Constitutive High-level Production of Human Lymphotoxin by CHO-K1 Cells Transformed with the Human Lymphotoxin Gene Controlled by a Human β-Actin Promoter

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To achieve high-level production of human lymphotoxin (hLT), a plasmid (pβLT-dhfr) containing the hLT genomic DNA, a mouse dihydrofolate reductase (DHFR) cDNA, and a bacterial Ecogpt gene was cotransfected with a plasmid (pβLTML) encoding only the hLT genomic DNA into Chinese hamster ovary (CHO-K1) cells at a 1:7 molar ratio. Subsequently one of the Ecogpt-positive clones (clone A31) was grown in stepwise increasing concentrations of methotrexate (MTX). A large amount of the hLT was secreted by cells resistant to increased levels of MTX as a result of coamplification of the DHFR cDNA and the hLT gene. A cell clone (clone M-1) resistant to up to 500 nM MTX constitutively expressed the hLT at a concentration of 80 μg per ml at an elevated level for about 2 months. The hLT was produced in glycosylated form the molecular mass of which was 23,000 daltons and the mRNA was normally spliced, so the protein molecules were probably homogeneous.

LT is a lymphokine produced by phytohemagglutinin or lectin activated lymphocytes. It specifically inhibits tumor cell growth in vivo and in vitro, and shows little anticellular activity on primary cell cultures and normal cell lines. Therefore, LT may have considerable potential in the treatment for tumors. The hLT gene was cloned at the cDNA level, and the cDNA clone was used to express recombinant hLT in E. coli. As measured by protein sequencing, mature LT is 171 residues in length (M, 18,660). The LT produced by E. coli has no carbohydrate, but a natural LT is a glycoprotein with a monomeric molecular mass of approximately 20,000 daltons. Since it was suggested that the carbohydrate groups might be required for the full spectrum of the biological activity of a natural LT, the use of mammalian cells would be preferable for expressing a natural LT. A large quantity of the LT close to the natural LT can be produced by complementing a powerful transcriptional unit with a high gene copy number.

β-actin is one of the most abundant cellular proteins in mammalian nonmuscle cells. Recently it was found that the 1.2 kb DNA fragment of the 5'-noncoding region of the human β-actin gene has stronger activity to promote transcription of genes than SV40 early promoter in some human or mouse cells. Therefore, this 1.2 kb human β-actin fragment will be useful for constructing mammalian expression vectors.

In this paper, to achieve high-level production of human LT, we constructed an amplifiable vector containing hLT genomic DNA under the control of the human β-actin promoter and a mouse dhfr cDNA. This vector also contains a bacterial Ecogpt gene as a dominant selectable marker. CHO-K1 cells were transformed with this vector, and then Ecogpt-positive clones were exposed to increasing concentrations of MTX. Consequently, MTX-resistant clones constitutively produced elevated levels of hLT.
Materials and Methods

Cell lines. Chinese hamster ovary cells (CHO-K1), BHK, FL, and Chang Liver were grown in Eagles' minimal essential medium (MEM) with 5% fetal calf serum (FCS). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Construction of plasmids. The structures of the hLT expression vectors are shown in Fig. 1. In constructing expression vectors, we introduced a NcoI site into the initiation site of the hLT gene by the method of Norris et al.⁸ to use all the useful sequences in the 5' flanking sequence plus the 5' untranslated region and the intervening sequence 1 of about 1.2kb of the human β-actin gene as stated later. The SalI-NcoI 1.2kb fragment of human β-actin gene was linked to the initiation site of the hLT gene (Fig. 1). Plasmids construction and DNA preparation were done as described by Maniatis et al.⁹ All plasmids were grown in the E. coli HB101.

