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Isolation of a functional human interleukin 2 gene from a cosmid library by recombination in vivo

(Recombinant DNA; DNA mediated gene transfer; expression plasmid; screening; packaging; bacteriophage λ)

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SUMMARY

A method has been developed that allows the isolation of genomic clones from a cosmid library by homologous recombination in vivo. This method was used to isolate a human genomic interleukin 2 (IL2) gene. The genomic cosmid library was packaged in vivo into λ phage particles. A recombination-proficient host strain carrying IL2 cDNA sequences in a non-homologous plasmid vector was infected by the packaged cosmid library. After in vivo packaging and reinfection, recombinants carrying the antibiotic resistance genes of both vectors were selected. From a recombinant cosmid clone the chromosomal IL2 gene was restored. After DNA mediated gene transfer into mouse L*tk*$^-$ cells human IL2 was expressed constitutively.

INTRODUCTION

IL2, also known as T-cell growth factor, plays a central role in the cellular immune response. It is a necessary signal for the proliferation of activated T lymphocytes and has been implicated in a number of immunoregulatory functions (for review see Robb, 1984). Human IL2 is a glycoprotein of apparent M_r of 16 500, and has been purified to homogeneity from stimulated normal lymphocytes and a human T leukemia line (Jurkat) (Robb et al., 1984; Conradt et al., 1985). The coding sequence of the human IL2 gene has been cloned from this cell line and from lymphoid tissues (Taniguchi et al., 1983, Devos et al., 1983, Maeda et al., 1983). Recently the isolation of chromosomal DNA fragments containing the IL2 sequences by conventional screening of phage λ libraries has been described (Fujita et al., 1983; Degrave et al., 1983; Holbrook et al., 1984). To study expression and modification of IL2 in homologous and heterologous animal cells in detail we decided to isolate the region containing the chromosomal gene and flanking sequences from a human cosmid library. For the isolation of specific cosmid clones we developed a method which uses the high specificity of homologous recombination

Abbreviations: Ap, ampicillin; bp, base pairs; ConA, concanavalin A; DMEM, Dulbecco's-modified Eagle medium; EtBr, ethidium bromide; FCS, fetal calf serum; HAT, selective medium containing hypoxanthine, aminopterin and thymidine (Szybalska and Szybalski, 1962); IFN, interferon; IL2, interleukin 2; kb, 1000 bp; Km, kanamycin; PHA, phytohemagglutinine; ^{R}, resistance; *tk*, gene coding for thymidine kinase; TPA, tetradecanoyl phorbol acetate; u, unit(s); [ ], designates plasmid-carrier state.

with the efficiency and *cis*-specificity of the λ packaging system. The development of this simple genetic screening procedure has been possible after in vivo packaging of cosmid gene libraries has been established (Lindenmaier et al., 1982). The procedure is selective and efficient enough to allow the isolation of single copy genes from a total genomic cosmid library. Handling of radioactively labelled probes of high specific activity can be avoided.

MATERIALS AND METHODS

(a) Bacterial strains

Escherichia coli HB101 (r^-, m^-, *proA*2, *leu*, *supE*44, *recA*13, *rpsL*20) and DH1 (r_k^-, m_k^+, *supE*, *gyrA*96, *recA*), kindly provided by H. Lehrach, were used as *recA*$^-$ hosts for hybrid cosmids. *E. coli* 1400 (*hsdS*$^-$, *met*$^-$, *supE*, *supF*, *recA*56, λL512; Cami and Kourilsky, 1978) was used for in vivo packaging of the human cosmid library. For recombination and in vivo packaging of recombinants BHB3169 (W3110 r^-, m^+, λ *b2red*$^-$ *imm*434ts*S*am7; Poustka et al., 1984), kindly provided by H. Lehrach, was used.

(b) Plasmids and cosmids

Cosmid and plasmid DNAs were isolated according to the method of Birnboim and Doly (1979). Vectors pAN26, pV34 (provided by B. Hohn), and pHC79-2cos/tk (Lindenmaier et al., 1982) were further purified by two cycles of CsCl-EtBr centrifugation. pCosIFNβ (Gross et al., 1981) was a gift of G. Gross. pAN26IF1 is described in Table I. The human cosmid library has been described (Lindenmaier et al., 1984).

