Expression of Human Adenosine Deaminase From Various Strong Promoters
After Gene Transfer Into Human Hematopoietic Cell Lines

By Randy A. Hock, A. Dusty Miller, and William R.A. Osborne

Adenosine deaminase (ADA) deficiency is associated with a fatal severe combined immunodeficiency. Because most patients do not have a suitable marrow donor, the introduction of a normal ADA gene into the patient’s marrow cells is a potentially useful alternative therapy. To identify vectors that provide optimal gene expression in human hematopoietic cells, we investigated retroviral vectors containing the ADA gene under the transcriptional control of the promoter/enhancers of Moloney murine leukemia virus, the simian virus 40 early region, the cytomegalovirus immediate-early gene, the lymphotropic papovavirus, and the human β-globin gene. ADA expression from these vectors was monitored in the ADA⁻ human histiocytic lymphoma cell line DHL-9, and in the multipotential chronic myeloid leukemia cell line K562. ADA expression in infected K562 cells was also measured after induction of megakaryoblastic differentiation by phorbol ester, and after induction of erythroid differentiation by sodium n-butyrate or hemin. In these hematopoietic cell lines, the vectors that contained ADA controlled by either the Moloney murine leukemia virus promoter (LASN) or the cytomegalovirus promoter (LNCA) expressed ADA at much higher levels than the other vectors tested. Furthermore, in K562 cells infected with LASN and LNCA vectors, induction of terminal differentiation resulted in the same or higher level expression of ADA. These cell lines have permitted the evaluation of transduced gene expression in proliferating and differentiating hematopoietic cells that provide a model for bone marrow-targeted gene therapy.

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MATERIALS AND METHODS

Cell Culture. Hematopoietic cells were grown in RPMI supplemented with 10% fetal calf serum at 37°C in an atmosphere...
containing 5% CO2. Adherent cells were grown in Dulbecco's modified Eagle medium with high glucose (4.5 g/L) supplemented with 10% calf serum (F2 cells) or 10% fetal calf serum (all other cell lines) at 37°C in an atmosphere containing 10% CO2. Cell lines used included PA31722 (ATCC no. CRL 9078), PES01 (a retrovirus packaging cell line similar to PA317 but with an ecotropic host range (ATCC no. CRL 9078), PE501 (a retrovirus at 37°C lines). ADA EXPRESSION IN HEMATOPOIETIC CELLS

**VECTOR TITER**

<table>
<thead>
<tr>
<th>VECTOR</th>
<th>TITER</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNL6</td>
<td>$2 \times 10^7$</td>
</tr>
<tr>
<td>LNSA</td>
<td>$1 \times 10^7$</td>
</tr>
<tr>
<td>LNL7</td>
<td>$7 \times 10^6$</td>
</tr>
<tr>
<td>LNBAB</td>
<td>$7 \times 10^6$</td>
</tr>
<tr>
<td>LNBBA</td>
<td>$7 \times 10^6$</td>
</tr>
<tr>
<td>LASN</td>
<td>$5 \times 10^6$</td>
</tr>
<tr>
<td>LNCA</td>
<td>$4 \times 10^6$</td>
</tr>
</tbody>
</table>

Fig 1. Vectors for transfer and expression of ADA. Promoters are indicated as large open boxes with arrows denoting the site of RNA transcription initiation; SV indicates a PvuII to HindIII fragment from simian virus 40 that contains the SV40 early region promoter and enhancers. CMV indicates a BamHI to Xmafl fragment from human cytomegalovirus that contains the CMV immediate early promoter and enhancers. LPV indicates a BglII to HindIII fragment containing the lymphotropic papovavirus enhancer linked to an enhancerless SV40 early promoter as described. GLOBIN indicates an Sp6l to NcoI fragment containing the human B-globin promoter, and LTR indicates the retroviral long terminal repeat that contains the retroviral promoter and enhancers. Large boxes with crosshatched or stippled regions indicate fragments of the neo or B-globin genes or an ADA cDNA, and the crosshatched or stippled areas represent the coding regions. Small boxes indicate the second intervening sequences (IVS2) or sequences downstream of the B-globin genes. Lines indicate retroviral sequences. Other symbols are: E3, exon 3 of the human B-globin gene; $\psi^+$, extended retroviral packaging signal; (A)m, polyadenylation site.
Expression in mouse erythroleukemia vector LNBAB donor to a cryptic splice acceptor just upstream of ADA does not yield virus at high titer, so LNBBA was constructed.