The pβLT-l contains the human genomic LT gene flanked by a human β-actin promoter on the 5' end and the SV40 polyadenylation sequence on the 3' end. It also contains a bacterial EcoGpt gene directed by a SV40 early promoter as a selection maker. Two expression vectors were constructed, based on this plasmid. The pβLT-ldhfr was constructed by cloning the XhoI/Sali fragment containing a mouse dhfr cDNA gene, which was under transcriptional control of the adenovirus major late promoter, into the SalI site of the pβLT-l. pβLT-ML was constructed by introducing the HindIII/BgIII fragment of the pβLT-l, which contained only the hLT DNA flanked by a human β-actin promoter, into the HindIII/BgIII site of pUC9.

The pSVεSmaILT-2 contains the SV40 early promoter at the upstream of the human genomic LT gene.

DNA transfection and selection of transformed cells. pβLT-l-dhfr (20μg for 1 x 10⁷ cells) and pβLT-ML (100μg for 1 x 10⁷ cells) were cotransfected into CHO-K1 cells at a 1:7 molar ratio by a modification of the methods of Chu and Sharp.¹⁰ Each plasmid DNA was coprecipitated with ethanol and suspended in 100μl of 10mm Tris-HCl (pH 8.0), 1mM ethylenediaminetetraacetic acid (EDTA). To the DNA solution, 0.3ml of 2M CaCl₂ and 2.1ml of H₂O were added and this solution was slowly dropped into 2.5ml of double-strength HBS buffer (HBS=280mM NaCl, 0.6mM NaH₂PO₄, 0.6mM Na₂HPO₄, and 50mM HEPES, pH 7.2) with continuous stirring. A calcium-phosphate-DNA complex was allowed to form for 30min at room temperature, and suspended with 10⁷ pelleted cells. After incubation for 15min at room temperature, the cocktail was diluted 10-fold with medium and plated on a 9cm dish at 10⁶ cells per dish. After incubation for 5hr at 37°C, the cells were washed twice with 0.8% NaCl solution, fed with 10ml of medium, and incubated at 37°C in CO₂-incubator. In 48hr, the cells were transfected to selection medium containing 25μg/ml mycophenolic acid, 0.1μg/ml aminopterin, 200μg/ml xanthine, 25μg/ml adenine, and 5μg/ml thymidine. Two weeks later, independent colonies could be observed. These were picked out and transferred to 96-well microplates.

For transient assays, pβLT-l and pSVεSmaILT-2 were transfected into BHK, FL, Change Liver, and CHO-K1 cells (5μg for 10⁴ cells). Cells were immediately transferred to 12-well plates. After cultivation for 4 days in MEM
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with 5% FCS, the medium was collected from each well and assayed for LT activity.

**LT assay.** Assay for the biological activity of LT was done in 96-well microtiter plates. LT-sensitive L-929 mouse fibroblast cells were added to each well at a density of $3 \times 10^4$ cells per well in 100 µl of D-MEM with 5% FCS. After incubation for 18 hr at 37°C, the medium was exchanged for 100 µl of D-MEM containing serially diluted samples and 1 µg/ml actinomycin-D. After incubation for 10 hr, the plates were stained with 0.036% neutral red solution, and the percentage of dead cells was measured. The LT activity of the sample was calculated as the reciprocal of the highest dilution that killed 50% of the cells. All LT assays were run in parallel with a laboratory standard and the titer was expressed in laboratory units.

**Genomic DNA blot analysis.** High molecular weight cellular DNA was isolated as described by Maniatis et al.9) A total of 20 µg of genomic DNA was completely digested with the indicated restriction endonuclease, and the same were electrophoresed on 1% agarose gels. The gels were soaked in 0.25 m HC1 for 7 min to insure complete transfer of large fragments, followed by denaturation with 0.5 M NaOH/1.5 M NaCl for 45 min. The DNA was then transferred from the gel to a natural nitrocellulose filter in 20 x SSC (0.3 M NaCl/0.03 M sodium citrate) by the method of Southern.11 The filters were then rinsed in 2 x SSC and baked for 2 hr at 80°C.

