Expression of Amplified cDNA Sequences Encoding Human Interleukin 2 in Human Cells

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Expression of human interleukin 2 (IL-2) at high levels has been achieved in human cells by cotransfection and subsequent coamplification of the transfected sequences. IL-2 production by these cells reached 2700 U/ml of culture medium. This productivity was higher than the 100 U/ml attained by concanavalin A (ConA) stimulated human leukaemic Jurkat, T cells. A plasmid, pSDI, containing human IL-2 cDNA and mouse dihydrofolate reductase (DHFR) cDNA was constructed, and cotransfected with plasmid pSV2neo at a 10:1 molar ratio, into Hela (human epitheloid carcinoma) cells by the DNA-calcium phosphate coprecipitation technique. Transformants producing IL-2 were easily obtained among antibiotic G418 resistant colonies. Cells resistant to increased levels of methotrexate (MTX) secreted a large amount of IL-2 as a result of coamplification of DHFR cDNA and IL-2 cDNA. Expression of IL-2 cDNA was stimulated by removing a G-C stretch which was present between Simian Virus 40 (SV40) early promoter and IL-2 cDNA. Plasmid pSDI was rescued from the transformed Hela cells and shown to be present in the intact form among the amplified cellular DNA.

Interleukin 2 (IL-2) is a lymphokine produced by lectin- or antigen-activated T cells.^{1,2)} It is required for the proliferation of activated T cells, the long-term *in vitro* growth of certain T-cell lines,^{1,3)} augmentation of natural killer cell activity,³³⁾ induction of cytotoxic T cell reactivity,³⁴⁾ and plaque-forming cell responses in culture of nude mouse spleen cells.³⁵⁾ IL-2 is important *in vivo* as an immunoregulatory substance and may have considerable potential in the treatment of various immunodeficiency syndromes and neoplastic diseases.^{33,36~38)} The human IL-2 gene has been cloned and characterized at both the cDNA^{4,5)} and genomic DNA^{6~8)} levels. IL-2 is initially synthesized intracellularly as a precursor polypeptide of 153 amino acids, the first 20 of which appear to constitute a signal polypeptide and do not appear in the secreted protein.⁴⁾ cDNA clones have been used to express recombinant human IL-2 in *E. coli*,^{5,9)} in insect cells,¹⁰⁾ and in transfected monkey cells.⁴⁾

Human IL-2 is glycosylated,²⁸⁾ but further

studies are required to characterize it. Bacterially produced human IL-2 is of course nonglycosylated, but it is biologically active.⁹⁾ It is still necessary to do comparative animal studies and possibly clinical trials with IL-2 produced in both animal and bacterial cells, because their pharmacokinetic properties may be different. Some human leukaemic T-cell lines produce IL-2 following stimulation by lectin, but studies on it have been impeded by limited availability of purified material. Recently several eukaryotic host/vector systems have become available for production of large quantities of cloned gene proteins, which are believed to be glycosylated and secreted. Usually rodent cells have been used as the hosts, such as in the efficient production of human interferons by Chinese hamster ovary cells^{11,12)} and mouse cells.¹³⁾ A human cell expression system is much more preferable to obtain products closely resembling their native forms, but there have been few reports of human cells being used as hosts for production of proteins *via* eukaryotic vectors. Yamada *et*

al. have tried to express the thymidine kinase gene of herpes simplex virus in human cells using adenovirus vectors.¹⁴⁾

Here we have used an amplifiable vector to produce human IL-2 in HeLa cells. We constructed a recombinant plasmid containing human IL-2 cDNA and mouse dihydrofolate reductase (DHFR) cDNA. Since there were no dihydrofolate reductase-negative HeLa cells, we could not use DHFR cDNA as a dominant selectable marker. Therefore, this plasmid was introduced into HeLa cells by cotransfection with a plasmid carrying a gene for resistance to the antibiotic G418. We were easily able to obtain cell lines containing both plasmids by this cotransfection method.

Cell lines containing multiple copies of the human IL-2 cDNA were selected by exposure to increasing concentrations of the DHFR inhibitor methotrexate (MTX). These cells secreted human IL-2 constitutively.

