COMMONWEALTH OF AUSTRALIA

Patents Act 1952-1969

CONVENTION APPLICATION FOR A PATENT

(1) Here insert (in full) Name or Names of Applicant or Applicants, followed by Address (es).

I/We (1) BEHRINGWERKE AKTIENGESELLSCHAFT

of D-3550 Marburg, Federal Republic of Germany

hereby apply for the grant of a Patent for an invention entitled: (2)

(2) Here insert Title of Invention.

THE GENERATION OF RECOMBINANT EUKARYOTIC CELLS WITH HIGH-LEVEL EXPRESSION

which is described in the accompanying complete specification. This application is a Convention application and is based on the application numbered (3)

(3) Here insert number(s) of basic application(s)

P38 06 617.3

for a patent or similar protection made in (4) Federal Republic of Germany on 2nd March 1988

(4) Here insert Name of basic Country or Countries, and basic date or dates

My/Our address for service is Messrs. Edwd. Waters & Sons, Patent Attorneys, 50 Queen Street, Melbourne, Victoria, Australia.

DATED this 28th day of February 19 89

(5) Signature (s) of Applicant (s) or Seal of Company and Signatures of its Officers as prescribed by its Articles of Association.

(5) BEHRINGWERKE AKTIENGESELLSCHAFT

by D. B. Mischlewski

Registered Patent Attorney

M00701 01/03/89

To:

THE COMMISSIONER OF PATENTS.

Edwd. Waters & Sons, Melbourne.

Form 8

COMMONWEALTH OF AUSTRALIA

Patents Act 1952

DECLARATION IN SUPPORT OF A CONVENTION APPLICATION UNDER PART XVI. FOR A PATENT.

In support of the Convention application made under Part XVI. of the Patents Act 1952 by BEHRINGWERKE AKTIENGESELLSCHAFT of D-3550 Marburg, Federal Republic of Germany for a patent for an invention entitled:

THE GENERATION OF RECOMBINANT EUKARYOTIC CELLS WITH HIGH-LEVEL EXPRESSION

We, Philipp Stein, Höhenweg 28, D-3550 Marburg, Heribert Bug, Amselweg 7, D-3551 Niederweimar, Federal Republic of Germany

do solemnly and sincerely declare as follows:

1. We are authorized by BEHRINGWERKE AKTIENGESELLSCHAFT the applicant for the patent to make this declaration on its behalf.

2. The basic application(s) as defined by Section 141 of the Act was (were) made at München in the Federal Republic of Germany under No. P 38 06 617.3

on March 2, 1988

by BEHRINGWERKE AKTIENGESELLSCHAFT

3. a) Gerd Zettlmeißl, Am Hofacker 15, D-3551 Lahntal
   b) Klaus-Dieter Langner, Lindenweg 14, D-3550 Marburg
   c) Manfred Wirth, Marktstraße 1, D-3340 Wolfenbüttel
   d) Hansjörg Hauser, Georg-Westermann-Allee 29, D-3300 Braunschweig
   a) - d) Federal Republic of Germany

~~I~~/are the actual inventor(s) of the invention and the facts upon which BEHRINGWERKE AKTIENGESELLSCHAFT is entitled to make the application are as follows:

The said BEHRINGWERKE AKTIENGESELLSCHAFT is the assignee of the said Gerd Zettlmeißl, Klaus-Dieter Langner, Manfred Wirth, Hansjörg Hauser

4. The basic application referred to in paragraph 2 of this Declaration was (were) the first application(s) made in a Convention country in respect of the invention the subject of the application.

DECLARED at Marburg, Federal Republic of Germany

this 10th day of February 1989

To the Commissioner of Patents

BEHRINGWERKE AKTIENGESELLSCHAFT

ppa. Stein
Prokurist

ppa. Bug
Prokurist

(12) PATENT ABRIDGMENT (11) Document No. AU-B-30858/89
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 617438

(54) Title
THE GENERATION OF RECOMBINANT EUKARYOTIC CELLS WITH HIGH- LEVEL EXPRESSION

International Patent Classification(s)
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C12N 015/67 **C12N 015/87**

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(74) Attorney or Agent
WATERMARK PATENT & TRADEMARK ATTORNEYS , Locked Bag 5, HAWTHORN VIC 3122

(57) Claim

1. A process for the expression of non-selectable genes in eukaryotic cells, which comprises two or more selection marker genes being transfected with the non-selectable genes, it being the case that the selection marker genes and the non-selectable genes are located on one or more vectors or DNA structures, and then selection being carried out for all the transfected selection markers by simultaneous addition of one or more appropriate inhibitory substances.

2. The process as claimed in claim 1, wherein selection is carried out for all the transfected selection markers simultaneously or within one hour.

5. The process as claimed in claim 1, 2, 3 or 4, wherein in successive selection steps the concentration of one or more inhibitory substances, preferably one inhibitory substance, is increased by a factor of 2 to 100, preferably by a factor of 8 to 12.