Before hybridization, filters were pretreated for 4 hr in hybridization solution (6 x SSPE, 0.5% sodium dodecyl sulfate (SDS), 5 x Denhart solution, and 250 µg denatured salmon sperm DNA per ml) at 65°C. Hybridization was done overnight at 65°C in hybridization solution and 3²P-labeled DNA probe prepared by nick translation kits (Amersham). Filters were then washed three times in 2 x SSC containing 0.1% SDS, and twice in 0.1 x SSC containing 1% SDS at 65°C before autoradiography.

**RNA analysis.** For dot-blot analysis, total RNA was prepared from transfected cells using the hot phenol technique, dissolved in 10 mm Tris–HCl, pH 7.5/1 mm EDTA and ethanol precipitated. The RNA was diluted serially in 2.2 M formaldehyde and spotted on a nitrocellulose filter. After it was baked for 2 hr at 80°C, the filter was hybridized with a nick-translated DNA probe. Then in the same manner as Southern blots, these filters were prehybridized, hybridized, and the autoradiography was performed. For Northern blots, poly(A)+ RNA was purified by oligo(dT) cellulose chromatography, separated on a 1% agarose gel containing 2.2 M formaldehyde, and transferred to a nitrocellulose filter. Finally the filter was baked for 2 hr at 80°C.

**Results**

**Comparison between β-actin and SV40 early promoters**

To evaluate the strength of the β-actin promoter, the activity of pβLT-1 was compared with that of pSVeSmaILT-2 containing the SV40 early promoter. First, these two plasmids were introduced individually into FL, BHK, Chang Liver, and CHO-K1 cell lines by calcium phosphate co-precipitation and the levels of hLT activity during the transient phase of expression were measured. The results are shown in Table I(A). Comparison between pβLT-1 and pSVeSmaILT-2 indicated that the human β-actin promoter was stronger than the SV40 early promoter in 4 cell lines. Especially the activity of β-actin promoter was about twelve-fold stronger than that of SV40 early promoter in BHK cells.

To achieve the high-level production of hLT, CHO cells are desirable because the MTX-induced gene amplification system can be used with the CHO-K1 wild-type cell line in our laboratory. Accordingly the advantages of the β-actin promoter in CHO cells were examined. The β-actin promoter was compared with SV40 early promoter in CHO cells after stable chromosomal integration. The stable transfectants were produced by selection with the selectable dominant Ecolgpt gene. Colonies produced by pβLT-1 and pSVeSmaILT-2 indicated that the human β-actin promoter was stronger than the SV40 early promoter in 4 cell lines. Especially the activity of β-actin promoter was about twelve-fold stronger than that of SV40 early promoter in BHK cells.
Table I(A). Transient Activity of Human LT Directed by Human β-Actin and SV40 Early Promoter

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>Cell type</th>
<th>FL</th>
<th>BHK</th>
<th>CHO-K1</th>
<th>Chang liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>pβLT-l</td>
<td>3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.5</td>
<td>7.7</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>pSVeSmaILT-2</td>
<td>0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5</td>
<td>2.5</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> LT activity (units/ml/10<sup>6</sup> cells). The different cell types were transiently transfected with the pβLT-l or pSVeSmaILT-2. After cultivation for 4 days, the activity in the medium was measured.

Table I(B). Stable Activity of Human LT Directed by the Human β-Actin and SV40 Early Promoter

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>Time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
</tr>
<tr>
<td>pβLT-l</td>
<td>494.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>pSVeSmaILT-2</td>
<td>109.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> LT activity (units/ml/10<sup>6</sup> cells/gene). CHO-K1 cells were transfected with the pβLT-l or pSVeSmaILT-2. Stable transformants by pβLT-l or pSVeSmaILT-2 were pooled (140 colonies each) and assayed for LT expression from 24 to 96 hours.

