Volume 14 Number 9 1986 Nucleic Acids Research

Cloning and expression of the rat interleukin-3 gene

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Received 11 March 1986; Accepted 11 April 1986

ABSTRACT

Genomic clones carrying the rat interleukin-3 (IL-3) gene have been isolated and the nucleotide sequence of the gene determined. Alignment of this sequence with that of the mouse IL-3 gene has allowed the structure of the rat IL-3 gene to be deduced. The intron-exon boundaries are conserved and extensive nucleotide homology (approx 90%) is present in the 5' flanking region and the portion of the gene coding for the signal peptide. Several proposed regulatory sequences are conserved and an analogous element to the tandem repeat in intron 2 of the mouse gene is also present. The predicted amino acid sequence for mature rat IL-3 shows surprisingly low homology (54%) with its murine counterpart, although all four cysteine residues are conserved. The rat IL-3 gene was expressed in monkey COS-1 cells and colony assays established that rat IL-3 is a multi-lineage haemopoietic growth regulator. There was little cross-reactivity of the respective IL-3 species on mouse and rat bone marrow cells suggesting that rat IL-3, in concert with its receptor, has evolved significantly away from the mouse IL-3/receptor system.

INTRODUCTION

Interleukin-3 (IL-3) is a member of the family of colony stimulating factors believed to regulate haemopoiesis[1-3]. It is involved in regulating growth and differentiation of pluripotent stem cells leading to the production of all the major blood cell types. The major natural source of IL-3 is the antigen-stimulated T lymphocyte, although it is also produced by a number of continuous cell lines[4-5]. IL-3 has been extensively characterized in the murine system, and cDNA[6,7] and genomic[8,9] clones have been reported recently. Little is known about IL-3 species in other mammals. There has been some biological characterization of mucosal mast cell growth factor in rats which may be equivalent to IL-3[10-13]. Pluripotent stem cell factor activity has been reported to be produced by the human bladder carcinoma cell line 5637[14], but it is still not established whether an exactly analogous lymphokine to murine IL-3 exists in man. Southern

hybridization analysis of mammalian DNA's, using a murine IL-3 cDNA probe, fails to detect homologous sequences in most mammalian species, even under conditions of relatively low stringency (unpublished data). This apparent low conservation of mammalian IL-3 genes contrasts with genes for other lymphokines such as IL-2, GM-CSF and γ-interferon which cross-hybridize between mouse and human[15-17].

As part of a study of the structure and biological role of IL-3 in other mammals, and in view of the possibility of studying the role of IL-3 using rat experimental models, we felt it was desirable to isolate the rat IL-3 gene and express it in animal cells. This is described in the present work together with the determination of some of the biological properties of recombinant rat IL-3.

MATERIALS AND METHODS

General Methods

(PVGxDA) male laboratory rats were used as the source of DNA for all experiments. Genomic DNA was prepared by homogenization and treatment with sarkosyl and proteinase K, followed by centrifugation in CsCl/ethidium bromide gradients[18,19]. Large scale λ DNA preparations were made from 1 litre cultures in Luria broth, lysed after 6 hr aeration at 37°C. Following addition of $CHCl_3$, phage DNA was purified as described[20]. Small scale preparations of λ DNA from plate lysates on Luria + Mg agarose plates were as described[8], and plasmid DNA minipreparations were made by the rapid boiling method[21]. Large scale plasmid preparations were carried out according to Clewell and Helinski[22] and plasmid DNA was purified using CsCl/ethidium bromide density gradients.

DNA fragments were isolated by electrophoretic separation on low-melting point agarose gels. Transformations were carried out using the high efficiency method of Hanahan[23].

The primers used for probe preparation and sequencing were synthesized by the phosphoramidite method. Autoradiography was carried out at -70°C using Fuji X-ray or Kodak XAR-5 film with Dupont Lightning Plus intensifying screens.

Construction and Screening of the Rat Genomic Library

Total genomic DNA from rat liver was partially digested with Sau3A and fragments in the size range 9-20kb were purified from low-melting point agarose. These fragments were ligated with λEMBL3A arms prepared by EcoRI/BamHI digestion of the phage DNA, and then packaged into bacteriophage particles[21,24]. Percentage recombinants was determined by spi selection[25].

The library was plated out for screening (using E.coli strain ED8655) on L top agarose/L agar in glass baking dishes (20cm x 30cm), approx 250,000 pfu per dish. Nitrocellulose replicas of plates containing plaques were prepared for hybridization as described by Benton and Davis[26].