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952-69

Form 10

61,438

COMPLETE SPECIFICATION

(ORIGINAL)

Class Int. Class

Application Number:
Lodged:

Complete Specification Lodged:
Accepted:
Published:

Priority :

Related Art :

Name of Applicant : BEHRINGWERKE AKTIENGESELLSCHAFT

Address of Applicant : D-3550 Marburg, Federal Republic of Germany

Actual Inventor: GERD ZETTLMEISSL, KLAUS-DIETER LANGNER, MANFRED WIRTH and HANSJORG HAUSER

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50 QUEEN STREET, MELBOURNE, AUSTRALIA, 3000.

Complete Specification for the invention entitled:

THE GENERATION OF RECOMBINANT EUKARYOTIC CELLS WITH HIGH -LEVEL EXPRESSION

The following statement is a full description of this invention, including the best method of performing it known to :- us

Description

The generation of recombinant eukaryotic cells with high-level expression

The transfection of DNA is a widely used method for transferring foreign genes into animal cells in culture. This technique has been used in recent years for, on the one hand, identifying a number of regulatory DNA regions of different genes and analyzing their function and, on the other hand, preparing proteins of pharmaceutical interest in their natural or substantially natural form.

A number of different techniques can now be used for gene transfer. Depending on the technique used, the transfected genes are integrated as a single copy or as associated concatemers in a statistical process in the chromosome of the host cell. In this connection, the expression rate for the transfected gene(s) depends on a number of factors: for example on the copy number, on the site of integration, on the strength of the promoter or enhancer used, and on the stability of the mRNA.

At the present state of the art, the specific incorporation of the foreign DNA into regions of the chromosome with high transcriptional activity represents a problem which is still substantially unsolved. It is possible, however, to manipulate the copy number. Since there is a connection between the copy number and the level of expression, systems which offer the possibility of gene amplification are of interest for the expression of a gene in high yields. A number of amplifiable eukaryotic genes have been described in recent years. The best characterized among the latter is the dihydrofolate reductase gene (DHFR) which is amplified in cells as a response to increasing concentrations of the inhibitor methotrexate (MTX). In this connection, a chinese hamster ovary cell line (CHO) which no longer possesses a functional endogenous DHFR gene (Urlaub und Chasin, 1980,

Proc. Natl. Acad. Sci. USA 77, 4216-4220) is particularly suitable for the amplification of an exogenous DHFR gene and genes coupled thereto (Kaufman und Sharp, 1982, J. Mol. Biol. 159, 601-621).

However, using this method the screening for satisfactorily producing cell lines is very costly in time and effort because, as a rule, 4 to 8 months are required for adequate amplification and expression. In addition, amplification via MTX has hitherto been confined to the CHO system or equivalent cell lines, because it is often accompanied by non-specific resistance in other cells which are not DHFR-deficient. An additional factor is that competing amplification of the endogenous DHFR gene cannot be ruled out, which may cut down the desired amplification effect.

The present invention is a method for the direct and rapid screening for cell lines of any desired origin, or wild-type cell lines, with high-level production. This method entails initially at least two selectable genes being cotransfected with the non-selectable gene(s) of interest into the appropriate cell or wild-type cell. It is known that cotransfection is a very efficient method for the simultaneous introduction of separate genes into animal cells. In this connection, cotransfection is also intended to mean quite generally the transfection of vectors or DNA structures. Cotransfected DNA fragments are, as a rule, integrated into the host chromosome as direct neighbors and, in many cases, undergo highly correlated expression. Subsequently, in a first step, selection for resistance with respect to all the markers is carried out by addition, simultaneously or in rapid succession, of the appropriate (inhibitory) substances to the medium. Simultaneous addition or addition within one hour is preferred, but the invention also extends to addition at intervals of 1 hour to 120 hours. This results in cell lines with markedly increased expression rates for the non-selectable gene compared with cells which have been

selected for only one marker gene. At the same time, the expression which is achieved reveals a multiplicative effect not merely an additive effect. The use of two or more selectable markers in this system avoids difficulties deriving from non-specific resistance. In this connection, the said "marker genes" can be located together with the non-selectable gene of interest on one vector, or can be distributed over several vectors.

Furthermore, it is now possible, by increasing the concentration of one (or several) of the selecting substances in the culture medium preferably by a factor of 2-100, more preferably by a factor of 8-12, while maintaining the other(s) constant, to select for cells with increased copy numbers for all transfected genes and, in particular, with high expression rates for the non-selectable gene.

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The advantage of this method lies in its wide applicability to any desired cell lines and the avoidance of lengthy selection and screening procedures. It is possible with it, for example, for protein mutants to be generated by genetic manipulation in a short time in the amounts required for activity studies and then to be tested.

Accordingly, the invention relates to the abovementioned process for the generation of recombinant cells with high-level expression, as well as to its use for screening mutants of non-selectable genes. Further embodiments of the invention are detailed in the examples which follow and in the patent claims.

Examples

1. Double selection of baby hamster kidney (BHK) cells

The number of transfected genes in the cotransfection method described above is not the limiting factor, so that it is perfectly possible for more than two genes to be introduced simultaneously into the cell. The example of the cotransfection of a transcription unit for human

antithrombin III with various selection marker combinations (neomycin resistance/DHFR; neomycin resistance/puromycin resistance) into BHK cells is intended to demonstrate the advantageous properties of the presented double selection system for the expression of a heterologous structural gene of interest.

