Retroviral-Mediated Transfer and Amplification of a Functional Human Factor VIII Gene

By David I. Israel and Randal J. Kaufman

Hemophilia A results from a deficiency in factor VIII (FVIII), a cofactor in the intrinsic pathway of blood coagulation. As an approach toward genetic therapy of this disease, we constructed a retroviral vector encoding human FVIII and a selectable and amplifiable genetic marker, human adenosine deaminase (Ada). A retrovirus packaging line was transfected with this vector and stable transformants were selected for Ada expression. Isolated transformants produced both FVIII activity in the conditioned medium and retrovirus capable of transferring the Ada selectable marker and FVIII expression to mouse 3T3 fibroblasts. Selection of virus-producer cell lines for increasing levels of Ada expression yielded a 20-fold increase in both FVIII expression and viral titer. Similarly, selection of infected 3T3 fibroblasts for Ada gene amplification yielded a 20-fold increase in FVIII expression. The results demonstrate the feasibility of retrovirus-mediated transfer of human FVIII, and also the utility of selection for gene amplification to increase retrovirus titers in producer cell lines as well as expression levels in infected cells.

Expression of FVIII activity in CHO cells containing amplified copies of the FVIII cDNA gene is reduced to 3 orders of magnitude relative to the expression of other recombinant proteins. Inefficient expression of FVIII can be accounted for by low levels of messenger RNA (mRNA) and inefficient processing and secretion of the primary translation product. In addition, accumulation of FVIII activity in the conditioned medium requires von Willebrand factor to promote stable secretion from the cell. FVIII molecules, which contain deletions within the B-domain, retain in vivo and in vitro procoagulant activity.

Although expression of B-domain-deleted FVIII is somewhat more efficient, it is still subject to the same factors that limit expression of the wild-type molecule. In this report we demonstrate the feasibility of retroviral transfer of functional FVIII.

MATERIALS AND METHODS

Cells and culture conditions. 3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum. Selection for adenosine deaminase (Ada) expression was in the same medium supplemented with 4 μmol/L xylol-A (4-XA) (obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment and Chemistry, National Cancer Institute, Bethesda, MD) and the indicated concentrations of 2'- deoxyuridine (Sigma, St Louis, MO). PA317 cells were obtained from Dusty Miller (Fred Hutchinson Cancer Research Center, Seattle, WA). The derivation and growth of CHO cell line LA3-5, which expresses B-domain-deleted FVIII, was previously described.

Construction of the retroviral expression vector. The starting retroviral vector was pAFVXM. pAFVXM is virtually identical to pEVX except that it lacks the Moloney murine leukemia virus splice donor site, and has more unique cloning sites (Michael Kriegler, personal communication, April 1985). The BglIII site within pAFVXM was converted to an Xhol site using synthetic linkers, yielding pAFVXM-Xho. The PvuII to HinFl fragment from pSV2Ada, containing the SV40 early promoter and human Ada cDNA, was ligated into the Clal site of pAFVXM-Xho. Before ligation, the CiaI and HinFl ends were rendered blunt with the Klenow fragment of DNA polymerase I in the presence of deoxyxynucleotide triphosphates. The desired product (pAFVXM-SVAda) contains the SV2Ada transcription unit in the same orientation as the long terminal repeat (LTR) promoter. The Xhol to SalI fragment encoding the LA B-domain-deleted FVIII was cloned into the Xhol site of pAFVXM-SVAda in the same orientation as Ada to yield pRetroLA-SVAda (Fig 1). All plasmids were checked.
Fig 1. Construction of the pRetroLA-SVAda vector used to generate FVIII retroviruses. The retroviral expression vector was generated from pAFVXM, pSV2Ada, and pLA-MT as detailed in Materials and Methods. The product of each step was verified by extensive restriction endonuclease analysis. Transcription from the promoters contained within the 5' LTR and the SV40 fragment is indicated by the horizontal arrows.

Transfection. PA317 cells were transfected by protoplast fusion as previously described. Two days later cells were selected for Ada expression as described above and independent stable derivatives were termed LAR.

Viral infections. LAR cells were plated at approximately 25% confluence in 10-cm tissue culture plates. The next day, the medium was replaced with 5 mL fresh medium. The conditioned medium was passed through a 0.45 μm filter 24 hours later and used as the viral stock. 3T3 cells were grown in 10-cm tissue culture plates and incubated with the viral stocks in complete medium supplemented with 8 μg/mL polybrene in a final volume of 3 mL. After 2 hours, 7 mL of medium was added. Cells were allowed to incubate an additional 48 hours, and then subcultured (1:10) into Ada selective medium. Colonies were counted 12 to 14 days later.

