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λ as a Cloning Vector

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1. Introduction

Genes can be identified and cloned by complementation of mutants, hybridization of nucleic acids, screening for their products, or systematic sequencing of genetic entities. The choice of the strategy to be applied for isolating a gene of interest has to be taken in a space of parameters including, for example, the availability of mutants and the stringency and specificity of selection procedures, the homology of nucleic-acid probes, the possibility to unambiguously identify an encoded function, the abundancy of the desired gene or its product with respect to the background (in other words, the size of the haystack in which to search for the needle), but also the effort and the amount of money that have to be spent to accomplish the task Evaluation of these parameters will then decide on the tools required, e g., DNA probes, antibodies, or functional assays, and help to determine the vector/host system and type of library to be used

The phage λ/*Escherichia coli* system has been one of the work horses in the field of gene cloning, and its applications reach from straightforward complementation of *E coli* mutants to the systematic determination of coding DNA sequences It is the aim of this chapter to briefly introduce some of the derivatives of λ as cloning vectors and to summarize examples for applications There is now a wide variety of λ vectors for almost any possible application, and examples for most of them are legion, therefore, this review can only cover selected examples

Since their first use as cloning vehicles more than 20 yr ago *(1)*, λ vectors and their bacterial hosts have been constantly improved, and now comprise a broad family of general or specialized vectors with features such as multiple-cloning sites, automatic subcloning of cDNA inserts for further manipulation, the capacity to accommodate large DNA fragments, or the possibility to produce recombinant proteins

Although it would at first seem paradoxical to use a large, approx 50 kb vector as a cloning vehicle, λ phages became a very successful, versatile, and robust tool in molecular biology Backed by a profound knowledge of the biology of the phage and its host, the in vivo and in vitro engineering of restriction sites *(2,3)* and the development of reliable in vitro packaging techniques *(4)* allowed the advantage of both in vitro cloning technology and the very high efficiency with which the phage can infect

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its host The inconvenience that preparing either genomic or cDNA libraries in λ is still more demanding than preparation of plasmid libraries is overruled by several advantages of the phage system First, even with the most sophisticated electroporation techniques, the efficiency of plasmid transformation (10^8/μg of DNA) is still well below the efficiency of infection with λ phages (up to 10^{12}/μg of DNA), even though a λ phage carries 10- or 20-fold more nonrecombinant DNA than a typical plasmid vector Second, phage libraries can be stored for many years without too high a drop in their titer (for example, we are still using with good success a λgt11 library prepared in 1985 *[5]*) Many λ vectors can be propagated both as phage particles and as lysogens, allowing to switch between a single-copy state during growth of the bacterial host and a highly amplified state during lytic growth An additional important advantage for screening experiments is the high density at which phage plaques can be plated as compared to bacterial colonies Whereas colonies will become confluent at densities above 10,000 per standard (9 cm) Petri dish, the same surface can accommodate 100,000 phage plaques, allowing screening of highly complex libraries, but also making the percentage of recombinant clones in a λ library less critical, in practice, this greatly reduces the amount of material and work that have to be invested in a screening experiment—screening 10 or 100 plates makes a huge difference

A definite advance, in the early 1980s, was the construction of the λgt series of vectors, with λgt10 and λgt11 as the most widely used examples *(6)* Cloning of DNA fragments in λgt10 takes advantage of the facts that a significant part of the λ genome is dispensable for propagation of the phage, and that insertion of foreign DNA into the *cI* repressor gene abolishes the phage's ability to become lysogenic Thus, large DNA fragments can be inserted and packaged into infectious-phage particles, and recombinants can be selected by plating a library on an *E coli hfl* host, where recombinant phages form clear plaques, whereas most wild-type λgt10 will enter the host genome as lysogens and become titrated out during an amplification step Extensions of this strategy are λEMBL and λCharon vectors, which take advantage of "stuffer" fragments inserted into the vector for propagation of the nonrecombinant phage. During cloning of DNA fragments, these stuffers are replaced by the cloned DNA Constructs lacking the stuffer or a cloned DNA fragment will be less efficiently packaged and are therefore counterselected The use of stuffers consisting of amplified short DNA fragments containing one or several restriction sites makes removal of these small fragments very easy Similarly, the Spi phenotype can been used to enrich for vectors carrying inserted DNA *(7)*