Filters were routinely pre-washed in 50 mM Tris-HCl pH8/1M NaCl/1mMEDTA/0.1% SDS at 42°C for 1-2 hr, pre-hybridized in 6xSSC/5xDenhardt's solution/ 10mM EDTA/0.5% SDS/50 μg/ml salmon sperm DNA at 65°C for 1-4 hr and hybridized in the same solution plus probe at 65°C for 18 hr. Filters were rinsed in 5xSSC/0.1%SDS and then washed twice in 2xSSC/0.1%SDS at 65°C for 45 min.

Probe Preparation

The 467 bp HindIII-NcoI fragment of the murine IL-3 cDNA-containing plasmid pILM3[6] was isolated and used as a template for primed synthesis of radioactively labelled cDNA, using a synthetic random decamer as primer. Probe preparation was as described[8], except that synthesized probe was routinely separated from un-incorporated label and very small labelled fragments by chromatography on Sephadex G-50 (fine).

Southern Hybridization

Restriction endonuclease digests of total genomic DNA (15 μg per lane) or recombinant bacteriophage DNA (0.5-1 μg per lane) were electrophoresed on 1% agarose/TAE (40mMTris-acetate/1mM EDTA) gels at 25mA for 16 hr. The gels were treated and blotted to nitrocellulose as described[27]. Pre-washing, pre-hybridization and hybridization of filters was as for genomic library screening. Following hybridization, filters were rinsed briefly in 5xSSC/0.1%SDS at room temperature, and then washed twice in 4xSSC/0.1%SDS at 65°C for 45 min. Stepwise reductions of salt

concentration (down to 0.1xSSC) were used for additional washes when necessary.

DNA Sequence Analysis

The sequence of the HindIII fragment covering the rat IL-3 gene was determined by the chain termination method of Sanger[28], using a universal flanking primer[29]. The DNA was prepared for sequencing by sonication of self-ligated fragment, T4 DNA polymerase repair and electrophoresis in low melting point agarose[30]. Fragments in size ranges 300-500 bp, 500-1000bp and 1000-2000bp were isolated from the gel and cloned into the SmaI site of M13mp10[31]. Competent cells of E.coli strain TG1, prepared by the method of Hanahan[23], were used for the transfection of the recombinant M13 DNA. Gels were routinely 5% acrylamide with "wedged" bottoms, and were dried to increase resolution[32]. Compressions were checked using gels containing 25% formamide[33]. DNA sequence data was entered directly from autoradiograms into the computer using a digitizer, and assembly and analysis of this data was performed using the computer programs of Staden[34-36]. The Genbank database was searched using the progam of Wilbur and Lipman[37].

Expression of Rat Interleukin-3

COS-1 cells were seeded at $5x10^5$ cells per 60mm diameter petri dish, grown overnight in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS), then transfected with the IL-3 expression vectors. 4 µg of plasmid DNA plus 16µg of carrier DNA was transfected per 10^6 cells by calcium phosphate precipitation and glycerol shock (15% glycerol for three minutes)[38,39]. The cells were washed in DMEM containing 10% FCS, and incubated in 5ml of the same medium at 37°C in 5% CO_2. Supernatants were collected at 72 and 144 hours post-transfection and filtered through Amicon 0.2µm filters. Samples were stored at 4°C prior to assay.

Assays for IL-3

1. Colony forming assays. Colony assays were done using bone marrow cells from WISTAR rats or BALB/c mice, scoring colonies on day 7 of culture[39]. The entire culture was fixed with 2.5% glutaraldehyde then stained with Lutol Fast Blue-hematoxylin to identify granulocyte-macrophage, erythroid, mixed erythroid and

megakaryocytic colonies. Each culture contained 75,000 bone marrow cells in 1.5 ml.

2. Bone marrow cell proliferation assays. Serial two-fold dilutions of test material were made in microtitre plates in 50µl RPMI 1640, 10% FCS. $5x10^4$ WISTAR rat or BALB/c mouse bone marrow cells were added to each well. Bone marrow cells from PVG, DA, PVG x DA, JC, PVG x JC and Fischer rats were also tested in this assay. Cultures were pulsed overnight on day 3 with [^{3}H]-thymidine. Activity is expressed as a reciprocal of the titration endpoint, the endpoint being defined as the supernatant dilution at which activity is no longer detectable[40].