Analysis of FVIII expression. Intracellular and secreted forms of FVIII were analyzed after labeling cells with [35S]-methionine as previously described. Cell extracts and conditioned medium were immunoprecipitated with an MoAb (F8-1.5.6) specific to the heavy chain of human FVIII. Immunoprecipitates were electrophoresed through an 8% polyacrylamide gel under reducing conditions and analyzed by fluorography. FVIII activity was quantitated in 24-hour conditioned medium from near-confluent cultures by measuring the FVIII-dependent generation of factor Xa from factor X (Kabi Coatest method).

Analysis of DNA and RNA. Total cellular RNA and high molecular weight cellular DNA were prepared as previously described from 3T3 cells, PA317 cells, and derivatives selected for Ada and FVIII expression. For Northern blot analysis, 10 μg/lane of RNA was electrophoresed through a 1% agarose/formaldehyde gel. DNA was digested with BamHI restriction endonuclease and electrophoresed through a 0.8% agarose gel (10 μg/lane). DNA and mRNA were transferred to nitrocellulose filters and hybridized to either FVIII or Ada cDNA fragments that were labeled by nick-translation.

RESULTS

Construction of retroviral vectors and packaging cell lines. Due to the size limitation for packaging retroviral genomes, a truncated form of the FVIII cDNA (LA VIII) was used in the derivation of the retrovirus. In addition to the size advantage, expression of LA VIII is also more efficient than the wild type molecule. LA VIII contains an in-frame deletion of 2,640 nucleotides and codes for a functional 130-Kd protein by both in vitro and in vivo activity analysis.

The plasmid pRetroLA-SVAda was constructed from the pAFVXM retroviral vector as indicated in Fig 1. In this vector, the truncated form of the human FVIII cDNA gene (LA VIII) is flanked by an Abelson leukemia virus/Harvey sarcoma virus hybrid 5' LTR and a Moloney leukemia virus/Abelson leukaemia virus hybrid 3' LTR (15, Michael Kriegler, personal communication, April 1985). A second transcription unit, which uses the SV40 early promoter to transcribe a human ADA cDNA gene, was introduced for selection and amplification of the plasmid and virus in recipient cells. The Ada and FVIII transcription units presumably use the same polyadenylation signal contained within the 3' LTR.

Bacterial protoplasts containing pRetro LA-SVAda were prepared and fused to PA317 cells, a 3T3-derived cell line that provides in trans the necessary components to package retroviral genomes. Transfected cells were selected for...
expression of Ada by growth in xyol-A and 5 nmol/L or 10 nmol/L dCF.\textsuperscript{12} Control-treated cells that did not receive bacterial protoplasts were killed on propagation in 5 nmol/L dCF. Individual clones, termed LAR (c1-c17, where each number represents a unique clone) were isolated and expanded.

All LAR clones produce FVIII activity in the conditioned medium as monitored by a chromogenic assay for factor Xa generation (Table 1). The FVIII activity was inhibited by a human anti-FVIII neutralizing MoAb (F8.1.5.6). No FVIII activity was detected in conditioned medium obtained from the original PA317 packaging line. Because all clones that exhibit the dCF resistance phenotype also express FVIII activity, it is unlikely that the plasmid DNA has undergone rearrangement during integration.

**Infection of 3T3 cells and amplification of virus titers.** Several LAR clones were tested for the production of virus capable of transferring the Ada marker to 3T3 cells. Infected and mock-infected cells were selected for Ada expression by growth in xyol-A and dCF. The results (Table 1, top) show that several LAR clones produce viruses capable of transferring the drug-resistant phenotype to 3T3 cells. Individual clones or pools of infected 3T3 cells, termed 3A8 (the number after 3A8 indicates which LAR packaging line was used to generate retrovirus; c or p indicates a clone or pool of infected cells, respectively), were isolated and expanded for further analysis.