In λgt11 the insertion of an *E coli lacZ* gene with a unique *Eco*RI cloning site near its 3' end allows the expression of coding DNA fragments (if inserted in the proper orientation and reading frame) as fusion proteins with an N terminal part of β-galactosidase, opening an avenue for isolating DNA clones by their encoded function Together with additional useful mutations (like an amber mutation in the *S* gene that allows phage products to be accumulated in nonlysed cell "carcasses") and a range of suitable host strains, λgt11 and its relatives have become a widely used system not only for the cloning of genes, but also for the expression of foreign proteins in *E coli* λgt10 and λgt11 may be considered the paradigms of the modern family of λ vectors, which now span a wide range of vehicles designed for a variety of special purposes like clon-

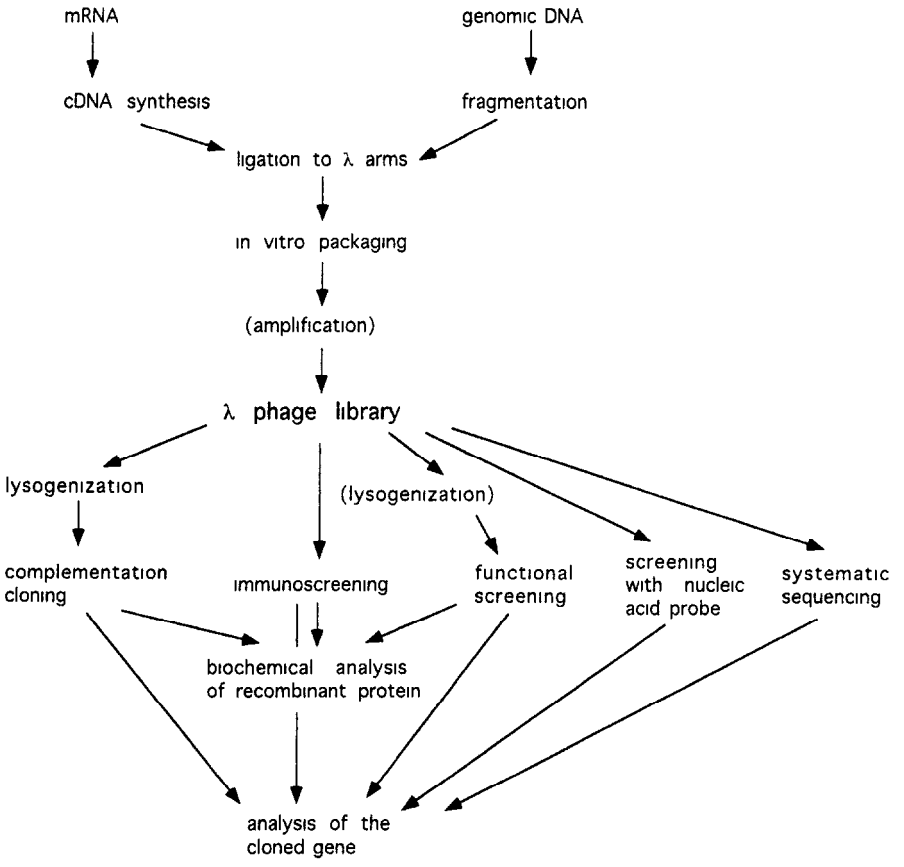

Fig 1 Strategies for cloning and analysis of genes with λ vectors mRNA (usually selected from total RNA by binding to an oligo dT matrix) is reverse-transcribed into cDNA *(35)*, genomic DNA is restriction-cut or mechanically trimmed into fragments of a size that can be accommodated by the chosen vector After rendering the ends compatible to the restriction site for cloning, the fragments are ligated to λ arms and packaged in phage particles in vitro The original library may be amplified and nonrecombinant phages (e g , in λgt10 libraries) counterselected by infection of a suitable host (however, extensive amplification may result in counterselection of phages harboring large fragments or genes whose expression can be deleterious to the host) An original or amplified phage library can then be transformed into a bacterial library by lysogenization (**22**, note the possibility to eliminate parental cells either by selecting for an antibiotic resistance encoded by the phage, or by challenging the lysogens with a λ *cI* mutant that will be lytic for cells that do not contain a lysogen and therefore do not express a functional λ repressor *[28]*) Details for the various techniques of isolating genes from λ libraries are described in the text

ing of DNA fragments as large as 20 kb (λ EMBL and λCharon vectors) to rapid, automatic subcloning of DNA inserts (e g , λZAP *[8]*) and to λYES-type shuttle vectors that allow to complement mutations of bacteria and yeast cells with a single cDNA library **Subheading 3.** (*See* **ref.** *4* for a concise overview on λ vectors and suitable *E coli* hosts). Applications of the use of λ vectors will be discussed later **Figure 1** gives a schematic overview of these applications