(c) Plating cells

Single colonies were streaked on LB-plates and grown overnight at 37°C (HB101 and DH1). In vivo packaging strains were streaked in parallel on two plates and incubated at 30°C and 42°C to verify the inducibility. NZ-broth containing 0.4% maltose was inoculated from the fresh overnight plates to an A_{600} of about 0.05 and grown with good aeration to $A_{600} = 2$.

(d) Animal cell culture and transfection

Mouse L*tk*$^-$ cells were grown in DMEM supplemented with 10% FCS. Transfection using the Ca · phosphate precipitation method was carried out as described (Hauser et al., 1982) employing HAT selection (Szybalska and Szybalski, 1962). pHIL49 was cotransferred with pHC79-2cos/tk in a molar ratio of 10 to 1. More than 100 HAT-resistant clones were combined and grown to confluency. For IL2 production the cells were induced by PHA, ConA, TPA and calcium ionophore A23187, or mock-induced and cultivated in RPMI 1640 supplemented with 10% FCS for 20 h.

RESULTS AND DISCUSSION

(a) Recombinant screening of cosmid libraries

The general strategy for gene isolation from cosmid libraries by recombination in vivo is shown in Fig. 1. In principle it is analogous to the method of Seed (1983) for recombinant screening of λ libraries (see legend to Fig. 1).

To allow screening by recombination in vivo a pair of cosmid and plasmid vectors has to be used that has no homologous sequences. For this purpose a number of vectors has been constructed that contain the basic replicon of pBEU17, a temperature-inducible copy mutant of plasmid R1 (Uhlin et al., 1979), and the Km^R gene of Tn*5* (A. Necker, to be published elsewhere). These R1-derived screening plasmids have no homology to pBR322 and are compatible with a large number of established pBR322-derived cosmid vectors (e.g., pHC79, pJB8) in contrast to the system of Poustka et al. (1984) which requires use of specific R6-K-derived cosmid vectors. Cointegrates should only be formed by recombination via the inserted sequence. Because formation of cointegrates leads to an increase of about 4 kb in cosmid size, packaging of the recombinant cosmid can be altered due to the size selection (38–52 kb; Feiss et al., 1977) exerted by the λ packaging system.

Cosmid packaging, however, in contrast to λ growth does not require repeated cycles of infection and lysis, therefore size selection should be less

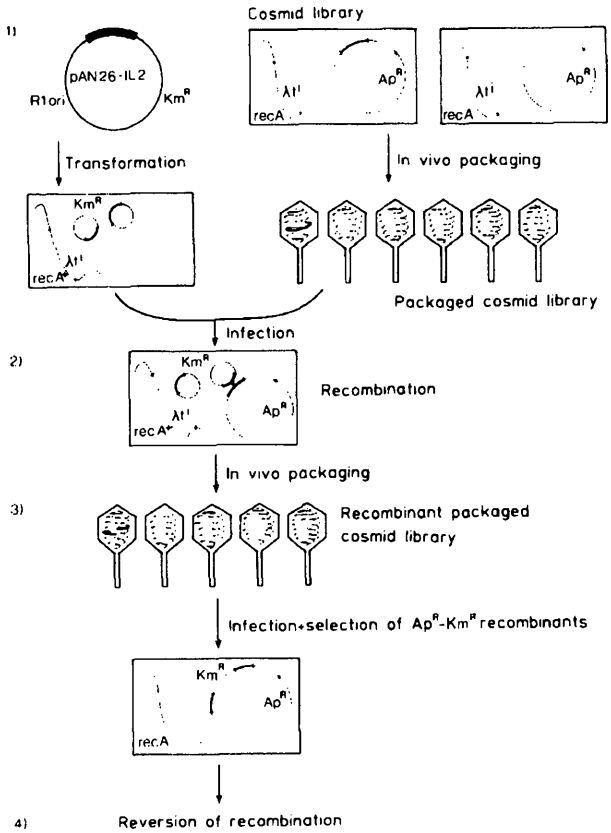

Fig. 1. Recombinant screening method using in vivo packaged cosmid libraries. (1) The sequence of interest (IL2) is cloned in pAN26, a small screening vector, and used to transform a *recA*$^+$ *E. coli* host strain carrying a temperature-inducible λ prophage ($\lambda t^i = \lambda imm434ts$). By heat induction the cosmid library is packaged in vivo forming transducing particles. (2) After transduction of the library into the screening strain recombination between homologous sequences can occur. A recombination event leads to the formation of cointegrates that carry the antibiotic resistance markers of both vectors in *cis*. (3) Recombinants are separated from nonrecombinants by in vivo packaging, transduction in a new host and selection of Ap^RKm^R colonies. (4) The genomic structure can be restored by reversion of the recombination event.