Translation from the correct start codon in experiments with SV40, and neo sequences, and terminates in the downstream complex is LASN where the ADA-encoding mRNA begins.

Enzyme assays. ADA and purine nucleoside phosphorylase (PNP: purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) were measured in cell extracts by spectrophotometric assays. Electrophoresis of ADA was performed in starch gels.

RESULTS

Construction and production of retroviral vectors. The vectors LNSA, LNCA, and LNLÀ contain simple transcription units for expression of ADA-encoding mRNA, consisting of a viral promoter, the ADA cDNA, and the retroviral LTR, which provides a polyadenylation signal. The ADA start codon is the first AUG in the mRNA. More complex is LASN where the ADA-encoding mRNA begins in the retroviral LTR (LTR), continues through ADA, SV40, and neo sequences, and terminates in the downstream LTR. The ADA start codon is not the first AUG in this mRNA, although the upstream AUGs have little effect on translation from the correct start codon in experiments with related vectors (A.D.M. and W.R.A.O., unpublished results). Occasional splicing from the normal MoMLV splice donor to a cryptic splice acceptor just upstream of ADA does occur in cells infected with this vector (data not shown). The vector LNBAB was based on a vector for expression of human β-globin (LNB*SA), which directs human β-globin expression in mouse erythroleukemia cells, human BFU-E, and in long-term reconstituted mice. The ADA cDNA was inserted in place of the S′ coding region and first intervening sequence of β-globin in an attempt to preserve the strong transcriptional potential of the β-globin gene. The first intervening sequence in β-globin appears to be dispensable while the second intervening sequence is essential for high-level gene expression. Unfortunately, this construct did not yield virus at high titer, so LNBBBA was constructed in which the β-globin promoter and the ADA cDNA are flipped in orientation in the hope that possible enhancer elements located in the second intervening sequence and in the region just downstream of the gene would activate the β-globin promoter.

Plasmid DNA containing each of the retroviral constructs depicted in Fig 1 was transfected into ecotropic retrovirus packaging cells and the virus from these cells used to infect PA317 amphotropic packaging cells. Clonal G418-resistant PA317 cell lines that contained single unrearranged copies (data not shown) of each of the vectors except LNBAB were isolated. All G418-resistant clones generated by using LNBAB contained rearranged proviruses and this virus was not used further. PA317 cell lines containing the remaining viruses that produced the vectors at high titer (10^6 to 10^7 colony-forming units (CFU) /mL) in the absence of helper virus (<1/mL) could be isolated (Fig 1). ADA expression in the virus-producing cell lines was measured to assess the strength of the regulatory elements in mouse fibroblasts. As an internal control, we measured the activity of PNP, a metabolically related enzyme. All of the infectants expressed ADA at 66- to 4.4-fold higher levels than uninfected control cells (Table 1). ADA expression was highest in the cells infected with LASN virus where ADA expression was promoted by the retroviral LTR. PNP activities were similar in control and in infected cells. Starch gel electrophoresis of cell extracts showed that the increased ADA activity was due to vector-derived human ADA of normal electrophoretic mobility and was not caused by overexpression of endogenous mouse ADA (Fig 2). Although not shown, a

Table 1. ADA Activity in Infected PA317 Cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>ADA Promoter</th>
<th>ADA</th>
<th>PNP</th>
<th>ADA/PNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>2.17</td>
<td>2.03</td>
<td>1.1</td>
</tr>
<tr>
<td>LNSA</td>
<td>SV40</td>
<td>4.80</td>
<td>1.77</td>
<td>2.7</td>
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<tr>
<td>LNLA</td>
<td>LPV</td>
<td>5.83</td>
<td>1.54</td>
<td>3.8</td>
</tr>
<tr>
<td>LNBBA</td>
<td>β-globin</td>
<td>3.58</td>
<td>1.45</td>
<td>2.5</td>
</tr>
<tr>
<td>LASN</td>
<td>MoMLV</td>
<td>9.50</td>
<td>1.67</td>
<td>5.7</td>
</tr>
<tr>
<td>LNCA</td>
<td>CMV</td>
<td>5.89</td>
<td>1.64</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Enzyme activity units are μmol/h/mg protein. The mean coefficient of variation for these values was 5.2% (range, 1% to 12%).

![Fig 2. Analysis of ADA from infected PA317 cells. Protein extracts from clones of infected PA317 cells were analyzed by starch gel electrophoresis. Samples having equal amounts of total ADA activity were loaded in each lane. The positions of mouse (M) and human (H) ADA are indicated. Mouse and human standards were from uninfected PA317 cells and human T lymphocytes, respectively.](image-url)
similar mouse/human ADA pattern was observed from PA317 cells containing the LASN vector.

