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Cloning and sequence analysis of the rat augmenter of liver regeneration (ALR) gene: Expression of biologically active recombinant ALR and demonstration of tissue distribution

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ABSTRACT **A full-length cDNA clone encoding a purified augmenter of liver regeneration (ALR) factor prepared from the cytosol of weanling rat livers was isolated. The 1.2-kb cDNA included a 299-bp 5′ untranslated region, a 375-bp coding region, and a 550-bp 3′ untranslated region. It encoded a protein consisting of 125 amino acids. The molecular weight of ALR calculated from the cDNA was 15,081, which is consistent with the size estimated by SDS/PAGE under reducing conditions. The molecular weight of the purified native ALR estimated by SDS/PAGE under nonreducing conditions was ≈30,000; thus ALR apparently has a homodimeric structure. The recombinant ALR produced by expression of the cDNA in COS cells was tested *in vivo* in the canine Eck fistula model and found to have potency equivalent to the purified native ALR. The 125-aa sequence deduced from the rat ALR cDNA shows 50% homology to the amino acid sequence of the gene for oxidative phosphorylation and vegetative growth in the yeast *Saccharomyces cerevisiae*.**

Heat-stable hepatocyte stimulatory activity has been described in the liver of weanling rats and in the regenerating liver or serum of adult rats and dogs after partial hepatectomy (1–6). We have previously reported the progressive purification to ×831,000 of the responsible factor (7–11), called augmenter of liver regeneration (ALR). This growth factor (originally called HSS), which is found in hepatocyte cytosol (4–6), does not affect the resting liver, but it augments the proliferative response to hepatectomy in rats (4) and dogs (5) and dramatically increases the hyperplasia caused by canine portacaval shunt (Eck fistula) (6).

Attempts also have been made by others to purify rat (12, 13) and human ALR (14, 15). We report here the complete amino acid sequence of a 30-kDa band from our purification product and the cloning and sequence analysis of its cDNA.‖ Full hepatotrophic activity of the recombinant ALR was then demonstrated with the Eck fistula model, and its mRNA expression was studied in the tissues of normal rats.

MATERIALS AND METHODS

Materials. The cDNA synthesis and cloning systems were purchased from Amersham and Pharmacia LKB. Enzymes for DNA manipulation were obtained from Toyobo (Osaka, Japan). Plasmid DNAs were purchased from Stratagene and Clontech. Radioisotope was purchased from Amersham.

Purification and Sequencing of Rat ALR. The method of purification of ALR from male Fisher weanling rats was as reported (9, 10). The procedures involved the following successive steps: ethanol precipitation, ultrafiltration through an Amicon PM30 membrane, cation-exchange FPLC on a Mono Q column, and nondissociating PAGE. The final product was applied on SDS/PAGE and contained residual bands of 14, 30, and 35 kDa. The 30-kDa band of the final product extracted from SDS/PAGE was sliced from the gel after staining with Coomassie brilliant blue, digested with lysyl endopeptidase (*Achromobacter lyticus* protease I) (16), and separated with an octyldecyl silica column. Each peak was sequenced using the Applied Biosystems 477A protein sequencer.

Synthesis of Oligonucleotides. Oligonucleotides encoding parts of the amino acid sequence of the rat ALR were synthesized on the Applied Biosystems 381A DNA synthesizer.

Extraction of mRNA and Construction of a cDNA Library. Total RNA was isolated from the livers of 2-week-old rats by guanidine isothiocyanate extraction (17). mRNA was purified by Oligotex dT30 (Nippon Roche, Tokyo). Three milligrams of total RNA was obtained from 1 g of liver, which yielded about 30 μg of mRNA. Double-stranded cDNA synthesized with oligo(dT) as a primer was ligated into a λgt11 vector with *Eco*RI adapters and packaged *in vitro* using packaging extract. Five micrograms of mRNA yielded 1×10^9 phages.