To increase virus titer in the LAR cell lines, we selected for increased Ada expression. The packaging line LARc17, selected and grown initially in 5 nmol/L dCF, was sequentially selected in 100 nmol/L and 1,000 nmol/L dCF. A stringent selection occurred at each concentration of dCF. LARc17 was selected for dCF resistance in the presence of hypoxanthine, aminopterin, and thymidine (HAT) to select for expression of retrovirus packaging functions. In the cells selected for increased Ada expression, there is a proportional increase in FVIII activity and viral titer (Table 1, bottom). These results show the titer of virus containing Ada can be amplified by selection for resistance to increasing concentrations of dCF. When amplification of virus titers was attempted in the absence of HAT, no increase in virus titer was observed, although FVIII expression levels increased in the infected cells. This finding is consistent with a loss in the retroviral packaging functions that can occur on prolonged propagation of a retroviral packaging cell line.\textsuperscript{22}

**Analysis and amplification of FVIII expression in infected 3T3 cells.** 3A8 clones were initially analyzed for their ability to produce FVIII activity. The results (Table 2) show that several 3A8 clones selected for Ada expression also produce FVIII activity. Thus, recombinant retroviruses are capable of transmitting a functional gene encoding human FVIII. Two of three clones (3A8c1 and 3A8c3) infected with virus from the LARc8 line produced no detectable FVIII activity (Table 2). The infected cells, which do not express FVIII but are resistant to dCF, may result from either infection by a subpopulation of viruses from the packaging line that have deleted the FVIII gene, or from a rearrangement of the viral genome in recipient 3T3 cells on infection.

The 3A8 clones that express FVIII activity were selected for growth in increasing concentrations of dCF. In the 3A8c4 cell line, cells resistant to 100 nmol/L dCF produce approximately 20-fold more FVIII activity than do the initial infected cells selected for growth in 10 nmol/L dCF (Table 2). Further selection for cells resistant to higher concentrations of dCF failed to produce higher amounts of FVIII. This

### Table 1. Generation of Cell Lines That Produce FVIII Transducing Retroviruses

<table>
<thead>
<tr>
<th>Sample</th>
<th>3T3 Cells in [dCF] nmol/L</th>
<th>Virus Titer (\dagger) Colonies of Infected 3T3 Cells in [dCF] nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5.0</td>
</tr>
<tr>
<td>PA317</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LARc1 (10)</td>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
<td>LARc1 (5)</td>
<td>0.3</td>
<td>1</td>
</tr>
<tr>
<td>LARc8 (5)</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>LARc12 (5)</td>
<td>0.6</td>
<td>1</td>
</tr>
<tr>
<td>LARc17 (5)</td>
<td>0.1</td>
<td>3</td>
</tr>
<tr>
<td>LARc17 (6)</td>
<td>2.9</td>
<td>ND</td>
</tr>
<tr>
<td>LARc17 (100)</td>
<td>27.2</td>
<td>ND</td>
</tr>
<tr>
<td>LARc17 (1,000)</td>
<td>55.7</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(\dagger\) Virus stocks from LAR cells or parental PA317 cells were prepared from 24-hour conditioned medium and passed through 0.45-\mu m filters. 3T3 cells were infected with 2 mL virus stock and 1 mL medium containing polybrene, and were subcultured into selective medium as described in Materials and Methods. The total number of drug-resistant colonies at each concentration of dCF were counted on single plates (top) or three plates (bottom) 12 to 14 days later. Cells initially selected for resistance to 5 nmol/L dCF were subsequently grown in 10 nmol/L dCF (see Table 2).

**Abbreviation:** ND, not determined.

\(\dagger\) Logarithmically growing cells were removed from selective medium and fed fresh medium. After 24 hours, conditioned medium was assayed for FVIII activity in the absence or presence of an FVIII MoAb (F8.1.5.6) that neutralizes FVIII activity. FVIII activity was determined by the Kabi-Coastest method.
Table 2. Amplification of FVIII Expression in Virally Infected Cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>dCF (nmol/L)</th>
<th>VIII Activity (mU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3 (uninfected)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3AB.17cl</td>
<td>10</td>
<td>3.0</td>
</tr>
<tr>
<td>3AB.17cl</td>
<td>100</td>
<td>3.2</td>
</tr>
<tr>
<td>3AB.17c2</td>
<td>10</td>
<td>3.8</td>
</tr>
<tr>
<td>3AB.17c2</td>
<td>100</td>
<td>3.9</td>
</tr>
<tr>
<td>3AB.8c1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>3AB.8c3</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>3AB.8c4</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>3AB.8c4</td>
<td>100</td>
<td>296</td>
</tr>
<tr>
<td>3AB.8c4</td>
<td>3,000</td>
<td>338</td>
</tr>
</tbody>
</table>