2. Systematic Sequencing of cDNAs

Systematic sequencing of expressed sequence tags (ESTs) from several eukaryotic organisms is currently under way (*see* **ref. *9*** for a list of examples), the vector of choice for preparing and analyzing the highly complex cDNA libraries required is λZAP, which combines the high efficiency of cDNA cloning and propagation in λ vectors with the convenience with which cDNA inserts in the bluescript-plasmid vector can be automatically subcloned and sequenced The most impressive example for the power of this approach is provided by Venter and his coworkers ***(9)***, who have assembled a database of 83 million nucleotides of human cDNA sequence, generated from more than 300 cDNA libraries prepared from 37 distinct tissues and defining nearly 90,000 unique sequences This approach is much more than just a shortcut to systematic sequencing of entire genomes, because it provides information not only about the number and nature of sequences present in a genome, but also about their expression pattern, tissue specificity and the abundancy of individual transcripts in a given cell type at a specific developmental stage

3. Complementation of *E. coli* and Yeast Mutants

A number of examples of cloning of genes by complementation of *E coli* and yeast mutants have been published For *E coli* mutants, the general strategy consists of introducing either the *hflA* mutation (which allows for efficient lysogenization of λ prophages) into the mutant of interest, or introducing the mutation of interest into a *hflA* strain, e g , Y1089 ***(6)***, suitable for further analysis of recombinant phages Bacteria are then lysogenized with the λ-phage library, and complementing phages identified by plating the lysogen library on selective medium Alternatively, λ vectors that allow in vivo excision of expression plasmids (e g , λZAP) can be converted to plasmid-expression libraries ***(10)*** Using these strategies, a cDNA for human holocarboxylase synthetase has been cloned by complementation of an *E coli* biotin auxotroph ***(11)*** and cDNAs for enzymes involved in purine synthesis have been cloned from different organisms ***(10,12)*** The approach of cloning GMP synthetase by complementation of the *E coli guaA* mutation has been extended by Kessin's group to cGMP phosphodiesterase by supplying a *guaA* mutant lysogenized with a λgt11 cDNA expression library with cGMP, allowing *guaA* mutants to grow if they can convert cGMP to 5' GMP ***(13)***.

λYES, a shuttle vector for use in complementation cloning both in *E coli* and *Saccharomyces cerevisiae*, has been described by Elledge et al ***(14)***; using cDNA libraries prepared in this vector, it was possible to clone the human *cdc28* homolog by complementation of a *cdc28* mutation of *S cerevisiae*, and enzymes involved in biosynthesis of amino acids in *Arabidopsis thaliana* by complementation of the corresponding *E coli* mutations ***(14)***

The bottleneck in this type of cloning experiment is the need for very tight selection based on the observation that a serine/threonine protein phosphatase from yeast could complement the *E coli serB* mutation ***(15)*** (serine biosynthesis in this mutant is blocked at the level of serine phosphate ***[16]***), we have tried to clone serine/threonine phosphatases from a *Dictyostelium* λgt11 expression library lysogenized in *E coli* Y1089 *serB* About 80 lysogens that grew on minimal medium without serine were identified in a total of 10^5 recombinant lysogens. However, none of the phages supported growth

when isolated and relysogenized in Y1089 *serB*, suggesting that the mutation could be readily by-passed by chromosomal mutations of *E coli* (Q Husain, M Ehrmann, and R. M , unpublished observations) Moreover, any corruption of a λ library with bacterial DNA will become apparent if the abundancy of complementing cDNA clones is low *(13)* Such a corruption would probably not be seen in a screening experiment with a DNA or antibody probe both for the low abundancy of contaminations and their lower degree of homology to the probe or for lower antigenic crossreactivity.