PCR. Single-stranded cDNA was synthesized from liver mRNA primed with 5′-AACTGGAAGAATTCGCGGCCGCAGGAA$(T)_{18}$-3′ (Pharmacia) and was amplified by PCR with the following mixture of 5′ primers: 5′-ATIGA(T/C)CGIAG(T/C)CA(A/G)CCIGA(T/C)AC-3′, 5′-ATIGA(T/C)CGITCICA(A/G)CCIGA(T/C)AC-3′, 5′-ATIGA(T/C)AG(A/G)AG(T/C)CA(A/G)CCIGA(T/C)AC-3′, and 5′-ATIGA(T/C)AG(A/G)TCICA(A/G)CCIGA(T/C)AC-3′, where I is inosine. The primers were synthesized on the basis of partial amino acid sequences of rat ALR-26 and the 3′ primer, 5′-GCCGCAGGAA$(T)_{10}$-3′. Then PCR was performed under the following conditions: 95°C for 1 min, 58°C for 28 min, and 75°C for 3 min per cycle for 40 cycles using *Thermus thermophilus* DNA polymerase. The PCR products were analyzed on an agarose gel, and bands were extracted from the gel and subcloned into the *Eco*RV site of pBluescript by TA cloning (18).

Screening of a cDNA Library. Replica filters were prepared and hybridized with a PCR probe that was generated by digestion with *Sal* I and *Xba* I and labeled with the multiprime DNA labeling system (Amersham). The hybridization was

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Abbreviations: ALR, augmenter of liver regeneration; FCS, fetal calf serum.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. D30735).

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ALR-19 Phe-Tyr-Pro-X-Glu-Glu-X-Ala-Glu-Asp-Ile
ALR-20 Leu-Gly-Lys-Pro-Asp-Phe-Asp-X-Ser-X-Val
ALR-26 X-Ile-Asp-Arg-Ser-Gln-Pro-Asp-Thr-Ser-Thr-Arg-
Val-Ser-Phe-X-Gln-X-Leu-X-X-Leu
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FIG. 1. Amino acid sequences of three peptide fragments derived from rat ALR.

carried out for 12 hr at 65°C in 4 × SSC containing 5× Denhardt's solution and 80 μg of denatured salmon testis DNA per ml. The filters were then washed at 65°C in 2 × SSC/0.1% SDS, air-dried, and autoradiographed on x-ray film (19).

Determination of the Nucleotide Sequence. The DNAs of the isolated phage clones were extracted (19), digested with *Eco*RI, subcloned into pBluescript, and subjected to nucleotide sequence determination by the dideoxynucleotide chain-termination method (20).

Expression by COS-1 Cells. The 5′ untranslated G+C-rich region (nucleotides 1–278; see Fig. 2) was eliminated, and a *Hin*dIII site was introduced by PCR-based *in vitro* mutagenesis. The generated 0.5-kb fragment, which includes the coding region of ALR cDNA, was inserted into the CDMmcs vector (21). The construct was transfected into COS-1 cells by the DEAE-dextran method (22) modified as follows: after the transfection, cells were incubated overnight in DMEM plus 10% (vol/vol) fetal calf serum (FCS), and the culture medium was changed from DMEM plus 10% FCS to DMEM without FCS and incubated for 2 days. Transfected cells were separated from culture medium by centrifugation. The cells were harvested by scraping and were homogenized in fresh DMEM without FCS and followed by centrifugation to get a cytosolic fraction. Both the culture medium and the cytosolic fraction were assayed for ALR activity.

Northern Blot Hybridization. To determine the tissue-specific expression, Clontech's multiple tissue Northern blot (MTN blot) was hybridized with the ^{32}P-labeled 1.2-kb *Eco*RI fragment of ALR26-5 cDNA for 15 hr at 42°C in 50% (vol/vol) formamide/5× SSC/10 mM sodium phosphate, pH 6.8/0.5%

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5′ -CGCGCGCTGGCGGTGGCATGCGCGCTGCTCTGTCCCGTCTCCTGCACGCCCTCTTGGCC      59

CCGCTGCTCGTACGCCAGCAATATGGCGGCGCCCAGCGAACCCGCAGGTTTCCCTCGCGGCAGTCGCTTCTCCTTCCTGCCGGGCGGCGCGCACTCGGAGATGACCGACGACCTGGTGAC   179

TGACGCGCGGGGCCGCGGCGCAAGGCATAGAAAAGACAACGCCCCTGCCGCGGCCCCGGCGCCGAAAGGTTTGGAGCACGGGAAGCACCGTGCCGGGCCTGCGTGGACTTCAAGTCGTGG   299

ATG CGG ACC CAG CAG AAG CGG GAC ATC AAG TTT AGG GAG GAC TGT CCA CAG GAT CGG GAA GAA TTG GGT CGC AAC ACC TGG GCT TTC CTT      389
 M  R  T  Q  Q  K  R  D  I  K  F  R  E  D  C  P  Q  D  R  E  E  L  G  R  N  T  W  A  F  L
 1                          10                          20                          30