MATERIALS AND METHODS

Cell lines. Medium for HeLa cell culture was Dulbecco's modified minimum essential medium (GIBCO Laboratories) containing 2% fetal bovine serum. Medium for COS-1 cell culture was Dulbecco's modified minimum essential medium containing 10% fetal bovine serum.

Construction of plasmids. Construction of human IL-2 expression plasmids is shown in Fig. 1. A plasmid pCEIL-2⁴⁾ (a gift from Dr. H. Matsui) contains a 0.8 kilobase (kb) *Pst*I fragment of human DNA containing IL-2 coding

FIG. 1. Construction of Human IL-2 Expression Plasmids.

sequences. The human IL-2 structural gene can be transcribed from the SV40 early promoter in appropriate host cells. The G-C stretch between the SV40 promoter and cDNA may hamper gene expression, as has already been reported for the expression of human gamma interferon cDNA.¹⁶⁾ Therefore, we removed it with nuclease *Bal* 31 (New England Biolabs) to form the plasmid pCEIL-2 (Bal). The IL-2 cDNA fragment was then inserted between *Hind*III and *Bgl*II cleavage sites in pSV2dhfr,¹⁷⁾ a plasmid containing mouse DHFR cDNA immediately downstream of the SV40 early promoter, and upstream of the SV40 early gene splicing and polyadenylation signals. During the insertion the DHFR sequence was lost, and the new IL-2-containing plasmid was called pSV2-IL2, whose structure is shown in Fig. 1. After cleaving this with *Pvu*II and *Bam*HI, a small fragment containing IL-2 cDNA and the SV40 early promoter was purified on an agarose gel, ligated with *Bam*HI linker (Takara Shuzo) and ligated into the single *Bam*HI site of pSV2dhfr to yield pSDI, a plasmid containing both DHFR and IL-2 cDNAs, as well as two SV40 early promoters, as shown in Fig. 1.

Assay for IL-2 activity. IL-2 activity was measured in the IL-2-dependent murine cell line CTLL-2 by measuring [methyl-³H] thymidine (³H-TdR) incorporation into CTLL-2 cells.¹⁸⁾ These cells (4000 per well) were seeded in 100 μ l of RPMI1640:Crick (1:1) medium containing 2% fetal bovine serum in 96-well flat-bottom microplates, together with 100 μ l of serially diluted assay samples. After 20 hr at 37°C, cells were pulse-labelled for 4 hr with 0.5 μ Ci of ³H-TdR, and collected onto glass fiber strips with an automated cell harvester; the incorporated radioactivity was then measured. One unit per ml of standard rat IL-2 caused 50% of the maximal stimulation of ³H-TdR incorporation.

DNA transfections and selection of transformed cells.

1) **Transient expression assay by monkey COS-1 cells.** To confirm IL-2 gene expression by plasmids constructed as shown in Fig. 1, transient expression assays were done using monkey COS-1 cells. Plasmids containing IL-2 cDNA were transfected into COS-1 cells by a modification of the method of Luthman and Magnusson.¹⁹⁾ One day before transfection, COS-1 cells were plated at a density of 10⁴ cells per cm² in 60 mm diameter plates with 5 ml of Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum. The medium was changed 4 hr before transfection. The cells were washed twice with tris-buffered saline (TBS)²⁰⁾ without MgCl₂ and CaCl₂. Plasmid DNA (5 μ g) dissolved in 0.8% TBS, containing 0.5 mg per ml DEAE-dextran (molecular weight 5 $\times$ 10⁵; Pharmacia Fine Chemicals), was added to the cell monolayers and incubated for 1 hr at 37°C. After removing the DNA cocktail, the cells were overlaid with 3 ml of medium containing 150 μ M chloroquine (Sigma), and were incubated for 3 hr at 37°C. The cells were then washed twice with TBS without MgCl₂ and CaCl₂, fed with 4 ml of

medium, and incubated overnight at 37°C. After 16 hr, cells were provided with 3 ml of fresh medium and incubated for a further 2 days at 37°C. Medium was then collected from each dish and assayed for IL-2.