1.a) Cotransfection of plasmid pSVATIII with the plasmids pAG60/pAdDHFR (neomycin resistance/DHFR cDNA)

BHK 21 (ATCC CCL-10) cells were grown for this experiment in Dulbecco's Modified Eagle's (DME) medium containing 10 % newborn calf serum (NCS).

For the transfection, the following circular plasmid DNAs were coprecipitated by the calcium phosphate technique (Graham und van der Eb (1973) Virology 52, 456-467) in a volume of 0.5 ml: 2 µg of pSVATIII (Zettlmeißl et al. (1987) Biotechnology 5, 720-725), 0.8 µg of pAdDHFR (Kaufman and Sharp, 1982, loc. cit.) and 0.2 µg of pAG60 (Colbère-Garapin et al. (1981) J. Mol. Biol. 150, 1-14). The coprecipitate was added with 5 ml of medium to 1.2 x 10^5 cells in a 25 cm^2 culture vessel and incubated at 37°C for 16 hours. After the cells had been incubated for a further 48 hours with 5 ml of DME/10 % NCS, the cells were transferred 1:3 in DME/10 % NCS medium + 500 µg/ml G418 (Geneticin, Gibco) + 0 - 300 nM MTX. After a selection time of 6 - days (medium changed every 3 - 4 days), the resistant clones were counted, trypsinized off and grown as clone mixture (mixed clone, i.e. pool of the resulting clones).

To determine the AT III expression rate, 5 x 10^5 cells of a mixed clone of this type were made up with 5 ml of medium in a 25 cm^2 culture vessel. After 24 hours, fresh medium was added for a further 24 hours.

The cells were counted, and the AT III content was determined using a specific enzyme-linked immunosorbent assay (ELISA) (proposed in German Patent Application P 36 24 453.8).

Fig. 1 shows that the number of clones per transfection decreases with increasing MTX concentration, whereas the amount of secreted AT III is up to four times higher with double selection than with single selection with G418.

The chromosomal DNA was isolated from BHK clones double-selected with different MTX concentrations and was hybridized in a dot-blot procedure (Maniatis et al. (1982) Molecular Cloning - A Laboratory Manual. Cold Spring Harbor, 158, 393-401) with a fragment from the AT III cDNA labeled with ^{32}P by nick translation. The results show that increasing resistance to MTX correlates with an increase in the mean AT III gene copy number of the clone mixtures and in the AT III expression rates.

1.b) Cotransfection of the plasmid pAB3-1 (AT III) with the plasmids pRMH140/pSV2 d hfr (neomycin resistance/DHFRcDNA)

This example is intended to show that the system presented in Example 1a) can also be carried out, with a slightly changed protocol, with vectors which carry other transcription regulation sequences for the relevant genes (AT III neomycin resistance, DHFR).

For these experiments BHK cells were grown in DME medium which contained 10 % fetal calf serum (FCS) (culture medium). For the transfection, the following circular plasmid DNAs were coprecipitated using the calcium phosphate technique (see above) in a volume of 1 ml: 20 µg pAB3-1, 5 µg pSV2dhfr

(Lee et al. (1981) Nature 294, 228-232) and 5 µg pRMH140 (Hudziak et al. (1982) Cell 31, 137-146). pAB3-1 is composed of the plasmid pSVATIII which has had the enhancer region of human cytomegalovirus position -147 to -598 (Boshart et al. (1985) Cell 41, 521-530) ligated into its EcoRI cleavage site. The coprecipitate was directly added to 5 x 10^5 cells in a 25 cm^2 culture vessel at 37°C for 30 minutes. Addition of 5 ml of culture medium was followed by incubation of the cells at 37°C for a further 5 - 6 hours. The cells were then treated with 15 % (v/v) glycerol in the culture medium at 37°C for 3 minutes. The cells were washed twice with culture medium and then incubated in culture medium at 37°C for 72 hours. For selection, the cells were subsequently transferred 1:3 to 1:4 into culture medium which contained 400 µg/ml G418 with and without 1 µM MTX.

After a selection time of 10 - 15 days (medium changed every 3 - 4 days), the resistant clones were counted, trypsinized off and grown as clone mixture. The AT III expression rate was determined as described in Example 1a). Table 1 shows that the number of clones occurring per transfection is a factor of about five lower with double selection than with selection with G418 alone. It is also evident that the AT III expression rates are a factor of about six higher after double selection.

Table 1

G418 [µg/ml]	MTX [µM]	Colonies/# transfection	AT III expression# [µg/10^6 cells/ 24 h]
400	0	300 ± 30	1 ± 0.2
400	1	60 ± 15	6 ± 0.3

mean of three independent transfection experiments

A double-selected mixed clone (3MK1; see Table 2) was exposed to stepwise increasing MTX concentrations in the medium (1 → 10 µM). It was possible by this to double the AT III expression rate. The mean copy number for AT III determined by a quantitative Southern blot method (Zettlemeißl et al., loc. sit.) likewise increases with this secondary MTX selection (Table 2).