CAT ACG CTG GCC GCC TAT TAC CCG GAC ATG CCC ACG CCA GAA CAA CAG CAG GAT ATG GCC CAG TTC ATA CAT ATA TTT TCC AAG TTT TAC      479
 H  T  L  A  A  Y  Y  P  D  M  P  T  P  E  Q  Q  Q  D  M  A  Q  F  I  H  I  F  S  K  F  Y
                          40                          50                          60

CCC TGT GAG GAG TGT GCA GAA GAC ATA AGG AAG AGG ATA GAC AGG AGC CAG CCA GAC ACA AGC ACT CGA GTG TCC TTC AGC CAG TGG CTG      569
 P  C  E  E  C  A  E  D  I  R  K  R  I  D  R  S  Q  P  D  T  S  T  R  V  S  F  S  Q  W  L
                          70                          80                          90

TGC CGC CTT CAC AAT GAA GTG AAC CGG AAG CTG GGC AAG CCT GAT TTT GAC TGC TCA AGA GTT GAT GAG CGA TGG CGT GAC GGC TGG AAG      659
 C  R  L  H  N  E  V  N  R  K  L  G  K  P  D  F  D  C  S  R  V  D  E  R  W  R  D  G  W  K
                          100                         110                         120

GAC GGC TCC TGT GAC TAA GGATTACCACAGACCGTGCAGGGCAACGCCGGGTTCTATGGGCAACAGCCTGACTGACGATTAAAGTGCATCTGAGCCAAAGCTTGTTTCTGTGGT      773
 D  G  S  C  D  *

GGGGGTGGGATCCCCTAGAACACTGCCTATGGGAACCCTACCCACAGACTCAGAAACGGAGGTGCCCACTATAGACAGTTGGGTGGCTTCCTCAGGTCTTAAAGCCCCATGGGACTGAAG      893

ATGAGAGGCAGGAGTGGTCCAGGGCACCCCATACCCCTTATGATACCCATTATACATTTGGGACATAGTTGCCTCAAAGGAAGGTGGGCTAGACCATTGCCTTCCTACTACATATCCCCA     1013

GCTGCCTACAGAACTGTGACCCAGGCAACTCTGCCATTTCAGAATTGAAGCAGGGTTCCAGCTCTAGTTGGGTTTTTCTCTTAGGGTAAACCAACCATGGTGCCCACTGTCAGCCTGGCA     1133

CATGGTCTTCTGCAGCCAGGACAAACATGTCAGCAGAGGATCCTGGGAAGGGCTTCCTTAGCGTTTGAGACCAAAATAAAATGAAGTGACTT  -3′                          1225
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FIG. 2. Nucleotide sequence of rat ALR cDNA and the deduced amino acid sequence. Amino acid residues are numbered below the sequence; nucleotide positions are numbered on the right. Chemically determined peptide sequences are underlined. The poly(A) additional signal is indicated with a double underline.

SDS containing 500 μg of salmon testis DNA per ml (19). The filter was then washed three times with 2× SSC/0.1% SDS for 30 min, air-dried, and autoradiographed on x-ray film for 2 days.

***In Vivo* Assay of Recombinant ALR.** The cytosolic fraction and the medium from the transfected COS cell experiment as well as the mock homogenate and supernatant (controls) were filtered through an Amicon PM30 membrane (30,000 molecular weight cutoff) and concentrated to a final volume of 0.2 ml. Aliquots were dissolved in normal saline containing 5 mM ammonium acetate and bovine serum albumin at 5 mg/liter (to prevent adhesion to the plastic tubing). After performing completely diverting portacaval shunt, an infusion catheter was inserted into the tied-off left portal vein for pump-driven constant infusion of the test substance over the next 4 days (6, 23). At the end of 4 days, the animals were injected intravenously with [^{3}H]thymidine (New England Nuclear) at 0.2 mCi/kg (1 Ci = 37 GBq) and killed 2 hr later. Specimens were obtained for comparison of the hepatocytes in the left (infused) and right (not infused) liver lobes using morphometric and autoradiographic techniques (6, 23). Autoradiography was carried out with Ilford K2 nuclear emulsion with an exposure time of at least 30 days. The number of replicating hepatocytes as an index of hepatocyte regeneration was determined by counting the number of [^{3}H]thymidine-labeled nuclei per 1000 hepatocytes.