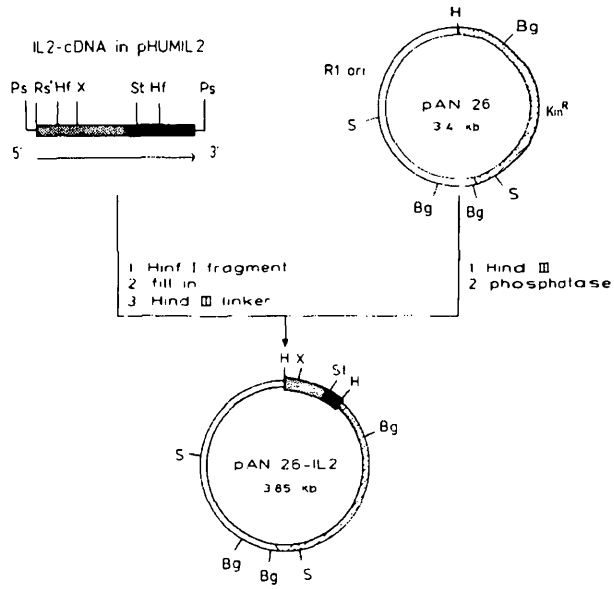

Fig. 2. Construction of the screening plasmid pAN26IL2. Sites for restriction enzymes are indicated as Ps, *Pst*I; Hf, *Hin*fI; X, *Xba*I; St, *Stu*I; H, *Hin*dIII; Bg, *Bgl*II; S, *Sal*I; Rs', *Rsa*I site created by connecting the G-tail to nucleotide 113 of IL2-cDNA (Taniguchi et al., 1983; W. S., unpublished data). IL2-coding region: dark-shaded bar; 3' noncoding region: black bar; R1 replicon: open bar; Km^R region from Tn*5*: hatched bar.

pronounced. For very large cosmids the recombination products might be packaged with decreased efficiency.

To test the specificity and efficiency of the recombination screening method, a small *Hin*dIII fragment (fragment G) of a cosmid containing the human IFNβ chromosomal gene cloned in pJB8 (Gross et al., 1981) was subcloned using pAN26 (Fig. 2) in *E. coli* BHB3169. pCosIFNβ was transformed into *E. coli* 1400 and packaged in vivo. The recombinant strains BHB3169[pAN26] and BHB3169-[pAN26IF1] were infected with cosIFNβ lysate, grown and induced for in vivo packaging as described in the legend to Table I. After infection of HB101 and selection for Km^R and Ap^R clones no colonies were found using BHB3169[pAN26], whereas doubly resistant recombinants between cosIFNβ and pAN26IF1 could be isolated with a frequency of about 1×10^{-4} (Table I). Restriction analysis of recombinant cosmids showed the expected structure (not shown).

For the isolation of the chromosomal IL2 gene from a human cosmid library by recombinant screening, human IL2 cDNA sequences cloned in pBR322 (W. S., unpublished data) were inserted into pAN26. The central 450-bp *Hin*fI fragment of the pHUMIL2 cDNA insert was subcloned into pAN26 (Fig. 2). The resulting plasmid, pAN26IL2 was transformed into *E. coli* BHB3169 for recombination screening. The approx. 300000 primary clones of the human cosmid library in *E. coli* 1400 were amplified as single colonies, resuspended and induced for in vivo packaging (Lindenmaier et al., 1982; 1984). The packaged library can efficiently be transferred to a host providing recombination and packaging functions for a limited period of time. About 10^9 packaged cosmids were used to infect

