from an agarose gel and subcloned into pGEM® plasmid (Promega, Madison, WI, USA). PCR amplification of the non-truncated FADD sequence did not produce a deleted fragment (data not shown), supporting the idea that the artifact described in this study could arise from a GC skipping mechanism in the RT reaction.

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Concentration of Recombinant Baculovirus by Cation-Exchange Chromatography

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Baculovirus are a diverse group of viruses with a host range restricted to insects. They are large enveloped viruses with a genome of double-stranded circular DNA. Recombinant baculovirus (rBV) vectors, primarily derived from Autographa californica M nucleopolyhedrosis virus (AcMNPV), are commonly used for the high-level production of heterologous proteins in insect cells (8). Recently, it was demonstrated that rBV can be used to express foreign genes in mammalian cells (3.5). This previously unobserved characteristic makes rBV potentially useful in human gene therapy. The potential advantages of rBV for gene therapy are (i) a very large DNA insert capacity, (ii) a fairly high viral titer, (iii) lack of a pre-existing immune response in humans, (iv) lack of replication or toxicity in mammals and (v) lack of expression of viral genes in mammalian cells due to the insect-specificity of the baculovirus transcriptional promoters.

Several circumstances require purified and concentrated high-titer rBV. In large-scale production of proteins in insect cells, it would be useful to have the baculovirus inoculum concentrated to high titer in a small volume. In direct in vivo gene therapy applications, it will be necessary to have the rBV at high titer in a physiological solution. High-titer rBV concentrations can be made by ultracentrifugation through a sucrose cushion followed by banding in a sucrose gradient and subsequent centrifugation and resuspension in a small volume (for examples of the use of this conventional concentration step, see References 2 and 9). However, virus aggregation is a widespread problem, as rBV preparations made in this manner generally tend to be badly aggregated. I found that this enveloped virus aggregated whenever ultracentrifugation was performed. Also, gradient centrifugation cannot be readily scaled-up. Because of the ability to scale-up chromatographic procedures, purification of viruses by column chromatography is an attractive alternative to the use of gradients and has been used with other gene therapy vectors such as adenoviruses (6). This report describes the concentration of rBV in a single step by column chromatography. The resulting virus is present in a physiological buffer in a relatively unaggregated state.

Construction of baculovirus vectors, generation of virus stocks and assay of virus by plaque titering were all done by conventional methods (4,7,8). The baculovirus Z4 (3) was used in these studies. This virus was shown to express high levels of the β-galactosidase (β-gal) reporter protein after transduction of human hepatoma cells and primary rat hepatocytes. Z4 was produced in Sf9 insect cells (Invitrogen, Carlsbad, CA, USA) grown in serum-free medium (SF-900 II SFM; Life Technologies, Gaithersburg, MD, USA) in 100-mL spinner culture vessels (Corning Costar, Cambridge, MA, USA). Sf9 cells at a density of 2 × 10^6 cells/mL were infected at a multiplicity of infection of 0.2, and virus was harvested 4 days later. The use of serum-free medium is preferred so that the total amount of protein in the conditioned medium will be low. If the virus is produced in serum-containing medium, the high concentration of bovine serum albumin and other proteins might bind to the column and decrease the binding of the virus. However, this protocol has been used successfully to concentrate rBV from serum-containing medium. The cells and debris were pelleted by centrifugation of the conditioned medium in a Model J-6M Centrifuge (Beckman Coulter, Fullerton, CA, USA) at 2500 rpm (1780×g) for 15 min at 4°C. The
supernatant, which contains the virus, was removed and either used immediately or stored at 4°C in the dark for later purification.

A strong cationic exchanger, the SP Sepharose® Fast Flow Column (Catalog No. 17-0729-01; Amersham Pharmacia Biotech, Piscataway, NJ, USA), was used to concentrate Z4. The virus binds to this column at low pH and readily elutes at a higher pH. The conditioned medium from the Z4-producing insect cells was acidic (pH 5.8–6.0). If a higher pH medium is used for virus production, the pH of the conditioned medium could be lowered by the addition of 1 M 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.8, to a final concentration of 25 mM just before loading the virus onto the column. Since this pH is close to that required to activate the fusion activity of the envelope protein gp64, a pH significantly lower than 5.8 was never used.

In this example, a small-scale, 2-mL SP column was used. The SP column was prepared according to the manufacturer’s instructions. All column steps took place at 4°C. Briefly, a small volume (ca. 6 mL) of SP Sepharose was withdrawn from the original container. Since the SP Sepharose was supplied in a pre-swollen 20% ethanol, 0.2 M sodium acetate solution, the withdrawn solution was allowed to sit for approximately 30 min to allow the Sepharose to settle. The ethanol-containing liquid was then removed from above the SP column, a large excess of 25 mM MES, pH 5.8, was loaded onto the column, and four 0.6-mL fractions were sequentially harvested.