The size of individual hepatocytes (index of hypertrophy or atrophy) was determined by tracing out at least 500 midzonal liver cells projected on standard-thickness paper, cutting out the individual silhouettes, and weighing each (6, 23). This method has been shown to be accurate for determining hepatocyte cell size and has been validated by planimetry and by studies of unicellular organisms, the size of which has been determined directly (24). End points indicating hepatotrophic activity were prevention of hepatocyte atrophy and augmentation of hepatocyte proliferation in the directly infused lobes only. This method has been shown to screen for hepatic growth factors with highly variable chemical structure and function (6, 23, 25–29).

RESULTS

After cleavage of the 30-kDa rat ALR with lysyl endopeptidase, the isolated peptides were subjected to amino acid sequencing (Fig. 1). We then took the following strategy to isolate rat ALR cDNA: (*i*) Single-stranded cDNA prepared from the livers of 2-week-old rats was amplified by PCR with a degenerate oligonucleotide primer as the 5′ primer and oligo(dT) as the 3′ primer. The ≈350-bp PCR product was detected by agarose gel electrophoresis when PCR was performed using 5′ mixed primers based on the ALR26 peptide sequence (Ile-Asp-Arg-Ser-Gln-Pro-Asp-Thr) shown in Fig. 1 and a 3′ oligo(dT) primer. (*ii*) The primer sequences of the candidate PCR products were confirmed. The region corresponding to the amino acid sequence of ALR26 as well as that of ALR20 was confirmed to be in the deduced amino acid sequence from the longest open reading frame. (*iii*) When this most promising PCR product was used as a probe to screen the cDNA library (7.5×10^5), three positive clones were confirmed.

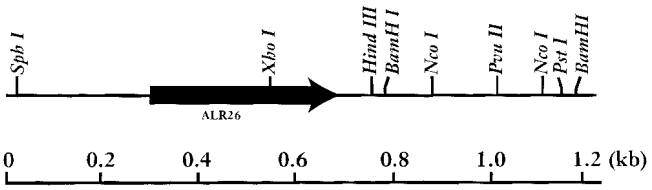

FIG. 3. Schematic representation of rat ALR cDNA (ALR26-5). Restriction sites are shown on the kilobase scale. The noncoding region is represented by a line, and the coding region is represented by the thick arrow.

By restriction enzyme mapping and sequence analysis, the three clones with insert sizes of about 1.2 kb, 0.95 kb, and 0.8 kb were found to cover the DNA sequence corresponding to the mRNA sequence for the coding region of rat ALR. The longest of these cDNA clones (ALR26-5) was characterized in detail. The complete cDNA and deduced amino acid sequences of rat ALR are shown in Fig. 2. The translation initiation site was assigned to the first methionine (ATG) codon (nucleotides 300–302). The entire 1.2-kb cDNA includes three parts (Fig. 3): (*i*) the 299-bp 5′ untranslated region, (*ii*) the 375-bp coding region starting from the putative ATG codon and ending at the GAC codon (nucleotides 672–674), and (*iii*) the termination codon TAA plus the 550-bp 3′ untranslated region.

The rat ALR consists of 125 aa and has a calculated molecular weight of 15,081. The molecular weight determined by SDS/PAGE is ≈15,000 under reducing conditions and ≈30,000 under nonreducing conditions. The native ALR has a homodimeric structure. There is no signal peptide sequence and no potential N-glycosylation site.

ALR mRNA Expression in Rat Tissue. The expression of ALR mRNA in rat tissues was examined by Northern hybridization with the entire cDNA as a probe using MTN blot paper. As shown in Fig. 4, rat ALR mRNA is expressed in almost all tissues in relatively low abundance but in high abundance in testis. The size of mRNA of ALR is about 1.2 kb, the same size as the cDNA.

Production and *in Vivo* Testing of Recombinant ALR. After the ALR expression vector was transfected into COS cells, its peptide product was collected from the supernatant and separately from the cytosolic fraction of COS cell homogenates. Both were tested in the Eck fistula model (Table 1). The vector-COS cytosol and supernatant contained no activity (negative controls). Recombinant hepatocyte growth factor, which also is produced in COS cells and is known to be hepatotrophic in the Eck fistula model (26), was a positive control.