2) **Transfection of HeLa cells and selection of transformed cells.** Before transfection, the plasmids pSDI and pSV2neo were linearized with *Pvu*II for efficient transformation. pSDI plasmid DNA (38 μ g for 1 $\times$ 10⁶ cells) was cotransfected into HeLa cells with pSV2neo (4 μ g)²¹⁾ by the calcium phosphate precipitation method.²²⁾ After 4 hr, the cells were treated with a 20% glycerol shock for three minutes. After 48 hr, the cells were transferred to selection medium containing 800 μ g/ml of the antibiotic G418 (GIBCO), which was replenished twice a week. After 3 weeks, independent colonies were picked and transferred to a 24-well plate. G418-resistant colonies were further selected by exposure to increasing concentrations of MTX as described by Ringold *et al.*²³⁾

Genomic DNA blot analysis. Total cellular DNA was prepared from parental and cDNA recipient cell lines by the method of Maniatis *et al.*²⁴⁾ DNA (10 μ g) was digested to completion and electrophoresed on 0.8% agarose gels for Southern blot transfer.²⁵⁾ After staining with ethidium bromide to ensure appropriate DNA sizing, the gel was left on a short (254 nm) wavelength UV transilluminator for 20 min. The gel was washed with 0.5 M NaOH–1.5 M NaCl for 45 min and then with 0.5 M Tris–chloride (pH 7.5)–3 M NaCl for 1 hr. The DNA was transferred to nylon membranes (Amersham) by blotting overnight in 10 $\times$ SSC (0.15 M NaCl plus 0.015 M sodium citrate). Blotted nylon membranes were wrapped in a plastic film (Saran Wrap; Asahi-Kasei) and placed, DNA side down, on a standard UV transilluminator for 5 min. Filters were then washed for at least 3 hr in hybridization solution (6 $\times$ SSC, 5 $\times$ Denhardt's solution,²⁴⁾ 0.5% sodium dodecyl sulfate (SDS), and 250 μ g of denatured salmon sperm DNA per ml) at 65°C. Fresh hybridization solution and ³²P-labeled DNA were added and incubated overnight at 65°C. Filters were washed twice in 2 $\times$ SSC, for 15 min each, once in 2 $\times$ SSC containing 0.1% SDS for 30 min, and once in 0.1 $\times$ SSC for 10 min at 65°C before autoradiography. Filters to be reprobbed with different DNA sequences were incubated at 45°C for 30 min in 0.4 M NaOH, then at 45°C for 30 min in 0.1 $\times$ SSC, 0.1% SDS, 0.2 M Tris–HCl (pH 7.5), and were autoradiographed overnight to ensure removal of all radioactivity before rehybridization.

DNA sequence analysis. The G-C stretch in IL-2 cDNA was removed by nuclease *Bal* 31. To confirm its removal, the nucleotide sequence of each terminal region of the cDNA was established by the M13 dideoxy chain-termination method²⁶⁾ using the M13 sequencing kit (Takara Shuzo).

Rescue of amplified DNA by bacterial transformation. DNA (10 μ g) from HeLa-pSDI-R200 was digested to

completion with either of the restriction enzymes *Pvu*II or *Bam*HI. After digestion, the reaction mixture was heated for 20 min at 68°C, extracted with phenol once and CHCl_3 /isoamylalcohol (24:1) once, and precipitated with ethanol. The DNA was dissolved in T4 DNA ligase buffer (66 mM Tris-HCl, pH 7.6, 6.6 mM MgCl_2 , 10 mM dithiothreitol (DTT), 1.0 mM ATP). T4 DNA ligase was added and incubated at 4°C overnight. The reaction products were used to transform competent *E. coli* DH1.