RESULTS

Southern Hybridization of Rat Genomic DNA

Rat genomic DNA was digested with the restriction enzymes EcoRI and HindIII, electrophoresed on an agarose gel, blotted to nitrocellulose and hybridized with a murine IL-3 cDNA probe. A single hybridizing fragment of appropriate intensity for a single-copy gene was observed in the HindIII digest at approx. 5.8kb (Fig.1). The EcoRI digest gave two bands, suggesting the presence of an EcoRI site within the rat IL-3 gene. The reason for the different intensity of the two EcoRI bands is not clear but may be due to a greater representation of sequences derived from particular regions of the template fragment in the random primer probe.

Screening of the Rat Genomic Library

A library of approx. 10^6 recombinant phage was generated by cloning 9-20 kb fragments from a partial Sau3A digest of total genomic Rat DNA into λEMBL3A arms created by BamHI/EcoRI digestion. The complete library was plated out onto 4 baking dishes (approx. 250,000 recombinant phage per plate) and duplicate lifts were taken from each dish. A total of 5 plaques were identified by hybridization to the 467 bp HindIII-NcoI fragment of murine IL-3 cDNA labelled with ^{32}P by randomly primed synthesis. These 5 clones were purified and small scale λDNA preparations were carried out. Digestion of the DNA with EcoRI showed the five clones to be overlapping, each containing one or both of the two expected EcoRI fragments (Fig. 2). Clone λR3, which contained

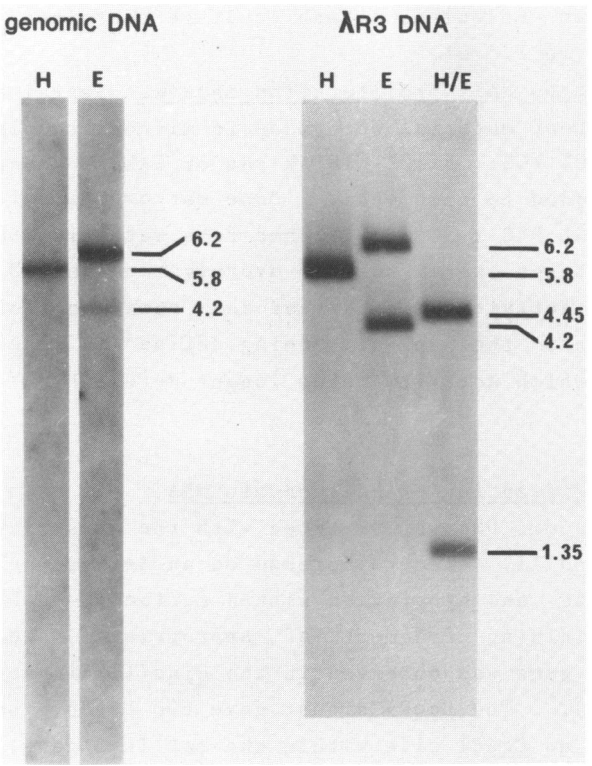


Figure 1 Southern blot analysis of rat genomic DNA and DNA from the genomic clone λR3, probed with an [α-^{32}P] dATP - labelled murine IL-3 cDNA fragment derived from pILM3[6]. Gel electrophoresis, blotting, probe preparation and hybridization conditions are given in Materials and Methods. Restriction endonuclease abbreviations are E, EcoRI and H, HindIII. Sizes given are in kilobase pairs (kb).

both fragments, was grown in large scale for further characterization. Southern hybridization of enzyme digests of λR3 DNA shows that it contains the fragments originally seen in the genomic DNA digests (Fig. 1) and allows localizaton of the rat IL-3 gene to a pair of fragments (3.3kb and 1.25kb) from a BamHI/HindIII double digest (Fig.2).

Nucleotide Sequence of the Rat IL-3 Gene

The nucleotide sequence of the 5.8 Kb HindIII fragment encompassing the rat IL-3 gene was determined on both strands by the chain termination method. The sequence of this region

