gene expression. Different vector systems (pAdHM4-hU6a and pAdHM4-hU6b) should be used according to the specific purpose.

To facilitate the construction of an siRNA expression plasmid, the U6 and H1 promoters, which contain *Apa*I, *Bbs*I, *Bgl*II, *Eco*RV, *Sal*I, and *Xba*I sites, etc., at the transcription start site, have been developed (Brummelkamp *et al.*, 2002; Lee *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; Sui *et al.*, 2002; Yu *et al.*, 2002; Boden *et al.*, 2003). All types of promoters worked efficiently, and could be widely used for efficient RNAi, although the efficiency (activity) of the mutated promoters described above has not been compared with that of the intact promoter. The present study clearly showed that the mutated hU6 promoter, at least one having a *Cla*I or *Swa*I site at the transcription start site and an *Xba*I site downstream of the promoter sequence, is similar in activity to the intact hU6 promoter and would not influence the function of the promoter.

The method using polymerase chain reaction (PCR)-based amplification of shRNA together with the U6 promoter followed by subsequent cloning of the complete expression cassette directly into the Ad vector genome is another strategy for one-step construction of recombinant Ad plasmids containing an siRNA expression cassette. In this method, however, the procedures described below are required for preparation of insert DNA: (1) ordering of the PCR primer, (2) PCR, (3) purification of the PCR product, (4) restriction enzyme digestion and purification of the PCR product, and (5) ligation. In our present system, only the following procedures are required: (1) ordering of the oligonucleotides, (2) hybridization of the oligonucleotides, and (3) ligation. Thus, the present method would be much easier and would allow any laboratory to easily construct

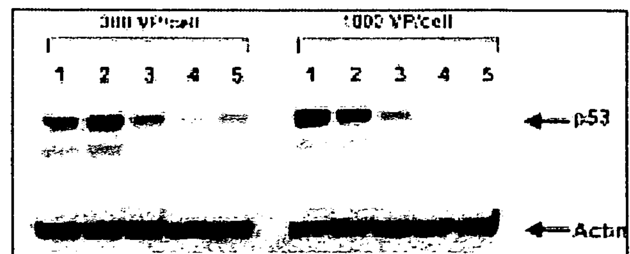

FIG. 4. Suppression of human p53 expression by Ad vector expressing siRNA. A549 cells were transduced for 1.5 hr with Ad-hU6 (lane 1), Ad-hU6-Lu (lane 2), Ad-hU6-p53 (lane 3), Ad-hU6a-p53 (lane 4), or Ad-hU6b-p53 (lane 5) at 300 or 1000 VP/cell, and then cultured for 3 days. Proteins were then extracted from the cells, and the levels of p53 expression were examined by Western blotting. The actin bands served as an internal control for equal total protein loading. The extra (lower) bands of p53 are nonspecific.