Table 2

G418 [µg/ml]	MTX [µM]	AT III gene copies/cell (3MK1)	AT III expression [µg/10^6 cells/ 24 h]
400	1	40	6 ± 0.2
400	10	150	12 ± 0.4

1.c) Cotransfection of plasmid pSVATIII with the plasmids pAG60/pSV2PAC (neomycin resistance/puromycin resistance)

BHK cells were cotransfected as described in Example 1a) using 0.8 µg of the plasmid pSV2PAC (Vara et al. (1986) Nucl. Acids Res. 11, 4617-4624) in place of the plasmid pAdDHFR. (pSV2PAV codes for an N-acetyltransferase from Streptomyces alboniger and, on expression in animal cells, confers resistance to the antibiotic puromycin.) The transfected cells were selected in DME/10 % NCS medium + 500 µg/ml G418 + 0 - 30 µg/ml puromycin, grown as mixed clones and assayed for AT III expression as described above. In this experiment too, the number of clones obtained per transfection decreases with increasing puromycin concentration. In contrast, the AT III expression rates are a factor of four to six higher in double-selected cells than in cells which have been selected only

with G418 (Fig. 2).

2. Double selection in other cell systems

The examples which follow are intended to show that the principle of double selection can also be applied to other animal cells in culture.

2.a) Cotransfection of plasmid pSVtss+ (contains cDNA for human interferon-β) with the plasmids pAG60/pSV2PAC (neomycin resistance/puromycin resistance) in CHO and mouse L TK$^-$ cells.

CHO dhfr$^-$ (Urlaub and Chasin (1980), loc. cit.) and L TK$^-$ (ATCC CCL1.3) cells were cotransfected as described in Example 1a) and 1c) with the following circular plasmid DNAs: 2 µg pSVtss+ (Reiser and Hauser (1987) Drug Res., 37, 482-485), 0.2 µg pAG60 and 0.8 µg pSVPAC. After selection in DME/10 % NCS + G418 (360 µg/ml for CHO cells; 700 µg/ml for L TK$^-$ cells) + puromycin (0 - 10 µg/ml), the number of stable transfectants was counted, the cells were grown as mixed clones, and the interferon-β expression rates were determined by an antiviral assay (Finter (1969) J. Gen. Virol, 5, 419-427). For this purpose, confluent cells were treated with poly(I):poly(C) for 5 hours. 15-hour supernatants were collected for the assay (Dinter and Hauser (1987) EMBO J. 6, 599-604). CHO mixed clones which had been double-selected with 10 µg/ml puromycin and G418 expressed 20 times more interferon-β than those subjected only to G418 selection (Fig. 3A). L TK$^-$ mixed clones which had been double-selected with 10 µg/ml puromycin and G418 expressed about 6 times more interferon-β than those subjected only to G418 selection (Fig. 3B).

2.b) Cotransfection of plasmid pAB3788 (contains cDNA for human factor VIII:C) with the plasmids pRMH140/ pSV2dhfr in HE7 cells (hamster)

To construct the plasmid pAB3788, a cDNA which codes for a deletion mutant of human factor VIII:C (deletion of amino acids 741 to 1689, as proposed in German Patent Application P 37 20 246.4) and is flanked by XhoI sites was cloned into the vector pAB3-1 (see Example 1b). For this purpose, pAB3-1 was cut with SalI/XbaI, the protruding 5' ends were filled in with the Klenow fragment of E. coli DNA polymerase I, and the vector fragment (3.3 kb) was purified. After the protruding 5' ends of the XhoI sites of the purified factor VIII:C DNA fragment had likewise been filled in, the vector pAB3788 was obtained by ligation. 20 µg of the plasmid pAB3788 were cotransfected as described in Example 1b) with the plasmids pRMH140 and pSV2dhfr in HE7 cells (Cook, J. L. and Lewin, A. M. (1979) Cancer Res. 39, 1455 - 1461). After selection as described in Example 1b, about 300 $\pm$ 30 clones were obtained per transfection mixture. Transfectants grown as a mixed clone expressed 0.7 $\pm$ 0. 1 units (U) of factor VIII:C per 10^6 cells per 24 h. Thus, the expression of the factor VIII:C mutant by double-selected cells was a factor of about 3 to 6 greater than in independent experiments with mixed clones selected with G418. The factor VIII:C activity was determined using the COA assay (Kabi Vitrum, Uppsala, Sweden).

3. Double selection as method for achieving high titers of recombinant retrovirus particles

To construct the retroviral expression vector pM5AT III, a 1.4 kb BglII/BamHI fragment (AT III cDNA) was cloned into the unique BamHI site of pM5neo which is flanked by a splice donor site and a splice acceptor site of the myeo-

proliferative sarcoma virus. The ATIII fragment was produced by attaching BglII linkers to the 1.6 kb SalI/EcoRI fragment from the vector pSVAT III (see above), followed by a BamHI/BglII cleavage. pM5neo contains, besides a pBR backbone, inter alia the abovementioned splice sites and the neomycin resistance gene as well as the LTRs (long terminal repeats) of the myeloproliferative sarcoma virus (Ostertag et al. (1980) J. Virol. 33, 573-582; Stacey et al. (1984) J. Virol. 50, 725-732).