A dose-dependent stimulation of DNA synthesis was detected only in the cytosolic fraction (dog 5, Table 1) but not in the culture supernatant (dog 4, Table 1). This activity was abolished by the same anti-ALR monoclonal antibody (dog 6, Table 1) that eliminated activity of the native purified peptide (11).

The recombinant ALR also prevented the hepatocyte atrophy of Eck fistula on the treated side of the liver but not the other (Table 1). The ultrastructure of the protected hepatocytes was normal, whereas hepatocytes in the control (untreated) liver showed a great decrease in the amount of

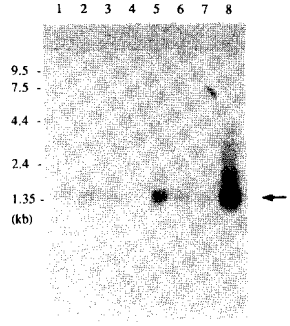

FIG. 4. Northern blot analysis of rat ALR: expression of the rat ALR mRNA in various adult rat tissues. Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, testis. The arrow indicates the location of rat ALR mRNA.

Table 1. Tests in the Eck fistula assay

Dog no.	Infusate	Side(s) infused	Hepatocyte size, size units		Labeled hepatocytes (per thousand)	
			Left	Right	Left	Right
1	HGF supernatant	Left	0.156	0.088	11.0	5.3
2	Vector-COS cytosol (NC)	Left	0.103	0.099	6.3	5.4
3	Vector-COS supernatant (NC)	Left	0.103	0.101	3.8	4.0
4	ALR-26 supernatant (40 ng/kg)	Left	0.079	0.103	6.4	6.6
5	ALR-26 cytosol (40 ng/kg)	Left	0.163	0.090	15.2	4.8
6	ALR-26 cytosol (20 ng/ml) to both + mAb to right	Both	0.150	0.104	10.1	5.1

Infusions were for 4 days immediately after the completely diverting portacaval shunt. The human hepatocyte growth factor (HGF) cDNA expression vector (del-HFG) was used for transfection (21). Hepatocyte growth factor previously was shown to be hepatotrophic (26), and this was a positive control of the assay. NC, negative control; mAb, monoclonal antibody.

rough endoplasmic reticulum and in the number of ribosomes on the membranes. The Golgi apparatus in these hepatocytes was poorly developed, and some of the mitochondria were enlarged and their cristae were disrupted.

The foregoing results were confirmed 2 months later with the next batch of COS cell ALR (data not shown).

DISCUSSION

The unique features of the Eck fistula assay, which was used in previous studies of native ALR and other growth factors (6, 10, 23, 25–29), assured accurate assessment of hepatotrophic activity in the current series of single dog experiments. With the Eck fistula model, the untreated portion of the liver provides an internal control for the treated part. The histopathologic end points are determined blindly and assembled without knowledge of the experiment. The effective dose range, 20–40 ng/ml, established in dogs 5 and 6 for the recombinant peptide was essentially the same as the 20–50 ng/kg range previously reported for the native ALR (10, 26).

The development of the crucial Eck fistula assay as well as our interest in ALR stemmed from research on auxiliary liver transplantation. It was observed that coexisting livers (one an allograft) (30), or alternatively the coexisting two fragments of an animal's own divided liver (24, 31), competed for unknown growth-modulating substances in the portal venous blood, which, when consumed by one liver or fragment, resulted in the acute atrophy of the other. The Eck fistula assay, which is a sophisticated double liver model, made it possible to prove in 1975 that insulin was the principal splanchnic hepatotrophic portal blood factor (23, 25). With the operation of Eck's fistula (portacaval shunt), the liver was deprived of portal venous blood, leaving it with an arterial supply only. Within 4 days, the hepatocytes shrink to half size, and the rate of resting cell renewal triples—an abnormal state that remains stable thereafter. Nonhypoglycemic doses of insulin infused into a principal branch of the tied-off central portal vein prevented the atrophy in the lobes supplied by this branch but not on the other side. In addition the insulin produced sustained unilateral proliferation (23, 25). Collectively, these two growth effects have been called hepatotrophic.

Our attention in 1978 turned upstream to the liver as a possible source of self-generated paracrine or humoral growth control factor(s) with the same hepatotrophic effects. When cytosol from regenerating dog livers was tested in the Eck fistula model, it protected the portaprival liver in the same way as insulin (6). Efforts to identify and purify the active constituent in the cytosol of rat livers were hampered by the inability to settle on an appropriate assay. Fractions prepared by other investigators were reported to be mitogenic in tissue cultures of hepatocytes or hepatoma cells (12, 13, 15), whereas ours were inert *in vitro* throughout purification to the final fraction used for amino acid sequence analysis and cDNA cloning (9, 10, 26).