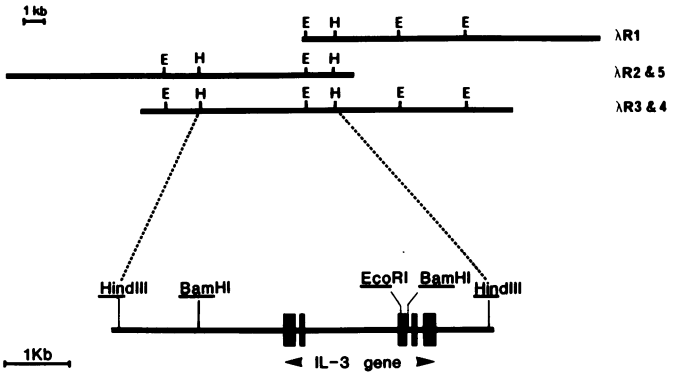


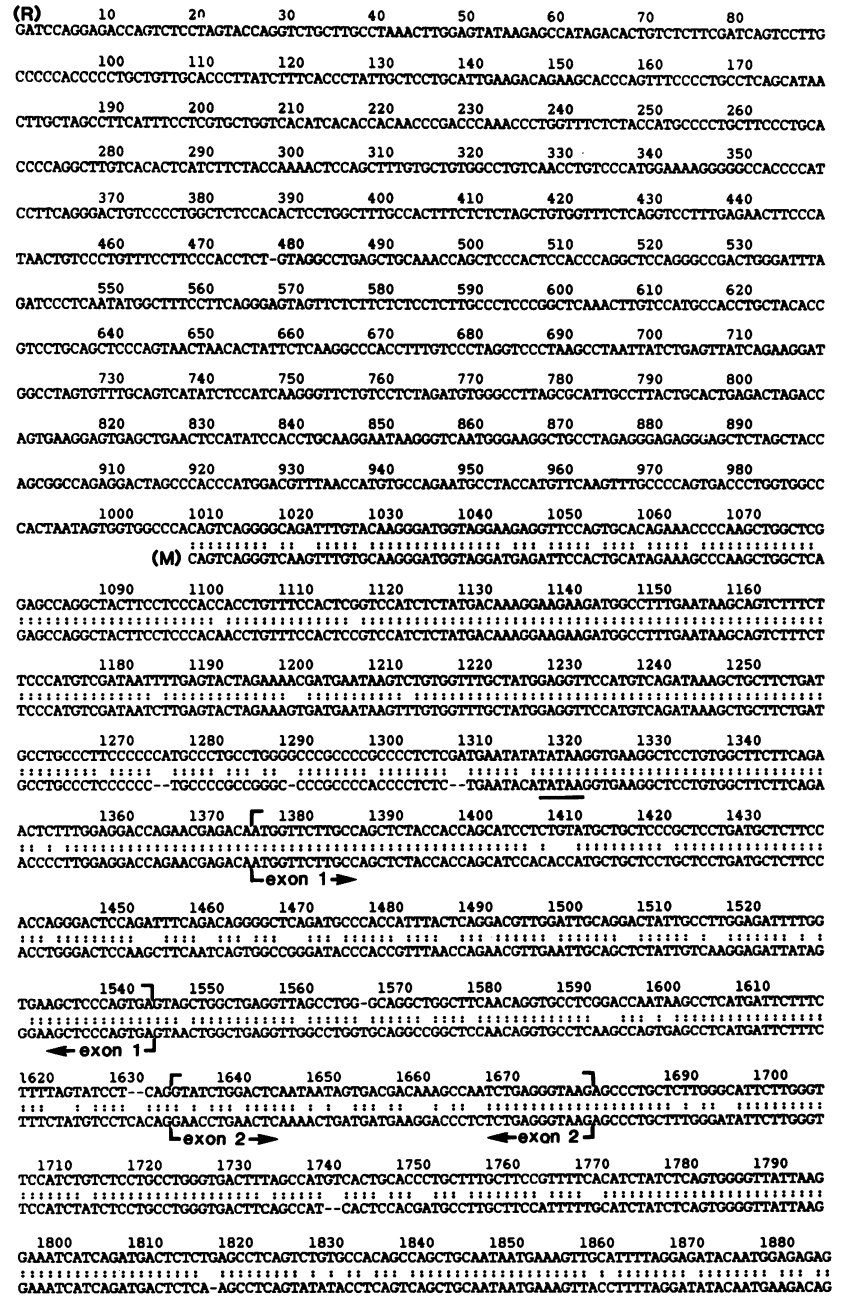
Figure 2 Restriction map of the rat IL-3 gene showing the regions covered by the 5 overlapping clones isolated from a λEMBL3A library of rat genomic DNA. The map of the gene was deduced from the characterization of purified λR3 DNA. Proposed exons are indicated by solid boxes, and cover the region from the AUG codon believed to be the start site of translation through to the translational termination codon UAA. Restriction endonuclease abbreviations are E, EcoRI and H, HindIII.

