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Structure of the murine Ia-associated invariant (Ii) chain as deduced from a cDNA clone

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The invariant (Ii) chain is a membrane-spanning glycoprotein found intracellularly associated with class II major histocompatibility complex (MHC) molecules. Using hybrid-selected translation and the Ii-specific monoclonal antibody In-1, we have isolated a cDNA clone (pIi-5) coding for most of the Ii chain. Sequence analysis of this clone reveals an open reading frame encoding 169 amino acid residues. The protein is rich in methionine and contains two potential N-glycosylation sites. No stretch of uncharged amino acid residues, characteristic for a membrane-spanning segment, is found close to the COOH-terminal end. There is one, however, close to the NH_2-terminal end. As it is known that ~20 amino acid residues of Ii chain are exposed on the cytoplasmic side, we conclude that the Ii chain spans the membrane exposing the NH_2 terminus on the cytoplasmic side and the COOH terminus on the luminal side.

Key words: major histocompatibility complex/invariant chain/cDNA cloning/transmembrane proteins

Introduction

The invariant (Ii) chain is a membrane protein associated intracellularly with class II major histocompatibility complex (MHC) molecules called Ia in mouse and HLA-DR in human (Klein, 1979; Benacerraf, 1981; Jones *et al.*, 1978). These molecules are involved in the presentation of foreign antigens on macrophage-like cells and B cells to a particular subclass of T cells (Benacerraf, 1981; Nagy *et al.*, 1981). They consist of an α chain non-covalently linked to a β chain. The Ii chain is a methionine-rich glycoprotein containing two carbohydrate side chains linked to asparagine (Sung and Jones, 1981; Charron *et al.*, 1983; Moosic *et al.*, 1980; Kvist *et al.*, 1982b; Swiedler, 1983; McMillan *et al.*, 1981). It has a mol. wt. of 31 K and is basic in character. Ii chains are synthesized in excess over Ia and HLA-DR α and β chains and assemble non-covalently with the latter in the membrane of the endoplasmic reticulum (ER) (Sung and Jones, 1981; Kvist *et al.*, 1982b). Intracellular transport of Ii chains from the ER to an as yet undefined intracellular compartment requires assembly with Ia α and β chains (Kvist *et al.*, 1982b). However, no Ii chain assembled with α and β chains can be detected on the cell surface. Whether Ii chain alone can reach the cell surface is still debated (Sung and Jones, 1981; Koch *et al.*, 1982).

Expression of Ii chains is closely linked to that of Ia chains. In certain cell lines Ii and Ia can be induced simultaneously by γ interferon (Koch *et al.*, 1984). Synthesis of Ii can be enhanced by the anti-mitotic drug mitomycin (Rahmsdorf *et al.*, 1983). However, the function of the Ii chain remains obscure. The chain may play an as yet undefined physiological role in the functioning of Ia molecules in the immune response, and in intracellular transport of Ia chains (Sung and Jones, 1981; Kvist *et al.*, 1982b; McMillan *et al.*, 1981). One way to test the function of the Ii chain is to isolate the encoding gene and express it together with Ia molecules in appropriate cells, follow its fate in the cell, and test the ability of these cells to present antigen to T cells (Nagy *et al.*, 1981). Here we describe the isolation and characterization of a cDNA clone coding for the Ii chain.

Results

In vitro synthesis of the Ii chain

For the selection of Ii cDNA clones by hybrid-selected translation it was necessary to characterize the Ii chain synthesized *in vitro*. mRNA was therefore isolated from mouse spleens (DBA/2 strain), a rich source of Ii and Ia polypeptides, and translated in a reticulocyte lysate, cell-free translation system. The Ii chain was immunoprecipitated with a previously characterized monoclonal antibody (mAb) In-1, and analysed by SDS-polyacrylamide gel electrophoresis (PAGE). The unprocessed Ii chain appears as a 25-kd protein, 6 kd smaller than the mature Ii chain (Figure 1, cf. lanes 1 and 2). The difference in mol. wt. probably reflects the presence of two