In vivo testing was difficult with the assay of LaBrecque and Pesch because the variability of the regeneration normally following partial hepatectomy (4, 9) made it difficult to quantitate the augmentation of this response with cytosol or its purified derivatives. Consequently, after establishing the species nonspecificity of our weanling rat fractions (9), we returned to the highly reproducible Eck fistula assay with which a comparison cell size and mitotic activity in treated versus nontreated liver fragments allowed each animal to provide its own control (10, 11).

The recombinant ALR eventually produced by the gene derived from the purified rat cytosol retained the same hepatotrophic potency as the native peptide in the Eck fistula assay while having no effect on cultured hepatocytes. No ALR could be found in the culture medium of COS cell transformants, indicating that the recovered ALR activity was intracellular in origin. Because ALR lacks a stretch of

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                    10         20         30         40         50
ALR26         1  MRTQQKRDIK FREDCPQDRE ELGRNTWAFL HTLAAYYPDM PTPEQQQDMA  50
SCERV1        1  MPGSRT---- YRKVDPPDVE QLGRSSWTLL HSVAASYPAQ PTDQQKGEMK  50

                    60         70         80         90        100
ALR26        51  QFIHIFSKFY PCEECAEDIR KRIDRSQPDT STRVSFSQWL CRLHNEVNRK 100
SCERV1       51  QFLNIFSHIY PCNWCAKDFE KYIRENAPQV ESREELGRWM CEAHNKVNKK 100

                   110        120        130        140        150
ALR26       101  LGKPDFDCSR VDERWRDGWK DGSCD..... .......... .......... 150
SCERV1      101  LRKPKFDCNF WEKRWKDGWD E---..... .......... .......... 150
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FIG. 5. Alignment of amino acid sequences of rat ALR (ALR26) and yeast ERV1 (SCERV1). Gaps were introduced to optimize the homology. Identical residues are in boldface type and are underlined.

hydrophobic amino acids, either N-terminal or internal, which could act as a signal sequence, we do not know if and how ALR is secreted from the cells of the liver. However, it is possible that ALR is secreted through a novel pathway, as has been suggested for interleukin 1B and thioredoxin, which may involve translocation of intracellular membranes, rather than the classical endoplasmic reticulum–Golgi route (32), depending on the cell types. Since ALR does not directly stimulate hepatocytes in culture, its powerful hepatic growth effects *in vivo* may be through the regulation of nonparenchymal cells, and, if so, this will be a link with the immune system.

As with insulin, the effects of ALR go beyond the augmentation of hepatocyte proliferation and maintenance of cell size. ALR and other major hepatotrophic substances identified with the Eck fistula model prevent disruption of the rough endoplasmic reticulum, mitochondria, and other organelles. These other agents are insulin (23, 25), insulin-like growth factor (26), transforming growth factor α (26), hepatocyte growth factor (26), the immunosuppressive agents cyclosporine (27) and FK 506 (28), and the recombinant immunophilin FKBP12 (29). The possibility must be considered that their remarkably similar end points are reached by a common intermediary molecular mechanism involving ALR.

While ALR has no structural relationship to any of these other hepatotrophic factors, it has an $\approx$50% amino acid homology with nuclear gene ERV1 (Fig. 5), which is essential for oxidative phosphorylation, vegetative growth, and life of the yeast *Saccharomyces cerevisiae* (33). This is one of the few dual-function nuclear genes that are not only part of the mitochondrial respiratory chain but also play a critical role in cell growth regulation (34). Disruption of the gene causes a severe growth defect and irreversible cessation of cell division after 3–4 days (33). A mammalian homologue of the ERV1 gene has not been reported, and the ALR gene may be it.

With the availability of the ALR gene and its recombinant product, a range of questions can be addressed about its production, secretion, mechanisms, and importance. Contrary to previous belief, the variable RNA expression in nonhepatic tissues suggests that ALR is not liver specific but would be expressed by other cells under the appropriate circumstances of proliferation.

The potential clinical implications of ALR for the treatment of liver disease, including fulminant hepatic failure, have been evident for some time (7).

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