The helper cell line Psi2 (Mann et al. (1983) Cell 33, 153-159) was multiplied in DME medium containing 10 % FCS. For the transfection, a mixture of circular plasmid DNA (5 µg of pM5AT III and 2 µg of pSV2PAV) and 8 µg of sheared DNA from mouse L cells in a volume of 0.5 ml was co-precipitated with calcium phosphate and, after incubation at room temperature for 30 minutes, added to 2 x 10^5 Psi2 cells in 5 ml of culture medium. After incubation at 37°C for about 12 hours, the medium was changed and, a further 24 h later, replaced by selection medium (single selection with 1 mg/ml G418, double selection with 1 mg/ml G418 and 2 or 5 µg/ml puromycin in DME medium plus 10 % FCS). The resulting clones were counted and pooled after 10-14 days.

To determine the virus titer, a culture of 5 x 10^5 Psi2 cells was set up, and the medium was changed after 24 hours. 24 hours later, the supernatant, which contains the recombinant retrovirus particles, or appropriate dilutions, was used to infect HIH 3T3 cells (ATCC CRL 1658; 1000-3000 cells per 24-well plate). The infection took place with the addition of 8 µg/ml polybren for about 12 hours. The medium was then changed and, after 24 hours, replaced by selection medium (1 mg/ml G418). The resulting clones were stained and counted 10 days after the change to selection medium. Clone mixtures deriving from the double selection achieve viral titers which are approximately 10 times those of single-selected clone mixtures (Fig. 4).

PATENT CLAIMS:
THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A process for the expression of non-selectable genes in eukaryotic cells, which comprises two or more selection marker genes being transfected with the non-selectable genes, it being the case that the selection marker genes and the non-selectable genes are located on one or more vectors or DNA structures, and then selection being carried out for all the transfected selection markers by simultaneous addition of one or more appropriate inhibitory substances.

2. The process as claimed in claim 1, wherein selection is carried out for all the transfected selection markers simultaneously or within one hour.

3. The process as claimed in claim 1, wherein there are intervals of 1 hour to 120 hours, preferably 2 to 72 hours, in the successive selection for the transfected selection markers.

4. The process as claimed in claim 1, 2 or 3, wherein a single non-selectable gene is cotransfected.

5. The process as claimed in claim 1, 2, 3 or 4, wherein in successive selection steps the concentration of one or more inhibitory substances, preferably one inhibitory substance, is increased by a factor of 2 to 100, preferably by a factor of 8 to 12.

6. The process as claimed in claim 1, 2, 3, 4 or 5, wherein the non-selectable gene codes for AT III, F VIII: C, F XIII a, t-PA, EPO, G-CSF, GM-CSF, PAI, protein C or IL-3.

7. The process as claimed in claim 1, 2, 3, 4 or 5 for screening mutants of non-selectable genes.

DATED this 28th day of February 1989.
BEHRINGWERKE AKTIENGESELLSCHAFT

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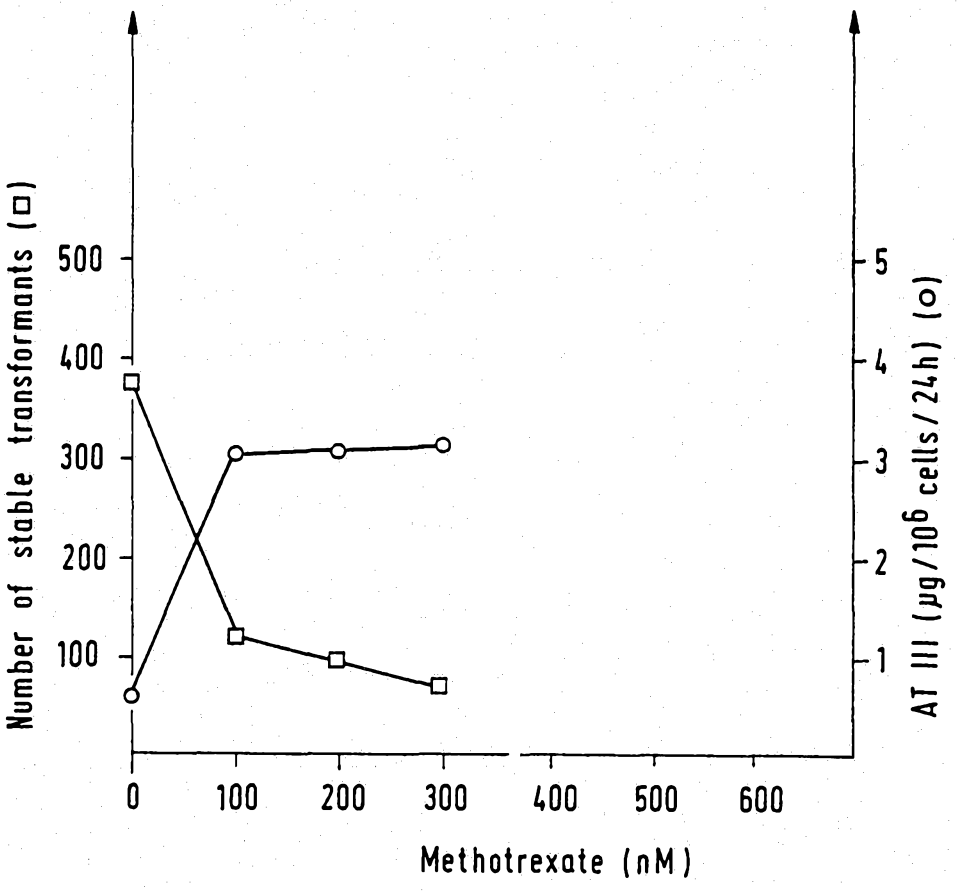
Number of stable transformants (□)
500
400
300
200
100
AT III (μg/10^6 cells/24h) (○)
5
4
3
2
1
0
100
200
300
400
500
600
Methotrexate (nM)