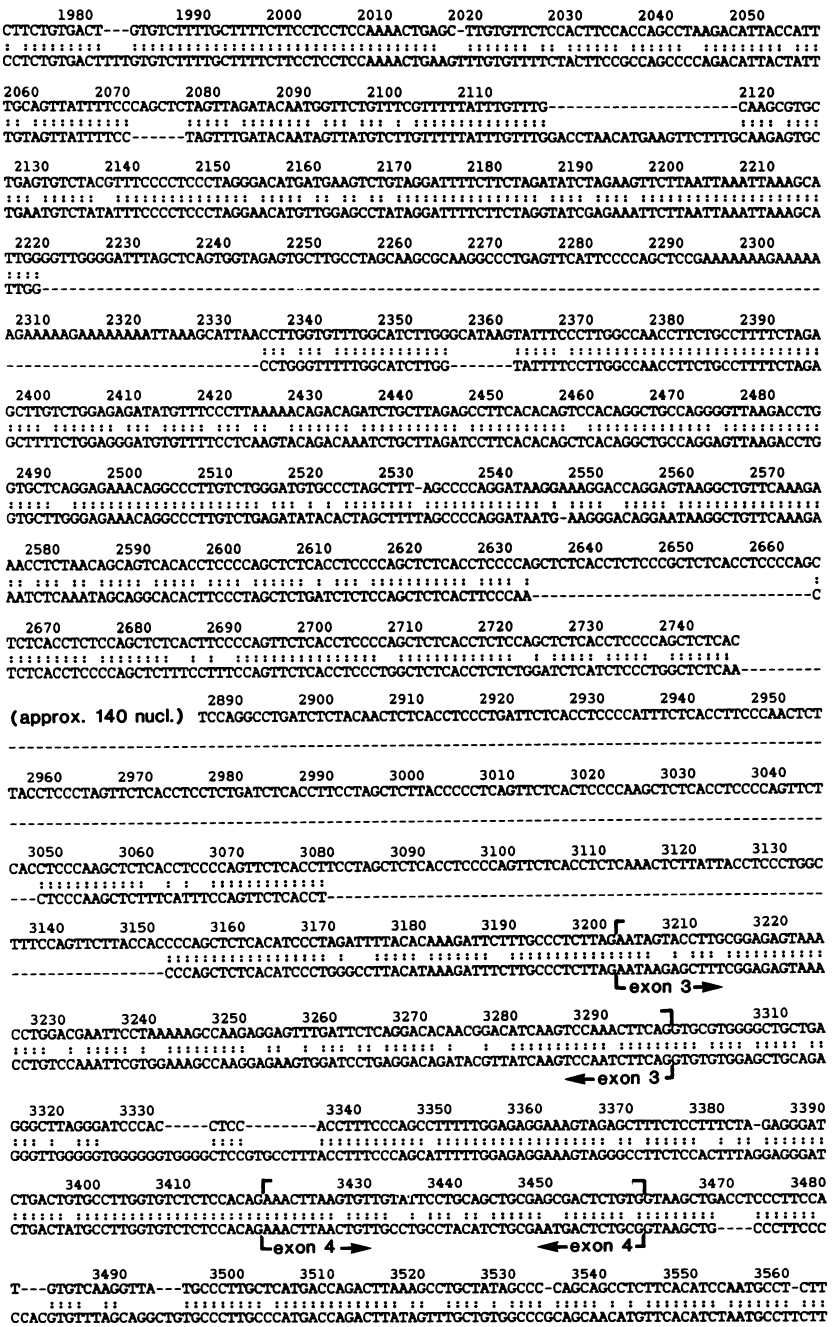
(excluding the 5' 1.25kb HindIII/BamHI fragment) is presented, together with an alignment against the murine IL-3 gene sequence, in Fig.3. The overall homology between the two nucleotide sequences in the coding regions is 76% ; the introns and flanking regions (approx. 200 nucleotides either side of the gene) are more highly conserved, with 80% and 90% homology respectively.

Structure of the Rat IL-3 Gene

Sufficient conservation of gene structure was present to allow the structure of the rat IL-3 gene to be deduced by alignment with the gene for murine IL-3 (Fig. 3). The rat IL-3 gene consists of five exons interrupted by four introns with conservation of the intron/exon boundaries between the rat and mouse genes. The deduction of the gene structure is greatly assisted by the high sequence homology through these regions. All introns interrupt the coding sequence between codons.