TABLE I

Efficiencies of in vivo recombination

Screening plasmid in BHB3169	Cosmid lysate[a] used for infection	Infected cells before addition of antibiotics	Titer of packaged cosmids[b] (Ap^R transductants/ml)	Titer of packaged cosmids[b] (Ap^RKm^R transductants/ml)	Recombination frequency
pAN26	pCosIFNβ	2.3×10^8	2×10^8	0	$< 5 \times 10^{-9}$
pAN26IL2	pCosIFNβ	1.8×10^8	3×10^7	0	$< 3 \times 10^{-8}$
pAN26IF1[c]	pCosIFNβ	2.9×10^8	1.0×10^8	10 000	1×10^{-4}
pAN26	cosmid library	1.3×10^8	9×10^8	70	7.8×10^{-8}
pAN26IL2	cosmid library	3.7×10^8	3.5×10^8	45	1.3×10^{-7}

[a] In vivo packaging of cosmid pCosIFNβ (Gross et al., 1981) and the human cosmid library were done essentially as described (Vollenweider et al., 1980; Lindenmaier et al., 1982).

[b] For recombinant screening, cells of BHB3169 harbouring the screened plasmid were prepared and infected with cosmid lysate by incubation at 30°C, 20 min. Infected cultures were diluted tenfold with L-broth and incubated for 40 min at 30°C. Infected cells were selected by adding Km and Ap to 15 μg/ml each. Incubation at 30°C was continued for 2 h more at 30°C. After induction and in vivo packaging, induced cells were pelleted by centrifugation, resuspended in 1/20 vol. of TM buffer (50 mM Tris·HCl pH 7.5, 10 mM $MgCl_2$) and lysed by addition of chloroform. Cell debris was pelleted by centrifugation and the supernatant used as cosmid lysate. HB101 plating cells were infected to determine the titer of Ap^R transductants and Ap^RKn^R recombinants.

[c] pAN26IF1 was constructed by inserting *Hin*dIII fragment G (0.55 kb) of pCosIFNβ into the unique *Hin*dIII site of pAN26.

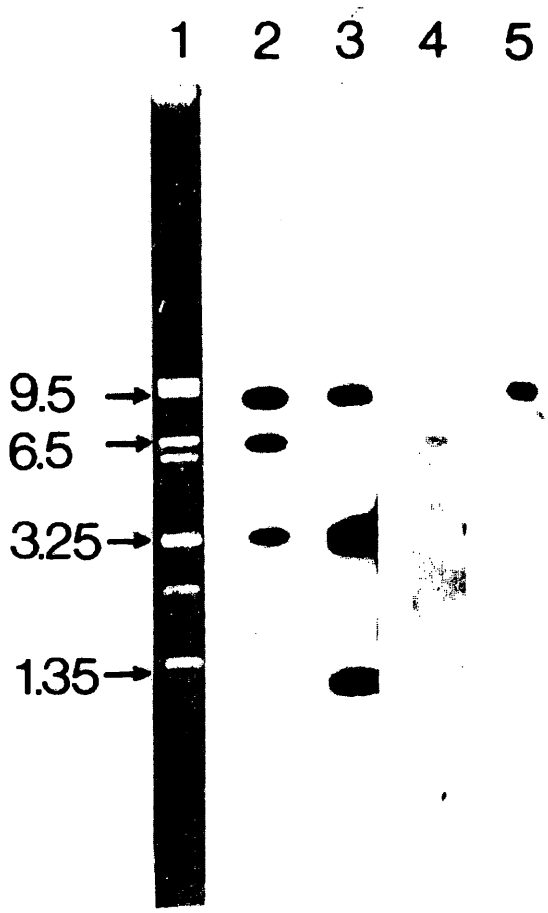

Fig. 3. Southern hybridization of *Bgl*II-digested DNA of recombinant cosmid cosHIL24. EtBr-stained agarose gel (1) and autoradiograms of hybridization to IL2-cDNA insert of pHUMIL2 (2), pAN26IL2 (3), *Hin*fI-*Pst*I fragment (4), and *Rsa*I-*Hin*fI fragment (5) of the pHUMIL2 insert (see Fig. 2). Sizes in kb are on the left margin.