Table I(C). Relative Values of LT mRNA Accumulation Directed by the Human β-Actin and SV40 Early Promoter

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>Time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48</td>
</tr>
<tr>
<td>pβLT-l</td>
<td>138.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>pSVeSmaILT-2</td>
<td>33.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Densitometric value/ml/10<sup>6</sup> cells/gene. Total cytoplasmic RNA was extracted from pools of stable transformants by pβLT-l or pSVeSmaILT-2. Two and a half μg was spotted on nitrocellulose and hybridized to a 32P-labeled probe prepared by nick translation of the 0.4 kb AccI–EcoRI fragment in the fourth exon of the human LT gene. Relative values of human LT mRNA accumulation were calculated from densitometric analysis of the autoradiograph.

Cotransfection of CHO cells with pβLT-1dhfr and pβLT-1ML

CHO-K1 cells were transformed with pβLT-1dhfr and pβLT-1ML at a 1:7 molar ratio and cultured for 48 hr. The cells were then transferred into selection medium containing 25 μg/ml mycophenolic acid and 0.1 μg/ml aminopterine. Two weeks later, 380 Ecogpt-positive transformants were obtained and transferred to 96-well microplates. To screen for clones that constitutively express the hLT gene and secrete the protein in the medium, these cells were grown to confluence in 96-well microplates. Then the medium was changed (100 μl per well) and the samples of the medium were used for the assay of LT activity after 24 hr. Of the 380 clones examined, clone A31 produced about 20,000 units per ml of LT. The levels of hLT produced varied from almost none to 20,000 units (7.0 μg) per ml.

Amplification of hLT production

To obtain a cell line that constitutively produced higher levels of hLT, the coamplification of hLT genomic DNA linked to a mouse dhfr cDNA was studied in CHO-K1 cells. As a parent cell, the clone A31 was grown in stepwise increasing concentrations of methotrexate (MTX). In two weeks, MTX-resistant colonies were selected and transferred to 96-well microplates for the assay of LT activity. To obtain clones that had increased hLT production, further selection was done by exposure to stepwise increasing concentrations of MTX. Then cell cloning was done to stabilize hLT activity of cells. Table II shows that the clones from clone A31 resistant to 200, 500, and 1,500 nm MTX constitutively produced increasing levels of hLT. The cell clone resistant up to 1,500 nm MTX expressed levels of hLT at a concentration of about 140 μg/ml for a few weeks, assuming that 1 mg of hLT was about 4–5 times higher than those directed by the SV40 early promoter. Next, total cytoplasmic RNA was extracted from these cells and hybridized to a radio-labelled hLT probe. Table I(C) shows that the transcriptional levels of hLT driven by the β-actin promoter were also about 3–5 times higher than those driven by SV40 early promoter.
Table II. Production of Human LT in Methotrexate-resistant CHO-K1 Cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MTX (nm)</th>
<th>LT production*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A31</td>
<td>0</td>
<td>20,000</td>
</tr>
<tr>
<td>A31-33</td>
<td>200</td>
<td>80,000</td>
</tr>
<tr>
<td>A31-33-7</td>
<td>500</td>
<td>160,000</td>
</tr>
<tr>
<td>M-1</td>
<td>500</td>
<td>220,000</td>
</tr>
<tr>
<td>MA-8</td>
<td>1,500</td>
<td>410,000</td>
</tr>
</tbody>
</table>

* LT activity (units/ml). Each cell was seeded at a density 1.0 x 10^5/ml in 12-well plates. After cells were cultivated for 5 days, cell supernatants were assayed for LT activity.

Fig. 2. Sodium Dodecyl Sulfate-Polyacrylamide (12.5%) Gel Electrophoresis of Purified LT from the Supernatant of M-l Cells.
Before it was put on the 20 μg sample was treated with 0.1% SDS and 0.1 M β-mercaptoethanol, and then heated at 90°C for 1 min. The gel was stained by Coomassie brilliant blue.

Fig. 3. Dot Hybridization Analysis of DNAs from Clone A31, A31-33 (Resistant to 200 nM MTX), M-1 (Resistant to 500 nM MTX), and MA-8 (Resistant to 1,500 nM MTX). Cells counted accurately were fixed to nitrocellulose directly and DNAs were extracted on it. Subsequently, the filter was hybridized to a 32P-labeled probe prepared by nick translation of a 0.4 kb AccI-EcoRI fragment in the fourth exon of human LT gene and prepared for autoradiography. As a standard, FL cells were put on, assuming they have two copies of the LT gene per cell.