RESULTS

Construction of the IL-2 expression vector

The G-C stretch in IL-2 cDNA plasmids was removed by nuclease *Bal* 31 from two clones, pCEIL-2(Bal)C1.7 and C1.14. To certificate its removal, the nucleotide sequence of each terminal region of the IL-2 cDNA was established as shown in Fig. 2. Plasmid pCEIL-2 contained more than 25 bp of G-C in both terminal regions. The IL-2 cDNA of pCEIL-2(Bal)C1.7 began 2 bp upstream of the first ATG triplicate, the initiation codon, and ended in a 6 bp G-C stretch. That of pCEIL-2(Bal)C1.14 began 47 bp upstream of the first ATG and ended in a 2 bp G-C stretch. Thus the G-C stretch which had been between the SV40 promoter and the IL-2 cDNA was completely removed from both plasmids. They were transfected into monkey COS-1 cells by

FIG. 2. 5' and 3'-Terminal Regions of the IL-2 cDNA of pCEIL-2(Bal).

the DEAE-dextran method. After 72 hr, supernatants from the transfected cells were assayed for IL-2 activity (Table I). Plasmid pCE-1, which was constructed by removing the IL-2 cDNA sequences from plasmid pCEIL-2, was used as a negative control. The IL-2 activity was increased by removal of the G-C stretch, so, pCEIL-2(Bal)C1.7 and C1.14 were used for further experiments.

The IL-2 cDNA fragments of pCEIL-2(Bal)C1.7 and C1.14 were inserted singly between the *Hind*III and *Bgl*II sites of pSV2dhfr to yield pSV2-IL2 C1.7 and C1.14, respectively. They contained human IL-2 cDNA immediately downstream of the SV40 early promoter and upstream of the SV40 early gene splicing and polyadenylation signals. Since pSV2-IL2 C1.7 was more active than pSV2-IL2 C1.14 in producing IL-2 by the transient expression assay in COS-1 cells (Table I), a small fragment of pSV2-IL2 C1.7 cleaved with *Pvu*II and *Bam*HI was used to construct the plasmid pSDI (Fig. 1), the hybrid

TABLE I. INCREASE IN IL-2 ACTIVITY IN THE CULTURE MEDIA OF COS-1 CELLS TRANSFECTED BY PLASMIDS FROM WHICH THE G-C STRETCH HAD BEEN REMOVED

Transfected DNA	IL-2 activity (U/ml)
pCEIL-2	5.4
pCEIL-2(Bal)C1.7	27.0
pCEIL-2(Bal)C1.14	19.7
pSV2IL-2(Bal)C1.7	22.9
pSV2IL-2(Bal)C1.14	18.6
pSDI	24.8

TABLE II. PRODUCTION OF HUMAN IL-2 FROM HELa CELLS

In all cases, cells were seeded at a density of 1.0×10^5 cells per ml. After 4 days, cell supernatants were assayed for IL-2 activity.

Cell line	IL-2 produced (U/ml)
HeLa	< 1
HeLa-pSDI-R20	110
HeLa-pSDI-R100	700
HeLa-pSDI-R200	1,500
HeLa-pSDI-R400	2,700

containing both IL-2 and DHFR cDNAs, which expressed IL-2 in COS-1 cells (Table I).

Production of IL-2 activity in HeLa cells

Plasmid pSDI was introduced into HeLa cells by cotransfection with plasmid pSV2neo, which conferred resistance to the neomycin analog G418. G418-resistant colonies (19 clones) were picked after 3 weeks in selection medium containing G418 (800 $\mu\text{g/ml}$); colonies were obtained only if pSDI was transfected with pSV2neo. HeLa-pSDI colonies were further selected by exposure to increasing concentrations of MTX, and were then examined for their IL-2 productivity. Since MTX might be harmful to CTLL-2 cells in the IL-2 assay, HeLa cell culture medium to be tested in the IL-2 assay was exchanged for fresh medium containing no MTX, and supernatants were collected at appropriate intervals. The supernatants of 12 actively growing clones were

assayed for IL-2 activity, and constitutive secretion was detected in 11 of them. Table II shows that HeLa-pSDI derivatives resistant to 20, 100, 200, and 400 nM MTX (designated HeLa-pSDI-R20, HeLa-pSDI-R100, HeLa-pSDI-R200, and HeLa-pSDI-R400, respectively) constitutively produced increasing levels of IL-2. HeLa-pSDI-R400 cells continued to produce IL-2 stably for at least 1 month.