Table 1. Concentration of Baculovirus Z4 by SP Sepharose Column Chromatography

<table>
<thead>
<tr>
<th>Sample</th>
<th>Vol. (mL)</th>
<th>β-galactosidase U/mL</th>
<th>Total Units</th>
<th>Yield (%)</th>
<th>Virus Titer pfu/mL</th>
<th>Total pfu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z4, starting</td>
<td>40.0</td>
<td>1.00</td>
<td>40.00</td>
<td>-</td>
<td>1.4 x 10⁹</td>
<td>5.6 x 10⁹</td>
</tr>
<tr>
<td>Flow-through</td>
<td>40.0</td>
<td>0.14</td>
<td>5.60</td>
<td>14.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>MES wash</td>
<td>2.0</td>
<td>0.23</td>
<td>0.46</td>
<td>1.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PBS eluate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fraction 1</td>
<td>0.6</td>
<td>1.79</td>
<td>1.07</td>
<td>2.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>fraction 2</td>
<td>0.6</td>
<td>5.18</td>
<td>3.11</td>
<td>7.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>fraction 3</td>
<td>0.6</td>
<td>43.71</td>
<td>26.23</td>
<td>65.6</td>
<td>6.3 x 10⁹</td>
<td>3.8 x 10⁹</td>
</tr>
<tr>
<td>fraction 4</td>
<td>0.6</td>
<td>1.82</td>
<td>1.09</td>
<td>2.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>0.5 M NaCl</td>
<td>1.0</td>
<td>0.03</td>
<td>0.03</td>
<td>0.1</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Notes:
- Hep G2 cells were treated with equal volumes of starting virus and column fractions. β-gal activity of treated cells was determined. All values are expressed relative to the level of activity in the starting virus.
- Not determined.
- 2.5 mL PBS were loaded onto the column, and four 0.6-mL fractions were sequentially harvested.

was driven by the increase in pH. After loading the PBS, small-volume fractions equal to approximately one-third of the total column volume were collected. After the PBS elution, PBS containing NaCl at a final concentration of 0.5 M was loaded onto the column to determine if this higher salt concentration could be used to elute more virus. This concentration of NaCl was used since it was the highest salt concentration that did not result in appreciable virus inactivation (data not shown).

Virus in the flow-through and elution fractions was assayed by transduction of the human hepatoma cell line Hep G2 (ATCC, Rockville, MD, USA) and quantitation of β-gal expression levels using a chemiluminescent β-gal assay. Cells were seeded in multiple wells of 6-well plates and treated with varying volumes of virus. On the next day, cell lysates were taken, and β-gal activity was quantitated by a Luminescent β-gal Detection Kit II (CLONTECH Laboratories, Palo Alto, CA, USA) according to the manufacturer’s instructions. The starting virus and concentrated virus titers also were quantitated using a conventional plaque-forming assay in SF9 cells (4.8). X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Sigma, St. Louis, MO, USA) was added to the agarose overlay at a final concentration of 0.15 mg/mL to allow for easier plaque detection. At seven days postin-
Benchmarks

Table 1 shows the results of one column-chromatography experiment. The numbers in Table 1 are β-gal levels relative to the starting virus, which was adjusted to 1.0. There was no detectable β-gal activity in cells treated with the column wash before the loading of virus (data not shown). Most of the loaded virus bound to this column. At ratios of virus load to total column volume of more than 20:1, a greater proportion of the virus could be found in the flow-through (data not shown).

The Z4 baculovirus was eluted from the column in one sharp peak, with most of the virus present in the third 0.6-mL fraction eluted. The peak of virus elution could be determined visually, because the eluate changed from perfectly clear to translucent when the high-titer virus came off the column. The titer of this concentrated virus was more than 40-fold above the starting virus titer. Quantitation by plaque assay correlated very closely to the quantitation by transduction of Hep G2 cells (Table 1). The total amount of virus in this fraction was 65.6% of the total starting virus. Most of the virus was removed from the column by the PBS elution, because the total amount of virus in all of the flow-through and elution fractions was equal to 94% of the starting virus. Treating the column with a PBS/0.5 M NaCl solution did not elute any more virus. The pH of the elution buffer was critical, since a pH gradient showed that virus remained bound to the column at pH values ≤ 7.0, began to elute at pH 7.2, and was removed from the column completely at pH 7.4 (data not shown).

An advantage of using an SP column is that only a low percentage of total protein (ca. 10%) will bind to this column. Thus, this one step will produce a concentrated rBV with very little contaminating protein. Since this is a physiological buffer, the eluted fraction can be directly added to cultured cells or injected into animals.

As stated above, purification of rBV by ultracentrifugation and sucrose gradient banding tended to cause aggregation of the virus. To assay virus aggregation, we performed a low-speed centrifugation at 7000 rpm (4000×g) for 6 min in a Model 5415C Table-Top Microcentrifuge; Eppendorf, Madison, WI, USA) followed by a β-gal assay of Hep G2 cells treated with samples of the supernatant. We found that rBV concentrated by ultracentrifugation was virtually completely pelleted by this low-speed spin (99.9% of virus in the pellet), indicating that it was aggregated. The presence of aggregates was confirmed by electron microscopy (data not shown). In contrast, column chromatography did not cause any apparent increase in virus aggregation, as the percentage of virus in the supernatant following centrifugation of the concentrated virus was roughly the same as that in the starting virus (ca. 75% of the virus was in the supernatant; data not shown).