Systematic sequencing of entire λ libraries and complementation cloning may be considered the extremes of how genes can be cloned from λ libraries there is either no search for any specific property of a gene, or this property is so specific that it can be selected for by complementation of a mutation in a heterologous host. In any other case, the gene of interest has to be searched for by screening a highly complex population of recombinant phages. There are two basic strategies for this, screening plaques formed during lytic growth of the phage and screening lysogens, that is, bacterial cells which harbor recombinant λ prophages, and the gene of interest can be screened for either by homology with a nucleic-acid probe, or by any function of its encoded protein, e g , binding of antibodies, its enzyme activity, or binding of a ligand

A typical screening experiment consists of infecting a suitable bacterial host with the λ library, plating on an agar surface at the highest acceptable density, and assaying the phage plaques or lysogen colonies after transfer to a filter support for the presence of the desired gene or its product, the original agar plate is saved as a source of viable phage or lysogens, which are isolated after identification of positive signals by autoradiography or with a chromogenic or chemiluminescent reaction, and rescreened at increasingly lower densities until every single clone will yield a positive signal

4. Screening with Nucleic Acid Probes

Isolating cDNAs from λ phage libraries with labeled nucleic-acid probes is a standard technique in molecular biology *(4)* Here, the high stability of cloned DNA fragments in λ vectors, the possibility to chose a λ derivative that can accommodate large pieces of foreign DNA, and the high density at which phage plaques can be plated and assayed, come into play Originally DNA probes used for screening by hybridization were labeled with ^{32}P either by nick-translation or end-labeling, with the advent of sensitive nonradioactive labeling and detection techniques the use of stable, nonradioactive probes that can be used over many months has proven advantageous In addition to avoiding the inconvenience of manipulating radioactivity, "second generation" chemiluminescence-detection techniques demand for very short exposure times, allowing performance of a screening experiment in <2 d

DNA probes can include anything from perfectly matching sequences to synthetic oligonucleotides designed by reverse translation of a piece of amino-acid sequence, to highly degenerated oligonucleotides derived from amino-acid sequence comparison of members of a protein family from phylogenetically distant organisms. The window for successful screening with short degenerated oligonucleotides is usually narrow, and the conditions that allow detection of complementary sequences with acceptable background hybridization to bacterial and λ DNA have to be largely determined by trial and error. PCR-based strategies may allow to speed up the procedure, either by amplifying first a specific DNA fragment from mRNA or DNA of the organism in question and

using this as a probe for screening the λ library, or by directly amplifying such a fragment from recombinant DNA present in the library and rescreening for complete clones, it is also possible to amplify specific probes from a λ library by using a specific primer and part of the known λ DNA sequence flanking the cloning site as a general primer *(17)*

4.1. Screening for Encoded Proteins

λ vectors have proven most useful for cloning and analyzing genes by assaying for a property of their encoded proteins, i.e , immunological crossreaction, binding of low-molecular weight, nucleic-acid or protein ligands, or even enzymatic reactions For discussion of these approaches, we will focus on λgt11, which has been widely used for this purpose

A prerequisite for expression of cDNAs in a phage vector is the presence of regulatory elements that allow transcription and translation of cloned DNA fragments in *E coli* cells In λgt11 cDNA fragments are cloned into a unique *Eco*RI restriction site located near the 3' end of a truncated *E coli* lactose operon (*lacP/O-lacZ*), allowing inducible, high-level transcription of *lacZ*-cDNA encoded fusion mRNAs that can be translated into fusion proteins if the cDNA coding strand is properly oriented with respect to *lac* transcription, and in phase with the β-galactosidase coding reading frame Full-length cDNAs including 5' noncoding sequence can also be obtained by screening for their protein products· eukaryotic 5' noncoding sequences can either harbor Shine-Delgarno like ribosome-binding sites (in this case translation is initiated on a fusion mRNA with the *lacZ* mRNA and the expression of the encoded protein is regulated by the *lac* promoter), or expression of cDNAs inserted in the opposite direction with respect to transcription of the *lac* gene can be driven by the λ *lom* promoter, which is located 3' from the *Eco*RI cloning site and drives transcription of the opposite DNA strand (*18,19*, in this case expression of the cloned DNA is unregulated). Some eukaryotic cDNAs may even harbor a complete set of regulatory elements for transcription and translation initiation *(20)*