E. coli BHB3169[pAN26IL2]. After recombination and repackaging, packaged cosmids were used to infect *E. coli* HB101. Ap^RKm^R colonies were found with a frequency of about 1×10^{-7}. In control experiments using BHB3169[pAN26] in combination with the human genomic library Ap^RKm^R colonies appeared with about the same frequency (Table I). We do not know whether this is due to a type of transient recombination as described for M13 phages (Dagert and Ehrlich, 1983) or to the presence of sequences homologous to pAN26 in the DNA library. But in spite of this nonspecific background, true recombinants should be highly enriched in the doubly resistant colonies.

From the recombination screening experiment with pAN26IL2 we analyzed 24 Ap^RKm^R colonies by digestion of cosmid DNA with *Bgl*II and *Pvu*II and Southern blotting. Two of the clones contained fragments hybridizing to IL2 cDNA different from the internal fragment of pAN26IL2. One of them (cosHIL24) contained three *Bgl*II fragments hybridizing to IL2 cDNA and was analyzed in more detail (Fig. 3). Hybridization to pAN26IL2 showed that it

contained the recombinant plasmid integrated into the cosmid DNA. A *Rsa*I-*Hin*fI fragment representing 5′ sequences and the *Hin*fI-*Pst*I fragment corresponding to 3′-untranslated sequences were isolated from pHUMIL2. These sequences are not contained in the recombinant plasmid pAN26IL2. When the 5′ and 3′ specific probe was hybridized to *Bgl*II-digested cosHIL24 DNA a 9.5-kb and a 6.5-kb fragment, respectively, hybridized specifically. This means that a cosmid clone containing human genomic IL2 sequences extending into the 5′ and 3′ direction from the recombinant sequence has been isolated by specific recombination.

(b) Restoration of the genomic IL2 gene structure

In the recombinant cosmid cosHIL24 the sequence of the gene is interrupted by the recombinant plasmid pAN26IL2. These sequences have to be removed to restore the genomic structure. In principle this can be achieved by reversion of the recombination event in vivo. The intramolecular recombination leading to excision of the recombinant plasmid spontaneously occurs in vivo with low efficiency even in *recA*$^-$ host strains, as can be seen by the appearance of the small recombinant plasmid. It can be enhanced by providing the functions for homologous recombination, e.g., by transfer into *recA*$^+$ *E. coli* strains or by superinfection with *λred*$^+$ phage (Poustka et al., 1984; our unpublished results). This second step probably does not occur at exactly the same position as the first one and could result in the formation of hybrid genes. In the case of cosHIL24 direct reversion was not possible. RecA$^+$ conditions resulted in a number of large deletions not confined to pAN26IL2. This instability of cosHIL24 is not a general feature of cointegrates. Other isolates were of normal stability.

To restore the chromosomal structure of the IL2 gene we had to use in vitro recombination. The IL2-coding sequence contains a single *Xba*I site (Taniguchi et al., 1983). In cosHIL24 the integration of pAN26IL2 occurred in the third exon, which contains the corresponding *Xba*I site leading to a duplication of this site. Removal of this *Xba*I fragment should restore the original structure. To do so the *Bgl*II fragments containing IL2 coding sequences have been subcloned into the *Bgl*II site of pV34 (Fig. 4). Restriction maps of the subclones confirmed the expected structure. Restoration of the genomic IL2 gene was achieved in two cloning steps (Fig. 4). The resulting plasmid (pHIL49) represents 12.8 kb of the genomic region. It was mapped with a number of restriction enzymes and showed the structure expected from the mapping of cosHIL24 and the subclones, except that pAN26IL2 sequences are deleted (Fig. 4B). It is also in agreement with restriction maps derived from published sequences of part of this region, except for one *Xba*I site (Fujita et al., 1983, Degrave et al., 1983, Holbrook et al., 1984). There are no discrepancies between fragments of human placental DNA digested with *Eco*RI, *Hin*dIII, *Bgl*II and *Pvu*II hybridizing to IL2 cDNA (not shown) and the fragments contained in pHIL49.