**Transfer of the ADA gene into ADA-human lymphoblasts.** Expression of ADA in human lymphoblasts was examined by infecting ADA− DHL-9 human lymphoma cells.14 Preliminary experiments with this cell line demonstrated that ADA expression of cells cocultivated with irradiated PA317 virus-producing cells. Stable populations of infected cells were obtained following G418 selection and analyzed for ADA expression. Measureable expression of ADA was observed from all of the vectors (Table 2). Considerably higher expression of ADA was obtained from infected ADA human lymphoma blast cells. Starch gel analysis demonstrated the human ADA was of normal electrophoretic mobility of ADA-1 phenotype (data not shown). Southern analysis of the integrated proviruses showed no major rearrangements (data not shown).

**Transfer of the ADA gene into K562 cells.** To test the ability of the vectors to express ADA in human hematopoietic progenitor cells, we infected K562 cells and measured ADA and PNP activity in the cells before and after infection, and after infection with a vector containing only the neo gene (LNL6) (Table 3). In the uninfected infected, the LASN and LNCA viruses produced the highest levels of ADA expression, with tenfold and ninefold increases in activity levels, respectively, in comparison to uninfected or LNL6 infected cells. The ADA/PNP ratios showed a similar increase for LASN and LNCA infectants of about tenfold. K562 cells respond to the phorbol ester TPA by differentiating into cells of megakaryoblastic phenotype.15,17,18 The effect on ADA expression of this induced differentiation of infected K562 cells is shown in Table 3. The LNCA infectants showed a fivefold increase in ADA expression after TPA induction whereas the LASN, LNSA, LNLA, and LNBBB infectants showed no marked change in ADA activity. These changes in total ADA activity were confirmed by the measurement of relative ADA values expressed as ADA/PNP ratios (Table 3). Uninfected control cells and LNL6 infectants both showed a similar 50% reduction in ADA activity after TPA treatment. This decrease in ADA activity can be related to the megakaryoblastic differentiation induced by TPA, as we determined that normal platelets have very low ADA activities (0.1 ± 0.01 μmol/h/mg protein, n = 5). ADA and PNP activities were also measured in K562 cells after incubation with sodium n-butyrate, which induces erythroid differentiation (Table 3).19,21 In LNCA infectants, a 70% increase in ADA activity was observed between induced and uninduced cells. ADA and PNP levels in all the other infected cells and in uninfected controls were not markedly different following exposure to sodium n-butyrate. K562 cells infected with the two best vectors, LASN and LNCA, were monitored for ADA expression after erythroid differentiation induced by hemin (Table 3).20,21 In LNCA infectants, an 85% increase in ADA activity was observed that is similar to the activity obtained from sodium n-butyrate treatment. In the LASN infectants, a threefold increase in ADA activity was observed in comparison to both uninfected cells and cells induced with sodium n-butyrate.

### DISCUSSION

We have systematically studied the efficiency of gene expression in cultured cell lines following retrovirus-mediated introduction of various transcriptional units containing a human ADA cDNA. Clonal cell lines that produced high-titer helper-free virus were established from PA317 packaging cells. All of the promoters efficiently expressed human ADA in PA317 cells. As anticipated from previous studies,26 the MoMLV LTR in LASN expressed the transduced ADA gene at the highest levels in these mouse-derived fibroblasts where a greater than fourfold increase over endogenous ADA was obtained. Also, with the exception of the β-globin-derived promoter, the SV40, CMV and lymphotropic papovavirus-SV40 promoters all expressed human ADA at levels at least twofold higher than the endogenous enzyme.

We used these vectors to infect human ADA− null histio-

### Table 2. ADA Activity in Infected DHL-9 Lymphoblasts

<table>
<thead>
<tr>
<th>Virus</th>
<th>ADA Promoter</th>
<th>ADA</th>
<th>PNP</th>
<th>ADA/PNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>0.01</td>
<td>2.66</td>
<td>&lt;0.0004</td>
</tr>
<tr>
<td>LNSA</td>
<td>SV40</td>
<td>0.15</td>
<td>3.08</td>
<td>0.05</td>
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<td>LNL6</td>
<td>LPV</td>
<td>0.33</td>
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<td>0.08</td>
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<tr>
<td>LNBBB</td>
<td>β-globin</td>
<td>0.03</td>
<td>3.24</td>
<td>0.009</td>
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<tr>
<td>LASN</td>
<td>MoMLV</td>
<td>3.86</td>
<td>3.01</td>
<td>1.3</td>
</tr>
<tr>
<td>LNCA</td>
<td>CMV</td>
<td>0.69</td>
<td>3.18</td>
<td>0.22</td>
</tr>
</tbody>
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Enzyme activity units are μmol/h/mg protein. The mean coefficient of variation for these values was 6.9% (range, 0% to 9%).