Sequence homology in the region corresponding to the leader sequence and extending 400 nucleotides upstream of the translational start site in murine IL-3 is extremely high. It is therefore likely that translation of rat IL-3 commences at nucleotide position 1374, making the first ten amino acids identical to those in the leader portion of murine IL-3. Transcriptional


(R)
(M)
exon 1
exon 1
exon 2
exon 2


(approx. 140 nucl.)
exon 3
exon 3
exon 4
exon 4

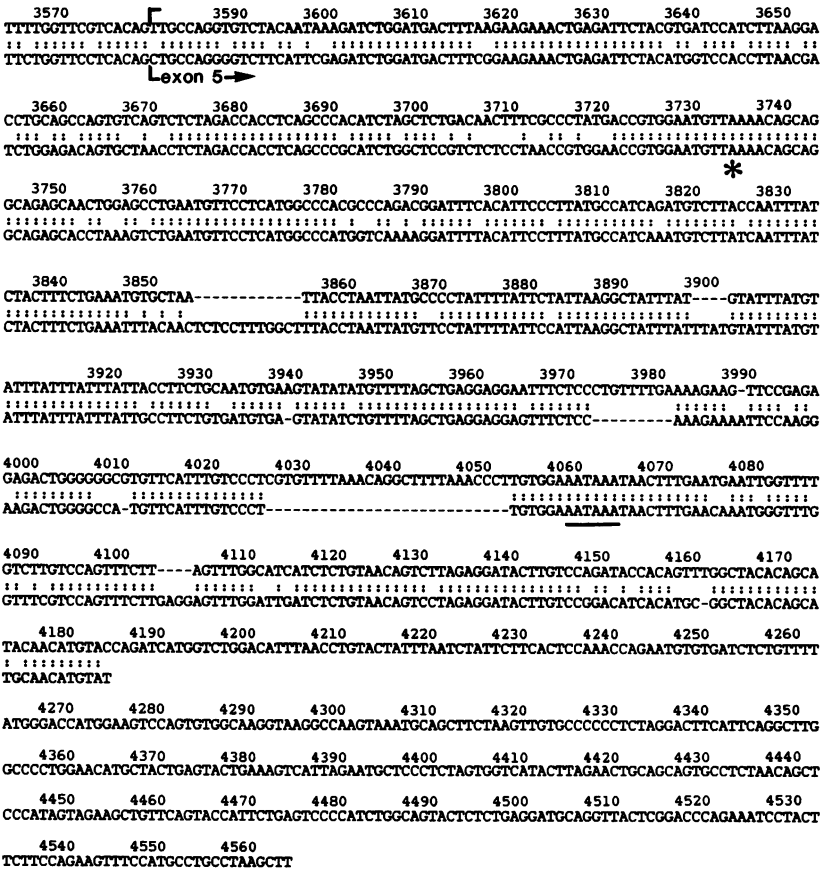


Figure 3 Nucleotide sequence of the rat (R) IL-3 gene covering the 3.3 Kb BamHI and 1.25 Kb BamHI/HindIII fragments, shown aligned against the sequence of the murine (M) IL-3 gene. TATA box and poly-adenylation signals are underlined. Intron/exon boundaries are shown as deduced for the murine gene[8,9].

control signals (including the potential enhancer hexanucleotide CCGCCC) described by Campbell et al[8] and Miyatake et al[9] are identical, although the proposed CAAT sequence would be a further 7 nucleotides upstream of the TATA box in the rat gene than is the case in murine IL-3.

Nucleotide sequence homology of the 3' end of the coding region is once again high, and there is an in-frame translational termination codon at position 3733. A single AATAAA sequence is found in the 3' untranslated region, 25 nucleotides nearer the

TAA stop codon than in murine IL-3. This sequence is likely to be involved in polyadenylation of the mRNA[41,42].

Other conserved features include two sets of tandemly repeated sequences. The first, a 14-15 bp element repeated about 12 times in intron 2 of murine IL-3, is also found in intron 2 of rat IL-3, except that in the latter case the repeat appears to extend for a further 400-450 nucleotides ie 25-30 more repeating units than in the murine gene. The complete sequence through these repeating units was not determined due to the difficulty in obtaining clones through this region. The amount of "missing" sequence was deduced by visual estimation from sequencing gel autoradiograms together with careful estimation of restriction fragment lengths. The consensus sequence of these repeats in the rat gene, AGCTCTCACCTCCC, is identical to the consensus sequence of the murine IL-3 intron 2 repeats[8]. The 9-bp inverted repeat which flanks these tandem repeats in the mouse IL-3 gene is not conserved in the rat gene. The second repetitive element occurs between the termination codon and the polyadenylation signal (nucleotides 3872-3922), and consists of nine copies of the sequence TATT[8].

One further feature of interest is the presence of sequences in the 5' untranslated region which show homology to murine B1, B2 and new Alu type repeating elements (data not shown). These elements represent major classes of short repeats dispersed throughout the mouse genome[43,44], and may each be present in up to 50,000 copies per haploid genome. Sequences homologous to rat poly (RY) repeats[45] are also found in this 5' region. The function of these sequences is unknown; there are suggestions ranging from a possible role in regulation of transcription/mRNA transport/translation[46-48] to the notion of "junk DNA" or pseudogenes[49].