FIG. 1

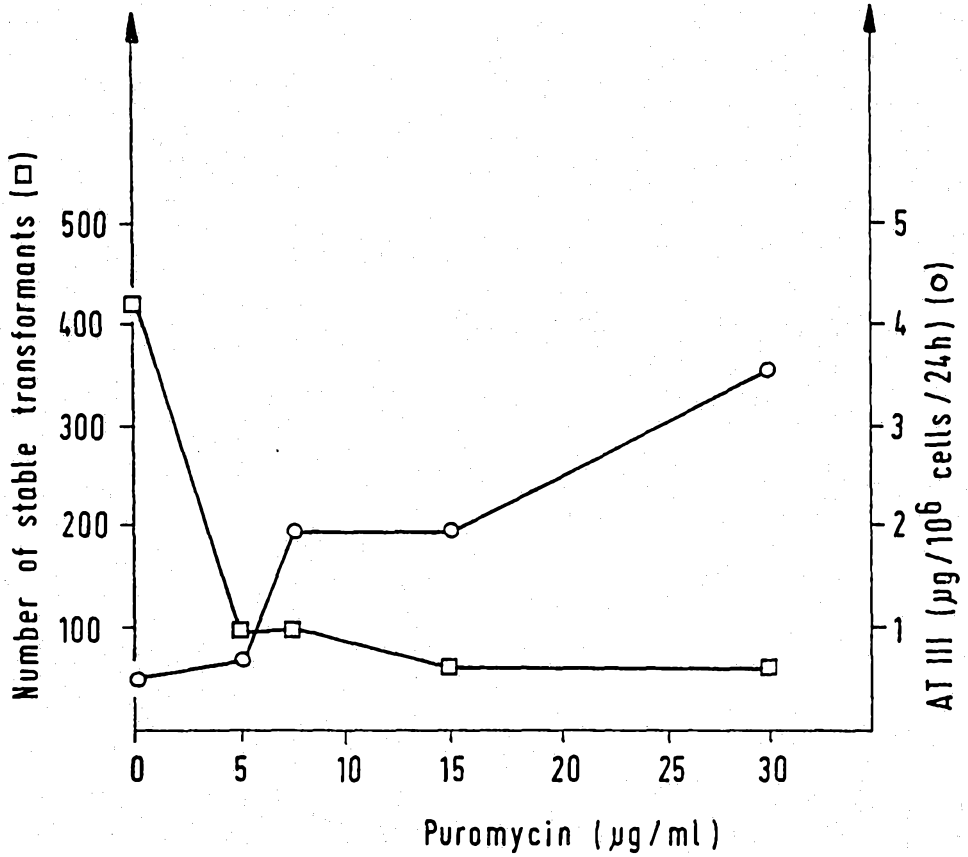
Number of stable transformants (□)
500
400
300
200
100
0
5
10
15
20
25
30
Puromycin (µg/ml)
AT III (µg/10⁶ cells/24h) (○)
5
4
3
2
1