Although the degree of virus concentration varies somewhat, yields in the range of 25-fold to 60-fold concentration are routinely achieved with Z4 and other rBV having the native baculoviral coat. Slight variability in the yield could be because of differences in starting viral titer and column geometry (long thin columns are superior to short fat ones). Recently, baculovirus having the vesicular stomatitis virus G glycoprotein in its viral envelope was generated (1). This virus, VGZ3, displays increased transduction capability in several cell types. SP Sepharose column chromatography of VGZ3 resulted in only an approximately 10-fold concentration of virus. The yield of VGZ3 was lower than Z4 due to the inability to remove all of the VGZ3 from the column. Better concentration of VGZ3 was achieved by eluting the virus from the SP column with a higher salt buffer (PBS/0.3 M NaCl) or by use of a weaker cationic-exchange CM Sepharose Fast Flow column (Catalog No. 17-0719-01; Amersham Pharmacia Biotech).

A protocol for the concentration of baculovirus is presented. The method is an easy and rapid single-step purification using column chromatography.
This protocol has advantages over other methods in that no ultracentrifugation is required, the virus does not aggregate appreciably during concentration, and the method can be readily scaled-up.

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Improved LM-PCR Procedure for In Vivo Footprinting Analysis of GC-Rich Promoters

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The mechanisms that lead to transcriptional control of promoters by DNA-binding proteins are usually determined by in vitro techniques such as electrophoretic mobility shift analysis (EMSA), in vitro footprinting and mutational analyses of reporter constructs. However, in many cases, these analyses do not accurately reproduce the true in vivo situation of endogenous promoters in the context of the cellular chromatin structure.

In vivo footprinting analysis of genomic DNA has been developed to address this issue. This type of analysis visualizes, at single-nucleotide resolution, DNA-protein contacts occurring in living cells, thus providing critical information on the presence of transcription factors on endogenous promoters. Currently, several protocols are available to perform this analysis (for examples, see References 1,3,5–7,9). All of them involve two steps: (i) in situ modifications of genomic DNA performed on cell cultures, tissues, whole embryos etc. with chemical, enzymatic or physical agents (e.g., methylaing agents, DNase I or UV irradiation, respectively) whose action on DNA is disturbed by the presence of DNA-bound proteins; and (ii) visualization of the DNA residues protected by proteins within a single-copy gene of interest. So far, the most sensitive technique used for step 2 involves a combination of ligation-mediated primer extension reaction (LM-PCR) amplification and genomic sequencing procedures that are performed with sets of primers specific for the studied gene (3,5,6). However, the success of this long and difficult technique is highly dependent on the GC-composition of the gene under study. Indeed, GC-rich, single-copy sequences are difficult to amplify by conventional PCR techniques, possibly due to rapid renaturation of the template and very stable secondary structures which cause elongation blocks during amplification by DNA polymerase. We repeatedly faced this problem during the study of cell cycle-regulated genes, which have promoters that are often highly GC-rich. To resolve this issue, we modified several steps of conventional in vivo footprinting protocols (1,3,5–7,9). Notably, we introduced dimethyl sulfoxide (DMSO) all along the procedure since this compound had been previously shown to improve PCR amplification of GC-rich DNA sequences by decreasing the rapid renaturation of GC-rich templates (8,10). Moreover, to reduce the duration of an experiment that usually requires two to three days with conventional protocols, we also used a quick-ligation step (4) and a RoboCycler® Gradient Temperature Cycler (Stratagene, La Jolla, CA, USA) allowing us to optimize and perform a complete experiment in 1.5 days.

To assess the efficiency of this protocol for analyzing GC-rich sequences, we used it to visualize in vivo DNA-protein contacts on the promoter of the human cyclin E gene (2) in exponentially growing MCF7 breast cells and in human IMR 90 fibroblasts. The investigated sequence is located -384/-175 nucleotides (nt) relative to the transcription initiation site, is 83% GC-rich (Figure 1A) and is “resistant” to analysis by standard genomic footprinting procedures (e.g., Figure 1B, lanes 1 and 2).

Living cells and control naked genomic DNA were treated with the guanosine methylating agent dimethyl sulfate (DMS; Sigma, St. Louis, MO, USA) (3,5,6). 2 × 10^6 cells per 14-cm diameter dish were treated with DMS at 0.2% for 5 min at room temperature in their cell culture medium (Dulbecco’s modified Eagle medium [DMEM]/fetal calf serum [FCS]) buffered with HEPES (20 mM final), pH 7.4. After DMS treatment, cells were washed 3× with cold PBS/2% β-mercaptoethanol and then collected in 1 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1% sodium dodecyl sulfate [SDS], 2% β-mercaptoethanol). Genomic DNA was isolated by three gentle extractions with phenol (pH 8.0) followed by two precipitations in 4 M ammonium acetate with 3 vol of absolute ethanol (with 70% EtOH washes). DNA was then redissolved in 1 mL of water. As a reference, genomic DNA