Amino Acid Sequence of Rat IL-3

The deduced amino acid sequence of rat IL-3 is shown in Fig. 4 compared to murine IL-3. Overall homology is 59% which reduces to 54% in the "mature" protein ie. after the removal of the putative leader sequence[50,51], which is the most highly conserved part of the protein. Rat IL-3 has two potential N-glycosylation sites : the second of these corresponds to one of

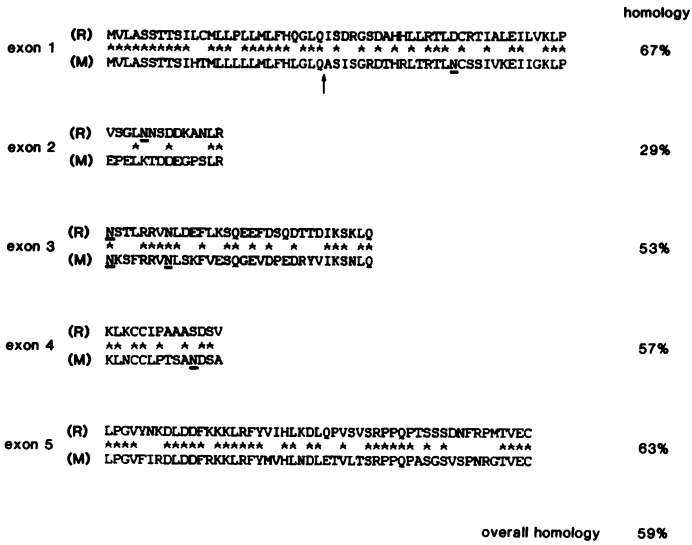


Figure 4 Comparison of predicted coding sequences of the exons of rat (R) and murine (M) IL-3 genes. Potential N-glycosylation sites are underlined. The arrow indicates the most likely site of cleavage of the signal peptide[50,51].

the 4 potential sites in murine IL-3. All four cysteine residues are conserved.

Expression of Rat IL-3 in Monkey COS-1 Cells

The 5.8kb HindIII fragment carrying the entire rat IL-3 gene (including transcriptional/translational control signals) was cloned into the HindIII site of the expression vector pSV2-neo[52]. Two clones were isolated, one for each possible orientation of the IL-3 gene relative to the SV40 promoter: pILR1 contained the IL-3 insert in the same orientation as the SV40 promoter and pILR2 had the insert in the reverse direction. Purified plasmid DNA from both constructions was used to transfect monkey COS-1 cells. Supernatants from the transfected cells were collected at 72 and 144 hours, based on previous experience with this expression system for murine IL-3. Murine IL-3 was prepared at the same time, using pSV2-neo vectors containing either genomic (pILM13) or cDNA (pILM4) murine IL-3 inserts. Supernatants from untransfected COS-1 cells were used as controls.

Colony Stimulating Activity of Rat IL-3

Recombinant rat IL-3 from both pILR1 and pILR2 stimulated

Table 1: Bone Marrow Proliferation Activity of Rat and Murine IL-3

Growth Factor	Source	Bone Marrow Proliferation[a] (Units/ml) Mouse	Rat
Mouse Il-3	WEHI-3 CM	1025	<10
Recombinant mouse IL-3	COS-1	.250	<10
		360	<10
		90	<10
Recombinant rat IL-3	COS-1	<10	725
		10	510
		50	630
Mouse GM-CSF	purified[b]	3100	210

(a) Proliferation was measured as described in Methods using bone marrow cells from either Balb/c mice or WISTAR rats. Activity is expressed as the reciprocal of the titration end-point. COS-1 conditioned medium showed no detectable activity.
(b) Purified murine GM-CSF was obtained from Genzyme (Boston, Mass.) Lot No. 01531 and was verified to be GM-CSF by its ability to stimulate growth of FDC-P1 but not 32Dcl-23 cells.

colony formation only with rat bone marrow cells, while expressed murine IL-3 stimulated colony formation only with mouse cells. Bacterially expressed murine IL-3 was also inactive on the rat bone marrow cells.

In these experiments, rat IL-3 produced the full range of colony types expected for this factor on rat bone marrow cells, although there was a much greater proportion of megakaryocytes and eosinophils amongst the colonies generated than in the corresponding assays of murine IL-3 on murine cells. (A similar elevated response of megakaryocytes and eosinophils has been observed when assaying bacterially expressed murine IL-3). The biological properties of recombinant rat IL-3 will be reported in detail elsewhere (A.J.Hapel, D.R.Cohen and I.G.Young, In preparation).