Clones of 3T3 cells infected with virus from LARc8 (3AB.8) or LARc17 (3AB.17) were grown in medium containing 4 μM xyol-A and the indicated concentrations of dCF (see Table 1). Conditioned medium was harvested and assayed as described in Table 1.

may result from the xyol-A + dCF selection protocol, in which only limited amplification of Ada genes is obtained due to deletion of adenosine kinase activity. Greater amplification has been obtained with other cell lines using an alternative protocol in which cells are selected for resistance to adenosine, alanosine, and uridine (11AAU), and increasing concentrations of dCF. However, we have been unsuccessful in obtaining either PA317 cells, LAR packaging cells, or 3T3 cells that can grow in 11AAU, even in the absence of dCF.

Southern blot analysis of DNA showed no detectable rearrangement of the expression vector after protoplast fusion into PA317 cells. The restriction pattern of integrated plasmid DNA is indistinguishable from the starting plasmid after hybridization to either FVIII (Fig 2A, lanes 3, 8, and 12) or Ada probes (data not shown), consistent with head-to-tail integration of a large number of plasmid multimers on protoplast fusion. DNA from the LARc17 packaging line was analyzed in more detail due to the unexpectedly high gene copy number seen in this and other LAR cell lines. Southern blot analysis of Hirt supernatant DNA versus high molecular weight chromosomal DNA indicated the vector is present predominantly within chromosomal DNA (data not shown). Furthermore, the methylation pattern, as determined by DpnI and MboI restriction enzyme sensitivity,
demonstrated that the retroviral DNA is unmethylated, ruling out the possibility of plasmid DNA contamination of the cellular DNA preparations (data not shown). Thus, PA317 cells selected for resistance to 5 nmol/L dCF contain approximately 1,000 integrated copies of the pRetroLA-SVAda vector arranged in a head-to-tail tandem array.

Infection of 3T3 cells with virus from LARc8 or LARc17 yields cells containing one or two copies of the retroviral genome. Each infectant shows a unique 5' and 3' integration site, as shown with FVIII (Fig 2B) or Ada probes (data not shown), respectively. In addition, each transfected or infected cell line contains an internal 2.8-kilobase (kb) LA-VIII BamHI fragment migrating slightly above a doublet of 2.5-kb. The 2.5-kb doublet presumably results from hybridization of the human FVIII cDNA probe to the mouse FVIII gene, since it is also present in 3T3 and PA317 cells (Fig 2B, lanes 1 and 2). Comparison of 3A8.8c4 in 10 nmol/L dCF versus 100 nmol/L dCF (Fig 2B, lanes 6 and 7) demonstrates approximately a 10-fold amplification of the retroviral genome on selection for drug resistance. Interestingly, the LAR packaging lines contain approximately 1,000-fold more copies of the RetroLA-SVAda genome than do the 3A8 infectants. Because the packaging cell line and the infected cell lines have both been selected for resistance to similar concentrations of dCF, expression of the genome via retroviral infection appears much more efficient than by protoplast fusion. The differences in the restriction pattern and gene copy number between the LAR packaging lines and the 3A8 infectants show that the 3T3 cells were not contaminated by the packaging cell line during the infection process.

RNA analysis by Northern blot hybridization detects two transcripts in LAR clones and 3A8 infectants from the pRetroLA-SVAda vector that are not observed in the parental cell lines (Fig 3). Hybridization with an FVIII probe shows a single transcript of approximately 8 kb. Hybridization with a probe to human Ada shows a transcript of 8 kb and a second transcript of approximately 3 kb that is present at several hundred-fold higher levels. These transcripts are the sizes expected from expression of the intact retroviral vector (Fig 1). The band migrating at 28S may represent cross-hybridization of the probe to 28S ribosomal RNA. The source of the mRNA migrating slightly below 28S ribosomal RNA that hybridizes to the Ada probe is unknown. Selection for increasing Ada expression resulted in amplified expression of the FVIII mRNA (Fig 3, lanes 3 and 4, 7 and 8). The greater abundance of Ada mRNA from the internal SV40 promoter may result from higher transcription from this promoter relative to the LTR promoter, and/or from instability of the FVIII/Ada transcript. Previous analyses demonstrated that FVIII mRNA is produced at much lower quantities than other transcripts expressed from the same vectors. These analyses, together with Southern blot analyses (Fig 2), demonstrate that the retroviral genome is largely intact and is expressed in most of the infected 3T3 cells and packaging cell lines.