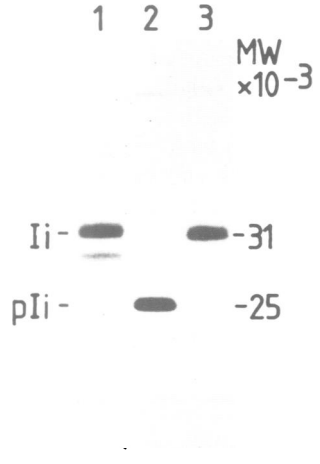

Fig. 1. *In vitro* synthesis and membrane insertion of Ii chain. mRNA from DBA/2 mouse spleens was translated in a reticulocyte lysate system in the absence or presence of dog pancreas microsomes. Antigens were immunoprecipitated with mAb In-1 and characterized by SDS-PAGE and fluorography. **Lane 2** shows Ii chain synthesised in the absence of microsomal membranes and **lane 3** in the presence of microsomal membranes. For comparison **lane 1** shows antigens immunoprecipitated from [^{35}S]methionine-labeled mouse spleen cell with monoclonal anti Ia-A monoclonal antibody 17/227 (Lemke *et al.*, 1979).

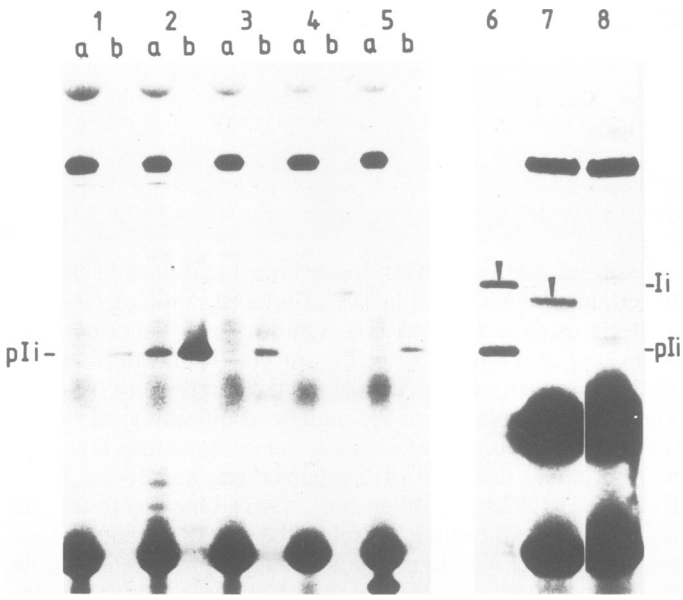

Fig. 2. Identification of a cDNA clone coding for Ii chain by hybrid-selected translation. After an initial screening of pools of recombinant plasmids constructed from spleen mRNA, DNA from one pool (number 11) selected mRNA coding for Ii chain. DNA from individual plasmids of this pool was then used in hybrid-selected translation. Filter-bound mRNA was translated in the reticulocyte lysate system and characterized by SDS-PAGE. **Lanes 1–4** show results with four of the plasmids, **lane 5** with no DNA bound to the filter. Antigens translated from hybrid-selected mRNAs are displayed either directly **(lanes a)** or after immunoprecipitation with mAb In-1 (Koch *et al.*, 1982) **(lanes b)**. mRNA hybridized to plasmid DNA used in **lane 2** was further translated in the presence of microsomal membrane and antigens immunoprecipitated with mAb In-1 **(lane 6)** or microsomal membranes were treated with proteinase K **(lane 7)** or proteinase K and 0.5% Triton X-100 **(lane 8)**. The faint band at the position of pIi in lanes 1b, 3b, 4b and 5b is derived from mRNA unspecifically adsorbed to the filter.