An *E coli* host strain is chosen that carries a $lacI^q$ allele on a high-copy number plasmid (pCM9), ensuring efficient repression of *lac* transcription in the absence of an inducer and minimizing possible deleterious effects of the expressed foreign protein Because λgt11 encodes a temperature-sensitive *cI* repressor (*cI*857), it can be lysogenized and stably maintained in a single-copy state as a lysogen at the permissive temperature, and subsequently switched to a "multi-copy vector" by inducing lytic growth of the prophage by a temperature shift Moreover, an amber mutation in the *S* gene (*S100*amb) renders the phage lysis-defective in a nonsuppressor host, allowing the accumulation of phage-encoded products inside the bacterial-host cells To maximize stability of *lacZ*-cDNA-encoded fusion proteins, a host strain is recommended *(6)* that carries a deletion in the gene for the *lon* protease

In a λ library, a nucleic-acid probe will detect any DNA fragment with sufficient homology, irrespective of its orientation, reading frame, and whether it would encode a complete protein or only a small peptide The number of target molecules will be approximately the same (about 10^6) for every phage plaque on a plate Probing a library for an expressed protein can not rely on the same set of relatively constant parameters The fraction of DNA fragments that can be detected is much lower, in λgt11 it is on average one sixth of the cDNAs encoding a particular protein, because they have to be

cloned in the proper orientation with respect to transcription from the *lac* promoter (the chance is 50% because there is no selection for the orientation of the insert), and they have to be inserted in frame with the reading frame for *lacZ* in order to be expressed as a C-terminal fusion part to *E coli* β galactosidase (the chance is one third) The expressed gene product must not be (too) toxic for *E coli* cells, even though tight repression of the *lac* promoter can be ensured by using a host strain that constitutively overproduces a $lacI^q$ gene, a few copies of a highly toxic product may significantly reduce the viability of infected bacteria and hence the yield of phages and phage-encoded proteins The assay for the protein in question should be highly specific because it has to be detected in a heavy background of *E coli* and phage proteins, and the assay should be highly sensitive because the amount of protein that can be expressed in a phage plaque is very low (typically about 1 fmol of a *lacZ*-cDNA encoded fusion protein in a single λgt11 phage plaque *[21]*)

4.2. Immunoscreening

Probably owing to the low amounts of cDNA-encoded protein that can be expressed in a phage plaque the original approach for screening λ cDNA expression libraries with antibodies consisted in screening of bacterial cells lysogenized with the recombinant phages *(22)*, however, it was soon realized that most antibodies can be readily detected after binding to their target protein in a phage plaque *(23)* This again allowed to take advantage of the high density at which phage plaques can be plated, a particularly important factor in this type of screening experiment (*see* **Subheading 4.1.**) The quality of the antibodies is of crucial importance for immunoscreening experiments polyclonal sera are preferred over monoclonal antibodies because they generally react with a number of different epitopes on the protein, allowing truncated or misfolded proteins to be detected Crossreaction with *E coli* or phage proteins can be a serious problem because positive clones have to be detected on a background of nonspecific binding Nonspecific antibodies against *E coli* or phage proteins can be removed from a serum either by affinity purification of the antibodies on the authentic antigen (coupled to a gel matrix or transferred to a filter support) or by simply neutralizing them with an excess of a crude extract from isogenic bacteria lysogenized with the nonrecombinant λ vector However, even when the antibodies recognize apparently common epitopes on *E coli* proteins and the cDNA-encoded products, it is sometimes possible to identify recombinant phages by the mere overexpression of the epitope, for example, we could isolate cDNA clones for *Dictyostelium* ribosomal proteins by using an antiserum that crossreacted with *E coli* ribosomal proteins (B Knoblach and R Mutzel, unpublished observation)