LA VIII protein produced by 3A8.8c4 (100 nmol/L dCF) was compared with that produced in CHO cells, in which the LA VIII gene was introduced by standard transfection procedures. The results (Fig 4) show that both the initial single-chain translation products (lanes 2 and 4) and processed secreted protein from the CHO (lane 10) and 3T3 (lane 12) cells are indistinguishable. A high molecular weight species of 200 Kd represents single-chain FVIII, which is obtained by expression of the LA deletion form of FVIII. In the conditioned medium (lanes 10 and 12), the processed heavy chain migrates as a heterogeneous species of approximately 90 Kd. The heterogeneity is due to glycosylation. The light chain of FVIII migrates as a doublet at 80 Kd. Comparison of the FVIII activity and the amount of radiolabeled FVIII in conditioned medium indicates that the specific activity of the protein secreted from CHO cells and 3T3 cells is similar. These results show that the LA human FVIII gene transmitted by a retrovirus infection into 3T3 fibroblasts is expressed as a functional protein and appears indistinguishable from LA FVIII produced in CHO cells from genes introduced by DNA transfection.

**DISCUSSION**

We used a derivative of the pAFVXM retrovirus to transmit a truncated human FVIII gene and an Ada select-
able marker into 3T3 cells. The results demonstrate that functional FVIII protein can be produced after stable transmission by retroviral infection. However, compared with results of other genes introduced into retroviruses, the titers obtained with the RetroLA-SVAda virus are very low. Several factors may account for the low titers of virus obtained with the RetroLA-SVAda vector. First, the amount of FVIII mRNA produced in both transient and stable expression systems is much lower than that obtained from other genes expressed in the same systems. In similar retroviral vectors that yield relatively high virus titers, the levels of full-length RNA transcribed from the LTR promoter are comparable with RNA transcribed from an internal SV40 promoter. Our results show the FVIII mRNA transcribed from the LTR promoter is reduced approximately 100-fold compared with the Ada mRNA transcribed from the SV40 promoter, and likely contributes to the low titers of virus reported here. Second, the expression level of Ada from the RetroLA-SVAda vector is marginally sufficient for producing the drug-resistant phenotype after either transfection or infection. For example, many noninfected 3T3 cells survive selection in 4-μmol/L dCF, but are completely killed by 5.0 μmol/L dCF. The number of drug-resistant colonies after infection of 3T3 cells decreases by 10-fold when the dCF concentration is increased from 5 μmol/L to 10 μmol/L (Table 1). Due to the narrow toxicity range of the drug selection, it is possible that some infected cells do not express enough Ada to survive 5 μmol/L dCF. This selection likely yields an underrepresentation of the virus titer. Finally, features of the retrovirus itself, such as an incomplete packaging site, may also contribute to the low titers reported here.

The levels of FVIII mRNA and secreted activity are similar in PA317 transfectants and 3T3 infectants selected for the same level of dCF resistance. However, the copy number of this vector is about 1,000-fold greater in the transfected cells. Therefore, the transcriptional activity per copy of the retroviral vector is much greater in infected versus transfected cells. A majority of the gene copies introduced by protoplast may not be expressed; alternatively, most copies of RetroLA-SVAda may be expressed at very low levels in the LAR transfectants. In either case, expression of the FVIII and Ada genes after retroviral infection is very efficient relative to expression of the same vector transfected by protoplast fusion. This result is consistent with previous observations.

The availability of a molecular clone for the human FVIII gene has lead to a tremendous advance in our understanding of the functional domains of this complex plasma glycoprotein. The availability of a high titer retrovirus capable of transferring an FVIII gene into a variety of cells will facilitate the studies of FVIII synthesis in heterologous cells. Such a virus will also make gene therapy of hemophilia A a future possibility. The viruses described here are not suitable for these purposes, but demonstrate the feasibility of retroviral gene transfer of a human FVIII gene and a gene encoding a dominant selectable amplifiable marker. Recently, Palmer et al. reported that the human factor IX clotting factor can be transferred into rodent and human fibroblasts using efficient retroviral vectors. Improvements in the design of FVIII-containing retroviral vectors will increase their utility in gene transfer experiments.

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**REFERENCES**