As natural LT has a molecular mass of approximately 60,000 daltons and is dissociated to a Mr 20,000 species as a monomer,6) the mobility of this protein indicates that the LT produced by M-1 cells was secreted as the glycosylated form.

Genetic characterization of the hLT producing cell lines
At first, clone A31 was started to select for resistance to 200 nM MTX, and 6 months later, the cell clone resistant up to 1,500 nM MTX was obtained. Consequently the cell clone showed constitutive production of LT. Dot blot hybridization analysis showed that clone A31, A31-33 (resistant to 200 nM MTX), M-1 (resistant to 500 nM MTX), and MA-8 (resistant to 1,500 nM MTX) contained respectively 8, 32, 64, and 128 copies of the hLT gene respectively (Fig. 3). It suggests that the increment of LT activity was due to amplification of the LT gene copy number. Cellular DNA of clone M-1 was isolated, cleaved with appropriate restriction enzymes, and analyzed by Southern hybridization. Figure 4 shows the relative number of NeoI-EcoRI or HindIII-EcoRI digested DNA sequences hybridizing to the nick-translated AccI-EcoRI fragment of hLT genomic DNA. In cellular DNA, fragments with the size similar to those of p/βLT-1dhfr and p/βLT-1ML were found to be equivalent to about 3 x 10^6 units. Moreover clone M-1 (resistant up to 500 nM) continued to produce 80 μg/ml hLT stably for about 2 months (about 30 populations doubling level). Figure 2 shows that the purified LT from the supernatant of M-1 cells was 23,000 daltons.
DNA was isolated from clone M-1, and 20 μg was digested to completion with either Ncol-EcoRI (lane c) or EcoRI-HindIII (lane f) and electrophoresed on a 1% agarose gel for Southern blot transfer. The filter was hybridized to a 32P-labeled probe prepared by nick translation of the 0.4kb AccI-EcoRI fragment in the fourth exon of the human LT gene and prepared for autoradiograph. One ng of pβLT-ldhfr digested with Ncol-EcoRI (lane a) or EcoRI-HindIII (lane d), and pβLTML digested with Ncol-EcoRI (lane b) or EcoRI-HindIII (lane e) was put on. The numbers on the right indicate the molecular weight markers (in kilobases).

abundant with a very little additional bands (about 1.5 kb size each). Therefore, most of pβLT-ldhfr and pβLT-1ML plasmid DNA sequences were intact in these cells, although a part of integrated plasmid might be rearranged during amplification. The hLT RNA produced by clone M-1 was analyzed by Northern hybridization. Approximately 3 μg of poly(A)+ RNA isolated from clone M-1 was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde, transferred to nitrocellulose, hybridized to a 32P-labeled probe prepared by nick translation of the 0.4 kb AccI-EcoRI fragment in the fourth exon of human LT gene, and prepared for autoradiography. The numbers on the right indicate the molecular weight markers (in kilobases).

Discussion

The β-isoform of actin, a major component of the cytoskeleton, is one of the most abundant proteins in many cells. Although human cells contain only one functional β-actin gene per haploid genome, β-actin protein content
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amounts to 5–10% of total proteins. Therefore, the β-actin promoter is expected to be a strong promoter. Human β-actin gene contains five introns, and the large one was found in the 5′ untranslated region 6 nucleotides upstream from ATG initiation codon. The first intron contains an enhancer core like sequence found in many enhancers; IgG heavy chain enhancer, histone H2B promoter, human U2 and amphibian U1/U2 small nuclear RNA gene promoter, and the SV40 enhancer.13'14'15'16)