(c) Expression of human IL2 in mouse L-cells

To show that the human IL2 gene in pHIL49 is functional, it was introduced into mouse L*tk*$^-$ cells

TABLE II

IL2 activity in supernatants of L*tk*$^-$ mouse cells transfected with pHIL49 containing the restored human IL2 gene

Medium	IL2 activity (u/ml)[a] Transfected cells		Control cells	Medium control
	Exp. 1	Exp. 2		
RPMI 1640	0.11	0.4	<0.01	<0.01
RPMI 1640 +10% FCS	0.13	0.25	<0.01	<0.01
RPMI 1640 +20 μg/ml PHA	0.12	0.21	nt	<0.01
RPMI 1640 +10 μg/ml ConA	0.12	0.12	<0.01	<0.01
RPMI 1640 +10 μg/ml TPA +10 μg/ml A23187	0.13	0.1	<0.01	<0.01

[a] IL2 activity was determined as the T cell growth factor-mediated [^{3}H]thymidine incorporation into the strictly IL2 dependent murine T cell line CTLL-2, which was kindly provided by Dr. H.H. Peter (Freiburg, F.R.G.). Briefly, IL2-containing supernatants were serially diluted on 96-well culture plates in RPMI1640 medium supplement with 10% heat-inactivated FCS. 4 x 10^3 cells were plated per well and incubated for 24 h at 37°C. After an [^{3}H]thymidine pulse (1 μCi/well; 1 Ci/mmol) for 4 h the cells were harvested with an automatic cell harvester. The IL2 activity was expressed as u/ml.