Increased IL-2 expression as a result of gene amplification

Schimke *et al.*²⁶⁾ have shown that the DHFR gene could be amplified by exposure of mouse cells to increasing MTX concentrations. Recombinant plasmids containing DHFR cDNA as well as DHFR genes within their normal host can be amplified.²³⁾ Southern blot analysis was done on DNA from HeLa and HeLa-pSDI-R200 cells to find whether gene amplification had occurred in

FIG. 3. Southern Blot Analysis of DNA from HeLa-pSDI-R200 Cells and HeLa Cells.

DNA was digested to completion with *Bam*HI (lanes a through h) and *Pvu*II (lanes i through l). Samples (10 μg) of genomic DNAs were put on a 0.8% agarose gel. Samples of 2.3, 0.23, 2.3, 0.23, 2.3, and 0.23 ng of plasmid pSDI were put in lanes a, b, e, f, i, and j, respectively. Panels A and C were probed with ^{32}P -labeled human IL-2 cDNA, and panel B was probed with ^{32}P -labeled mouse DHFR cDNA.

these cells. *Bam*HI and *Pvu*II-digested genomic DNA was electrophoresed and analyzed by Southern hybridization. The probe used was either a radioactively labeled human IL-2 cDNA fragment or a mouse DHFR cDNA fragment (both about 0.8 kb). Figures 3A and 3C show that human IL-2 cDNA was abundant in HeLa-pSDI-R200 cells. On the other hand, human IL-2 DNA could not be detected in ordinary HeLa cells by autoradiography for 5 days. HeLa-pSDI-R200 was found to contain about 200 copies of human IL-2 cDNA by inspection of the autoradiograms (pSDI DNA was put onto the gels at concentrations corresponding to 10 and 100 copies per cell) and by another DNA dot-blot analysis (not shown). The nylon filters used in Fig. 3A were stripped of hybridizing IL-2 cDNA fragments and reprobbed with a labeled mouse DHFR cDNA fragment. Figure 3B shows that the mouse DHFR cDNA was also amplified in HeLa-pSDI-R200. Several minor sequences were detected in HeLa-pSDI-R200 in addition to bands of the same size as pSDI DNA (Figs. 3B and 3C). Human IL-2 mRNA in HeLa-pSDI-R200 was detected by dot-blot analysis (not shown) with a 32 P-labeled probe prepared by nick translation of the 0.8 kb IL-2 cDNA fragment.

Rescue of the transforming DNA

Since pSDI plasmid DNA sequences appeared intact by blot analysis, attempts were made to rescue these sequences by bacterial transformation. Genomic DNA was digested with *Pvu*II or *Bam*HI, circularized with T4 DNA ligase, and used to transform *E. coli* DH1 to ampicillin resistance. Using HeLa-pSDI-R200 cell DNA, 24 resistant colonies were recovered (16 plasmids rescued from *Pvu*II-cleaved DNA and 8 plasmids rescued from *Bam*HI-cleaved DNA.) Five plasmids rescued from each restriction enzyme-cleaved cellular DNA were randomly selected and screened. All five plasmids rescued from *Bam*HI-cleaved DNA were identical to the large fragment of *Bam*HI-digested pSDI plasmid DNA. Of five plasmids rescued from

*Pvu*II-cleaved DNA, three were identical to pSDI plasmid DNA, but the other two were larger than the pSDI DNA plasmid. Due to differences in restriction maps these plasmids were thought to contain DNA not derived from the original plasmid pSDI (data not shown).

DISCUSSION

The purpose of this investigation was to apply gene amplification to the development of a human cell line producing high levels of IL-2. Plasmid DNA containing the human IL-2 cDNA and mouse DHFR cDNA was transfected into human (HeLa) cells. The cells resistant to increased levels of MTX, in other words, those expressing the DHFR gene at a high level, secreted large amounts of IL-2 as a result of coamplification of the DHFR and IL-2 genes. Thus, the HeLa-pSDI cell, supplemented with plasmids containing DHFR and IL-2 genes, could be induced to become resistant to 400 nM MTX, and produce at the same time up to 2700 U/ml IL-2. The rate of production of human IL-2 from HeLa-pSDI may increase further following selection of resistant cell lines in higher concentrations of MTX. Production of human IL-2 by Chinese hamster ovary cells has also been attempted using these gene amplification vectors. IL-2 titers of 95000 U/ml (about 2 mg/l) medium were achieved by a genetically derived cell line resistant to 40000 nM MTX. The productivity by this cell line increased more than 20 fold above that of a cell line resistant to 400 nM MTX (unpublished data).