Upstream from the initiation codon AUG, there are 15 base-repeat sequences. The sequences are A[CCGCCGAGACCGCGUCCGCCCGCGCGCUUUGCCGA][CCGCCGCCCGUCCACCAUG (AUG: Met codon). The last two repeat sequences near the codon AUG are highly homologous to each other and they are apparently complementary to the sequence of the 3′ terminal of 18S rRNA (3′ GGCGUCGUCCAAGUGGAUG 5′).17) The sequences corresponding to the SD sequence18) in prokaryotic mRNA has been unknown in eukaryotic mRNA, but Yamaguchi et al.19) showed that an apparent complementary sequence to the 3′ terminal of 18S rRNA in the 5′ noncoding region of the eukaryotic mRNA enhanced the rate of initiation complex formation and protein-synthesizing ability. Therefore, it is possible that these 15 base-repeat sequences may enhance the rate of human β-actin translation or mRNA stability. Also unique sequences found in the 5′ untranslated region of about 1.2 kb are two CArG boxes,20) multiple hexanucleotides and several potential Z-form nucleotide sequences.12) Sugiyama et al.7) demonstrated that the promoter activity of the 1.2 kb DNA fragment was greater than that of the SV40 early region in human FL cells and in mouse NIH 3T3 and L cells. Accordingly, to evaluate the effects of the human β-actin promoter on productivity of hLT, we constructed expression vectors containing the hLT gene fused to the β-actin promoter or the SV40 early promoter, which was then introduced into cell lines including CHO-K1. It was found that the transient hLT expression by the β-actin promoter is 3–5 times greater than that by the SV40 early promoter in FL, BHK, Chang Liver, and CHO-K1 cells. Moreover, the β-actin promoter was compared with the SV40 early promoter in CHO-K1 cells after stable chromosomal integration. Since LT gene expression of each transformant must be diverse due to difference of chromosomal integration sites (position effect) in spite of a single transfection experiment, stable transformants were pooled all together and assayed for LT expression, to minimize position effect. As a result, through the 5 days' culture, stable activity of the hLT and accumulation of the LT mRNA directed by the β-actin promoter were about 4 times higher than those directed by the SV40 early promoter.

Amplification vectors based on DHFR mouse cDNA can be used to obtain high-level expression of a variety of heterogenous genes, because genes linked to it can be coamplified by increasing the MTX concentration. However amplification of dhfr-based vectors is generally limited to DHFR-deficient CHO cells. To overcome this defect, Wigler et al.21) used genomic DNA from a MTX-resistant variant cell line (CHO-A29) to transfer the mutant gene to wild type MTX sensitive cells. Furthermore, Simonsen et al.22) isolated cDNA clone encoding a DHFR that has a reduced affinity for MTX from mouse 3T6 cells and used it as a dominant selectable maker. For this report, however, we tried to amplify normal mouse DHFR cDNA in wild-type CHO-K1 cells. Constructing an expression vector containing an EcoGpt or a Neomycin-resistance gene as a dominant selectable marker besides mouse DHFR cDNA, we must obtain clones containing exogenous dhfr genes and amplify them by addition of MTX.

We constructed an amplifiable vector containing a mouse DHFR cDNA and a bacterial EcoGpt gene besides the hLT genomic DNA under the control of the β-actin promoter. In addition, we constructed a vector containing only the hLT genomic DNA under
the control of the β-actin promoter. CHO-K1 cells were cotransfected with the vectors at a 1:7 molar ratio to integrate as many hLT genes as possible near the exogenous dhfr gene. Ecgogpt-positive transformants were picked up and grown in stepwise increasing concentrations of MTX. Clone A31 produced increasing levels of hLT during acquisition of resistance to increasing doses of MTX. The cell clone resistant to up to 500 nM MTX (named M-1) constitutively expressed elevated levels of hLT at a concentration of about 80 mg/l for 2 months. The increased titer of hLT reflected concomitant coamplification of the exogenous dhfr gene and the hLT gene copy number (data not shown). Furthermore as the result of Northern hybridization the LT mRNA of clone M-1 was normally spliced and the protein molecules were probably homogeneous.

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References