FIG. 2

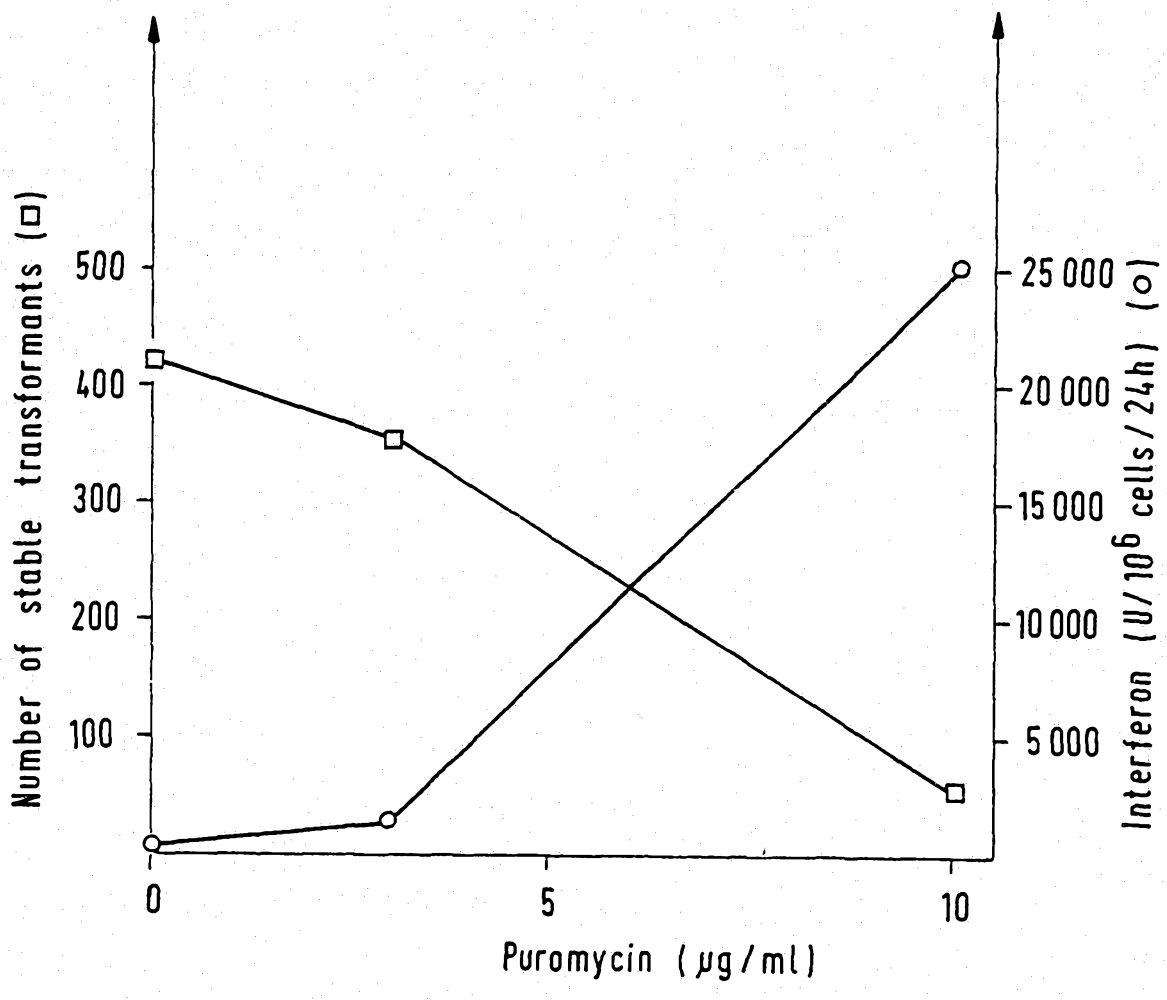
Number of stable transformants (□)
500
400
300
200
100
25 000
20 000
15 000
10 000
5 000
Interferon (U/10^6 cells/24h) (○)
0
5
10
Puromycin (µg/ml)

FIG. 3A

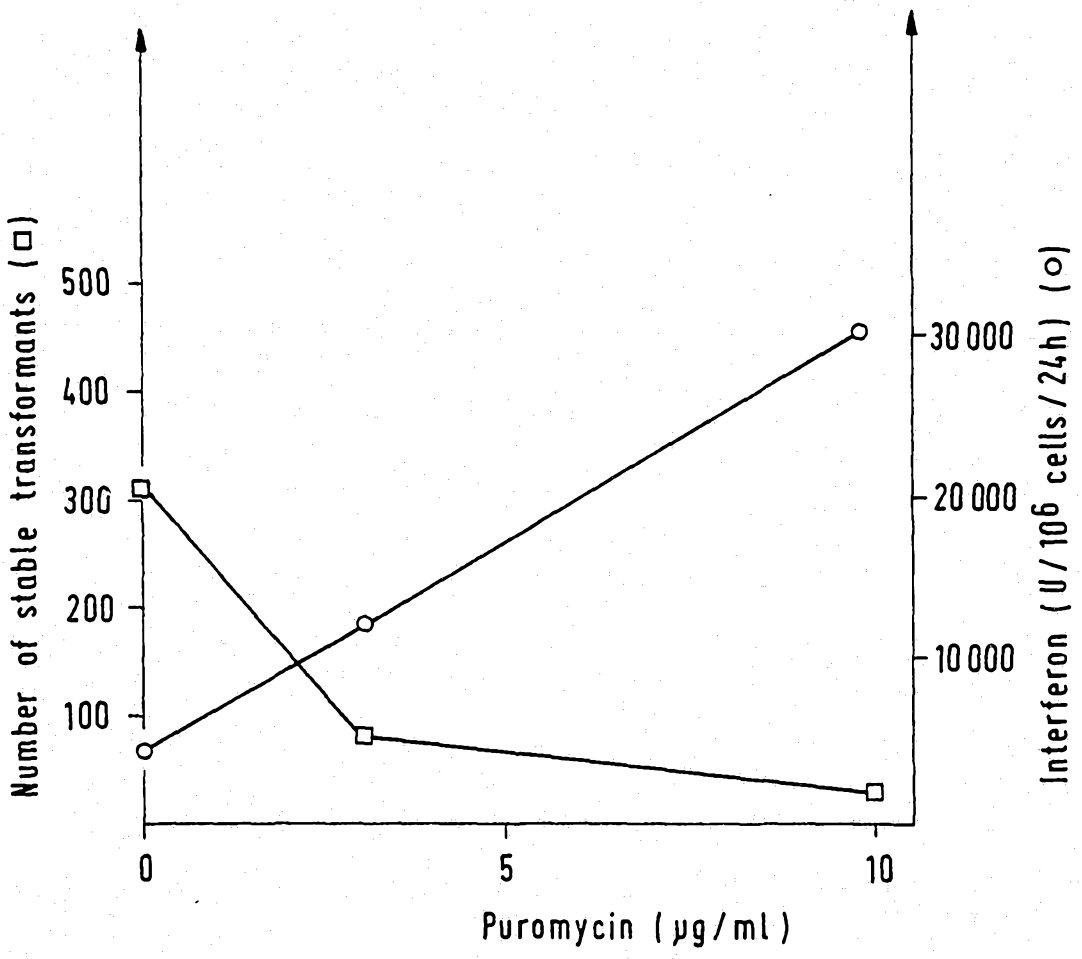
Number of stable transformants (□)
500
400
300
200
100
30 000
20 000
10 000
Interferon (U/10⁶ cells/24h) (○)
0
5
10
Puromycin (µg/ml)