4.3. Functional Screening

Functional screening of λ libraries became a possibility once it was realized that many *lacZ*-cDNA encoded fusion proteins retain their ligand-binding or even enzymatic functions when expressed in λ lysogens or in phage plaques (e g., *24–26*) Functional isolation of a gene can be undertaken when specific antibodies or oligonucleotide probes are not available, or heterologous antibodies or DNA probes fail to recognize specifically their counterparts from evolutionary distant organisms. What has been said in **Subheading 4.1.** on the limits of detection applies in an even more stringent manner to this technique More-

over, if the function depends on a post-translational modification of the protein that cannot be performed by the *E coli* host, or when several subunits that are encoded by individual genes are required for functionality, screening will not be possible Background problems will be encountered if *E coli* cells or the λ vehicle express a similar enzyme

Although some enzymes may interact strongly enough with their substrates to make interaction cloning feasible *(27)*, functional screening has been mainly applied to "simple" high-affinity ligand-binding proteins in which the binding function is confined to a well defined, stable-binding domain For example, cDNA clones for the high-affinity cAMP-binding subunit of protein kinase A could be identified by *in situ* binding of ^{32}P-labeled cAMP to λgt11 phage plaques *(21)*, but also by ^{3}H-cAMP binding to the protein expressed in bacteria lysogenized with the same λgt11 library *(28)* In both cases binding of the labeled nucleotide to the *E coli* cAMP receptor protein was very low owing to its 1000–10,000-fold lower affinity so that recombinant clones could be readily detected In a similar experiment, isolation of a cDNA clone for a monomeric GTP-binding protein was reported *(29)* Here the original library was divided into sublibraries containing each 1000 individual phages; total protein synthesized in each of them was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, probed with labeled GTP, and sublibraries containing phages that encoded a GTP-binding activity were further analyzed by the same procedure

A classical example for screening a λ library by using a protein ligand is the work of Sikela and Hahn *(30)*, who used iodinated bovine-brain calmodulin to identify clones encoding a rat-brain calmodulin-binding protein in a λgt11 expression library Repeating their experiment with *Dictyostelium* calmodulin to isolate calmodulin-binding proteins from a *Dictyostelium* λgt11 cDNA library, we failed to detect any positive signals when phage plaques were screened, however, detection of calmodulin-binding proteins such as the catalytic subunit of a calmodulin-dependent protein phosphatase (calcineurin A *[31]*) was possible when the cDNAs were expressed in bacterial colonies lysogenized with the same λgt11 library, demonstrating that the success of this type of screening approach can be critically dependent on the affinity of the target for its ligand, the choice of the labeled probe, and the amount of recombinant protein expressed in an individual clone *(28)* An elegant extension of this type of interaction cloning has been proposed by Germino et al. *(32)* who constructed plasmids encoding genetic fusions of protein ligands with part of the biotin carboxylase carrier protein, which labels the fusion protein with biotin in vivo Bacteria transformed with this constructs were then infected with a λ library containing cDNAs fused to a functional *lacZ* gene, and protein complexes formed between the ligand and its binding proteins were immobilized on filter supports containing bound streptavidin, avidin, or antibiotin antibodies, and detected by the β galactosidase activity of the λ-encoded fusion protein.

Finally, labeled nucleic-acid probes have been used in a number of cases to detect DNA-binding proteins with modified "Southwestern" assays *(33)*, taking advantage of the high affinity and specificity of binding of these proteins to their target nucleic acid sequences

5. Analysis of Positive Clones

Recombinant λ DNA clones obtained from a selection or screening experiment are a very convenient material for further analysis With the rapidly increasing number of

known primary structures that are readily accessible from various databases, determination of part of the sequence of the clone will often be helpful to decide whether the "good" gene has been obtained Such sequence tags can be obtained very rapidly even from recombinant DNA cloned in λ vectors that do not contain excisable phagemids of the bluescript type by PCR amplification of the cloned insert using primers directed against λ sequences flanking the cloning site and direct sequencing of the amplified fragment Partial clones isolated by immunoscreening may be used as specific DNA probes for the isolation of full-length cDNA clones or the entire gene in question, but can also be directly used to assess the expression pattern of the gene, e.g , on Northern blots The λ system also offers direct access to the biochemistry of the encoded protein a recombinant λ lysogen can serve as a model to establish a purification protocol for the encoded foreign protein (e.g., *see* **ref. *31***). High molecular-weight fusion proteins with β galactosidase can be rapidly purified from SDS polyacrylamide gels and used to raise antibodies against the cDNA-encoded protein (e g , *see* **ref. *34***)

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