### Table 3. ADA Activity in Infected K562 Cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>Uninfected</th>
<th>TPA</th>
<th>Butyrate</th>
<th>Hemin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADA</td>
<td>PNP</td>
<td>ADA/PNP</td>
<td>ADA</td>
</tr>
<tr>
<td>None</td>
<td>0.85</td>
<td>2.70</td>
<td>0.3</td>
<td>0.39</td>
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<td>LNL6</td>
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<td>2.72</td>
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<tr>
<td>LNSA</td>
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<td>2.62</td>
<td>0.4</td>
<td>1.42</td>
</tr>
<tr>
<td>LNLA</td>
<td>1.38</td>
<td>3.67</td>
<td>0.4</td>
<td>1.85</td>
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<tr>
<td>LNBBA</td>
<td>1.03</td>
<td>2.88</td>
<td>0.4</td>
<td>0.74</td>
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<tr>
<td>LASN</td>
<td>8.37</td>
<td>2.85</td>
<td>2.9</td>
<td>11.6</td>
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<td>LNCA</td>
<td>7.03</td>
<td>2.38</td>
<td>3.0</td>
<td>35.4</td>
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</table>

Enzyme activity units are μmol/h/mg protein. The mean coefficient of variation for these values was 5.0% (range, 1% to 19%).

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cytic lymphoma cells (DHL-9). Although all the vectors provided ADA expression in DHL-9 cells, only the LTR promoter in LASN virus expressed ADA at potentially therapeutic levels. In these LASN-infectants, the ADA activity was 3.86 μmol/h/mg, which is about threefold higher than the ADA level found in normal human bone marrow or B cells. The PNP activity in DHL-9 cells is similar to that reported for human B and T cells. The LNSA, LNLA, and LNBBB vectors gave low levels of ADA expression. The K562 cell line can be induced by the phorbol ester TPA to terminally differentiate to phenotypically megakaryoblastic cells, which provided a means of evaluating transduced gene expression in hematopoietic cells after terminal differentiation. Our data showed that TPA induction of cells infected with the LNCA vector resulted in a fivefold increase in ADA expression in the megakaryoblastic-differentiated cells in comparison to their infected but uninduced progenitors (Table 3). In contrast, the ADA expression of LASN, LNSA, LNLA, or LNBBB infectants was relatively unchanged following TPA induction. We also induced erythroid characteristics in K562 cells by incubation with either sodium n-butyrate or hemin. The LNCA infectants nearly doubled their expression of ADA following sodium n-butyrate and hematin treatment. In the LASN infectants, sodium n-butyrate did not increase ADA expression in comparison to uninduced control cells, whereas hematin treatment resulted in a nearly threefold increase in ADA activity. ADA activity in LNSA, LNLA, and LNBBB infectants was relatively unchanged following sodium n-butyrate treatment. We had hoped that linkage of the ADA cDNA to the β-globin promoter would allow high ADA expression in erythroid cells and attribute our lack of success with this approach to disruption of β-globin regulatory elements in the LNBBB vector. The result that induction of differentiation in cells infected with any of the vectors does not decrease ADA expression is significant because alterations in gene expression during stem cell differentiation has been suggested as the cause of the low expression of genes transferred into bone marrow cells in vivo.

These studies show that vectors using promoters derived from SV40, β-globin, and lymphotropic papovavirus provide limited expression of ADA in the lymphoid and myeloid cell lines we have tested. Low expression found by using the SV40 promoter in this model system corresponds to the undetectable expression of ADA found following transplantation of mouse bone marrow infected with a retroviral vector containing ADA linked to the SV40 promoter. In contrast, the LNCA or LASN vectors provide relatively high-level expression of ADA in cultured hematopoietic cells. A recent report shows that transplantation of mouse bone marrow infected with a retroviral vector containing ADA linked to the retroviral LTR allows significant ADA expression in hematopoietic cells of reconstituted animals. However, while these limited observations suggest a correlation between vector expression in cultured cells and in animals transplanted with infected bone marrow, several reports have shown a lack of correlation. We are currently testing these vectors in animals to determine their usefulness for directed long-term expression in hematopoietic cells.

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