FIG. 3B

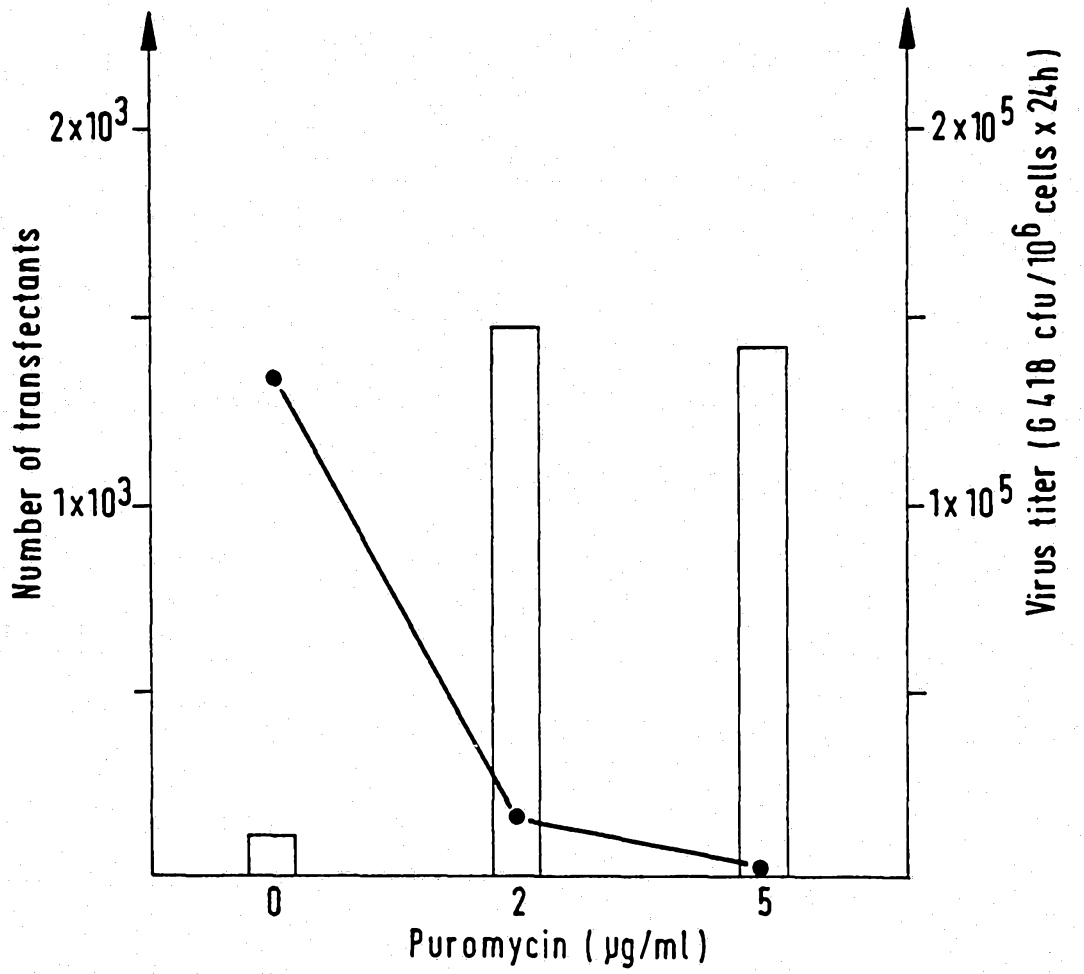
2x10^3
1x10^3
Number of transfectants
2x10^5
1x10^5
Virus titer (G418 cfu/10^6 cells x 24h)
0
2
5
Puromycin (µg/ml)

FIG.4