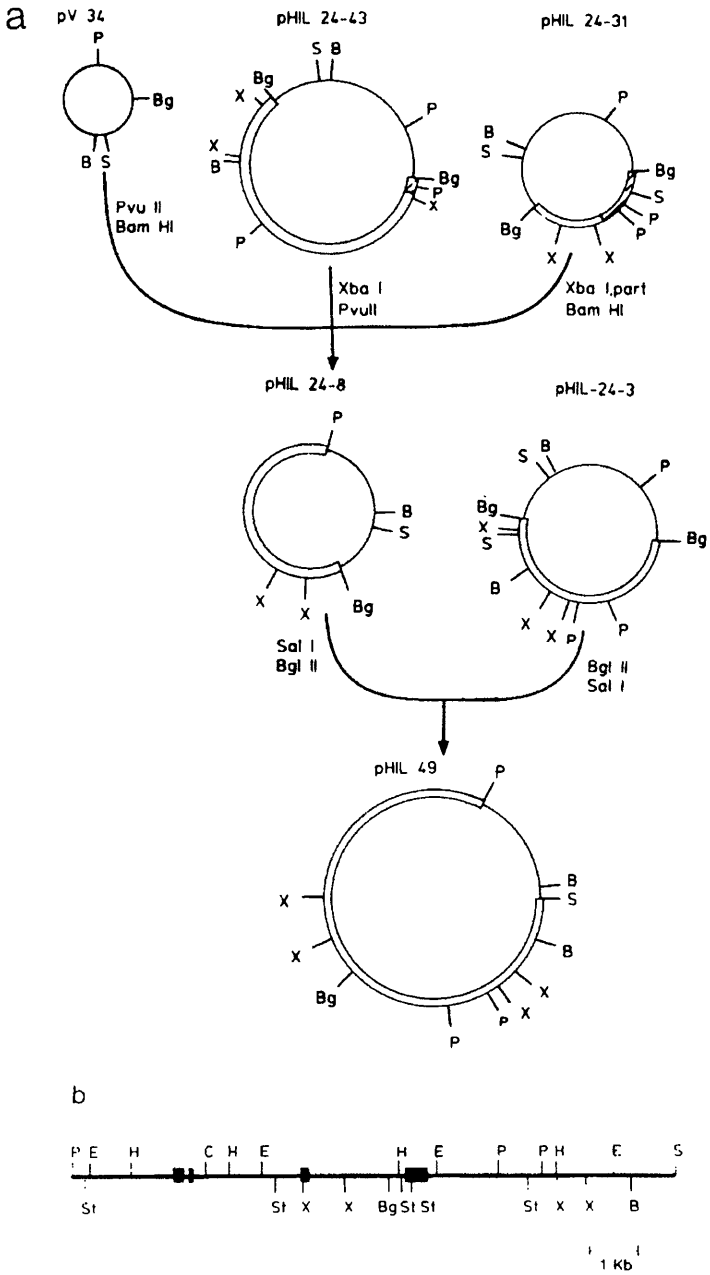

Fig. 4. Restoration of the chromosomal IL2 gene structure. (a) Subclones pHIL24-43, pHIL24-31 and pHIL24-3, containing the 9.5-kb, 3.1-kb and 6.5-kb *Bgl*II fragments of cosHIL24, were digested with the enzymes indicated and the fragments corresponding to genomic IL2 sequences isolated. In the first step the *Pvu*II-*Xba*I fragment of pHIL24-43 and the partial *Xba*I + *Bam*HI fragment of pHIL24-31 were cloned into *Pvu*II + *Bam*HI-digested pV34 to remove the recombinant plasmid sequences. The resulting plasmid pHIL24-8 was digested by *Bgl*II + *Sal*I, and the 3' fragment isolated from pHIL24-3 by digestion with the same enzymes was inserted. Open bars: human genomic sequences; hatched bars: pAN26 sequences. (b) Restriction map of the restored region of the human IL2 gene contained in pHIL49. Sites for B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II; S, *Sal*I; St, *Stu*I and X, *Xba*I are indicated. Black bars indicate coding regions.

using the cotransfer technique (Wigler et al., 1979). Stable HAT-resistant clones were selected and supernatants tested for IL2 activity on the strictly IL2-dependent mouse cell line CTLL2. Supernatants of clones stably transfected with pHIL49 showed low but clearly detectable levels of constitutive expression of IL2 activity (Table II). When mouse L cells transfected with the human IL2 gene were treated with PHA, ConA, TPA or TPA plus calcium ionophore A23 187, agents that induce IL2 production in peripheral blood lymphocytes and Jurkat cells (Robb,1984), no increase in the amount of IL2 produced could be detected (Table II). This lack of inducibility could be due to the absence of regulatory DNA sequences on pHIL49 controlling the induction and/or the lack of other regulatory factors from the host cells which might be species- and/or differentiation-specific.

The IL2 activity produced by mouse L cells transfected with pHIL49 gene was further characterized by gel filtration. It eluted at exactly the same position

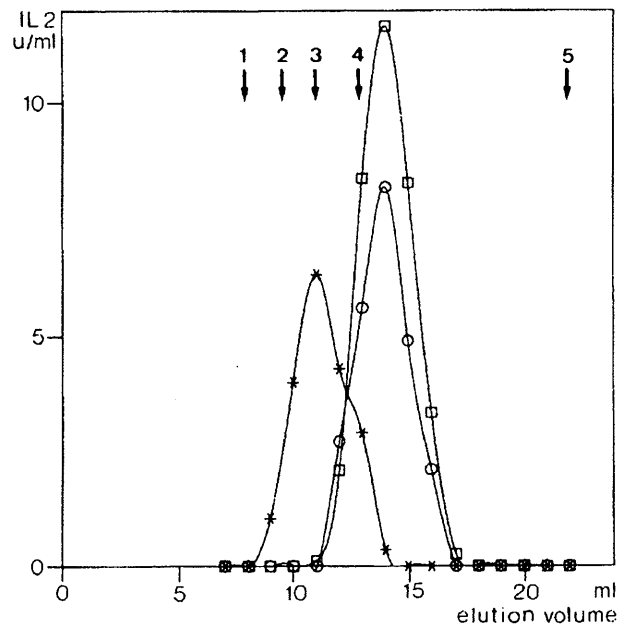

Fig. 5. Gel filtration on Sephadex G75. IL2 activities produced by L*tk*$^-$ mouse cells transfected with pHIL49 (○) were compared to mouse IL2 (*) and human IL2 (□). M_r markers were dextran blue (1), bovine serum albumin (2), ovalbumin (3), myoglobin (4) and phenol red (5). Serum-free culture supernatants were concentrated by $(NH_4)_2SO_4$ precipitation (80% w/w). The pellet was dissolved in 10 mM Hepes pH 7.4, 100 mM NaCl and fractionated on Sephadex G-75 column. The eluted fractions were collected over FCS (final concentration 10% v/v), filter-sterilized and tested for IL2 activity.

as authentic human IL2 from Jurkat cells or normal human lymphocytes and was clearly distinguishable from mouse IL2 (Fig. 5). To see whether posttranslational processing and modification is identical to authentic human IL2, which is *O*-glycosylated at threonine in position 3 (Robb et al., 1984, Conradt et al., 1985), more efficient production, purification and analysis will be required.

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