Proliferation of Rat and Mouse Bone Marrow Cells in IL-3

Bone marrow cells are generally as sensitive as FDC-P1 cells for detecting IL-3 and GM-CSF activities in the mouse when used as at 10^5 cells per well in the standard microtitre cell proliferation assay. As there are no established cell line assays for

rat IL-3 we used bone marrow cells from a variety of rats and BALB/c mice to measure the growth factor activity of expressed rat and mouse IL-3.

The data in Table 1 show that rat IL-3 had good activity in promoting the proliferation of WISTAR rat bone marrow cells and slight activity on murine cells, while expressed murine IL-3 was active only on cells from murine bone marrow. This is in contrast to murine GM-CSF which has significant activity in the rat system. A similar result has been obtained using bone marrow cells from other strains of rats, except that PVG, PVG x JC and PVG x DA rats gave end-points about 4-8 fold greater than WISTAR, Fischer and JC rats. More extensive characterization of the biological relationship between rat and murine IL-3 will be described elsewhere (A.J.Hapel, D.R.Cohen and I.G.Young, In preparation).

DISCUSSION

Previous work on the biology and molecular biology of IL-3 has been predominantly with the mouse system. It is clear from the work described above that the rat genome carries an IL-3 gene which is closely related in overall structure to that found in the mouse genome. The cloning and sequence analysis of the rat IL-3 gene has enabled a detailed comparison to be made between the rat and mouse genes. The flanking regions (200 nucleotides either side of the gene) and introns of the mouse and rat IL-3 genes show 90% and 80% nucleotide homology, respectively, with the intron/exon junctions fully conserved. The portion of exon 1 encoding the putative signal peptide is also highly conserved (90%). Surprisingly, the nucleotide homology between the coding regions is only 76%. This is reflected in the amino acid homology which is only 54% for mature rat and mouse IL-3 (59% including the signal peptide). This is atypical of other lymphokines characterized to date. For example, γ-IFN shows an amino acid homology of 87% between mouse and rat[53], while IL-2 and GM-CSF show mouse : human amino acid homologies of 60% and 54% respectively[15,16,54]. The very high conservation of the signal peptide region observed between rat and mouse IL-3 is also not typical of the other lymphokines[15,16,53,54].

The conservation of the two sets of tandomly repeated units is also of interest. The TATT sequence, repeated nine times between the translational termination codon and the poly-adenylation signal in both murine and rat IL-3, is also found in a similar position in all IL-2, GM-CSF and Y-IFN genes sequenced to date[15-17,53-58]. The consensus sequence of the other repeat, a 14-15 bp element in intron 2, is identical to that of the tandem repeats in intron 2 of mouse IL-3. This element shows homology to a human BKV enhancer sequence homolog[8,59], and also to a portion of the consensus sequence for a 33-bp human myoglobin gene tandem repeat[8,60]. Similar repeats are found in the human insulin gene[61] and globin gene complex[62] and are associated with "hypervariable regions" of DNA.

Expression experiments using monkey COS-1 cells have demonstrated that the rat IL-3 gene encodes a multi-lineage haemopoietic growth regulator, which appears to have an analogous biological role to mouse IL-3[63]. The low amino acid homology between rat and mouse IL-3 correlates with the demonstration that rat and mouse IL-3 show little cross-reactivity. Other lymphokines and CSF's are somewhat varied in this regard. For example, human IL-2 is about 5 times more active on mouse cells than is murine IL-2 on human cells[15], whereas human and murine GM-CSF's do not cross-react either way[16]. Rat Y-IFN is active on mouse cells but not on human cells[53], and human M-CSF can stimulate growth of murine macrophages[64]. The low sequence homology and poor cross-reactivity suggest that rat IL-3, together with its receptor, have evolved significantly away from the murine IL-3/receptor system.

The availability of the rat IL-3 gene together with recombinant rat IL-3 should assist in studying the biological role of IL-3 in rat experimental models such as adjuvant-induced arthritis[65], graft rejection[66] and megakaryocyte proliferation[67,68]. The rat gene sequence also provides useful information for continuing studies on the regulatory elements controlling IL-3 expression.

ACKNOWLEDGEMENTS

We would like to thank G. Mayo for synthesis of oligonucleotides, R.M. Johnson for some DNA preparations, A. George

for preparation of packaging extracts and K. Ridgway, W. Tucker and P. Townsend for technical assistance. We are also grateful to H. Campbell for helpful discussions and to G. Quinn for programming assistance.

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