Application of the transformed HeLa cells to practical production of human IL-2 has advantages over the method using ConA-stimulated human leukaemic Jurkat T cells, for the following three reasons. First, IL-2 productivity by the transformed HeLa cells was 2700 U/ml, compared to 100 U/ml by ConA-stimulated Jurkat cells. Secondly, the transformed HeLa cells produced IL-2 constitutively, but Jurkat cells needed ConA induction. This property would be favorable for

long-term production using hollow fiber culture systems. Thirdly, scale-up of the IL-2 production system would be easier and more practical due to the absence of an induction process, thus avoiding large scale replenishment with freshly prepared medium. Medium costs would be double in ConA-stimulated Jurkat cells, since fresh serum-containing medium is required twice at the growth- and induction-phases.

Human cells deficient in the DHFR gene would be preferable as a host cell, although DHFR-positive HeLa cells were employed in our experiments. Cells containing only transfected DHFR genes could be easily selected on the basis of their ability to grow in the absence of nucleosides, thus avoiding antibiotic resistance cotransfection procedures. Furthermore, subsequent amplification of the DHFR gene in such cells would always result in coamplification of the adjacent IL-2 gene. In the case of naturally DHFR-positive cells, on the other hand, the native DHFR genes can also be amplified in the presence of MTX, resulting in inefficient coamplification of the IL-2 gene. Therefore, isolation of human cells deficient in the DHFR gene might be required as an initial step in the genetic breeding of IL-2 overproducing cells. Such an attempt would be extremely difficult in diploid eukaryotic cells.

Chinese hamster ovary cells which are deficient in DHFR²⁹⁾ have been used as hosts to produce high levels of gamma interferon,^{11,30)} beta interferon¹²⁾ and tissue-type plasminogen activator³¹⁾ by application of gene amplification vectors. As it is not known whether glycosylation in hamster and human cells leads to identical structures, it may be more appropriate to generate and use a human DHFR-negative cell line for the production of human glycoproteins. However, since it is tedious to generate a human DHFR-negative cell line, a cotransfection method was used in our experiments to transfect plasmid DNA containing IL-2 cDNA and DHFR cDNA into DHFR-positive HeLa cells. As a result, transformed HeLa cells producing high levels of IL-2 were obtained following gene amplification.

Southern blot analysis showed that selection for DHFR gene amplification by increasing MTX resistance provided for the coamplification of IL-2 sequences. Hybridization studies of HeLa-pSDI-R200 DNA with IL-2 cDNA and DHFR cDNA probes detected several fragments in addition to bands of the same size as pSDI DNA (Figs. 3B and 3C). This result implies the existence of at least two independent integration points for the plasmid pSDI into this cell line's genome, or rearrangement of integrated pSDI DNA during amplification. Such rearrangement of transforming DNA has been observed in amplification processes.³²⁾

Amplified sequences containing plasmid pSDI DNA can be rescued by bacterial transformation. The amplification of DNA sequences by MTX increased the level of plasmid DNA in favor of rescue. Some rescued plasmids were thought to contain DNA sequences not derived from the original plasmid pSDI, and were probably complementary to locally repeated HeLa chromosomal DNA sequences, which were also amplified along with the pSDI DNA. Previous experiments have shown that rescued plasmid DNA from transformed cells contained host DNA.³²⁾

The G-C stretch which was present between the SV40 promoter and the IL-2 cDNA hampered gene expression. Its removal gave a 4~5 fold increase in IL-2 secretion. Since the G-C bond is stronger than the A-T bond, it is likely that the G-C stretch hampers the movement of RNA polymerase during transcription.

It is now possible to obtain a large amount of IL-2 from both *E. coli* and human cells. Comparative studies on the pharmacokinetics and immunogenicity will find which preparation is preferable for medical use.

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