N-linked carbohydrate moieties in the mature Ii chain (Charron *et al.*, 1983; Swiedler *et al.*, 1983). Indeed when microsomal membranes from dog pancreas, which are known to support N-linked glycosylation, were added to the *in vitro* translation system, a polypeptide of 31 kd was precipitated with mAb In-1, indistinguishable from the form found in cells after pulse-labeling (Figure 1, lane 3). To compare *in vitro* synthesized Ii chain with Ii chain made *in vivo*, we labeled spleen cells for 15 min with [^{35}S]methionine and immunoprecipitated antigens with monoclonal antibody against Ia-Aβ chains (Figure 1, lane 1). The Ii chain was found as a polypeptide of 31 kd having an identical mol. wt. to the Ii chain synthesized *in vitro* in the presence of dog pancreas microsomes (compare Figure 1, lanes 1 and 3).

Identification of Ii chain cDNA clones by hybrid-selected translation

Size-fractionated (12–16S) spleen mRNA was converted into double-stranded cDNA and cloned into the *Pst*I site of pBR322 by the homopolymer tailing method. The transformation yielded 1500 independent recombinants from one-tenth of the culture that was plated directly onto agar plates. For the initial screening, 240 of these clones were picked and aliquots combined into 30 pools of eight clones each. Plasmid DNA was prepared from the pools and immobilized on nitrocellulose filters for hybrid selection as described (Kvist *et al.*, 1982a). DNA from one of the pools (pool 11) selected spleen mRNA which directed the translation of Ii chains as verified

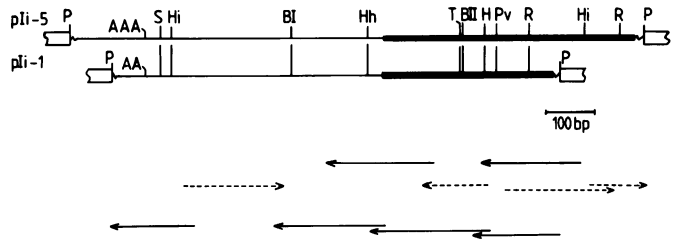

Fig. 3. Restriction map and sequencing strategy. Complementary DNA clones, pIi-1 and pIi-5, identified to code for Ii chain by hybrid-selected translation or colony hybridization are oriented 3′-5′ with respect to the mRNA sequence. The coding region (solid bar), non-coding region (thin line) and poly(A) sequences (AAA) are indicated. Restriction sites are BI, *Bgl*I, BII, *Bgl*II; H, *Hinf*I; Hi, *Hind*III; Hh, *Hha*I; P, *Pst*I; Pv, *Pvu*II; R, *Rsa*I; S, *Sau*3a; T, *Taq*I. Regions sequenced by the deletion subcloning method (for clone pIi-1) are indicated by the lower set of arrows. For clone pIi-5 restriction fragments were isolated and sequenced from either the *Hind*III, *Bgl*II or *Pvu*II sites; upper strand sequencing (solid lines) lower strand (broken lines).

by immunoprecipitation with the mAb In-1 and SDS-PAGE (data not shown).

Based on the above results, plasmid DNA from the individual clones in pool 11 was prepared and used for the hybrid selection assay. Translation products from the reticulocyte lysate system were characterized by SDS-PAGE (Figure 2, lanes 1–5), either directly (lanes a) or after immunoprecipitation with mAb In-1 (lanes b). In lane 2a the 25-kd unprocessed form of the Ii chain is seen clearly as the major translation product and is specifically immunoprecipitated with mAb In-1 (lane 2b). The clone selecting this mRNA was thus identified as containing the Ii-encoding cDNA sequence, and was named pIi-1.

To characterize further the translated product, mRNA selected by clone pIi-1 was added to the reticulocyte lysate system along with microsomal membranes. Figure 2, lane 6 shows that in the presence of microsomal membranes, a processed 31-kd product, indistinguishable in mobility from *in vivo* synthesized Ii chain is produced. When proteinase K is used to digest the cytoplasmically exposed portion of this protein, a polypeptide ~2 kd smaller that the mature Ii chain was found (Figure 2, lane 7). When the lipid bilayer of the microsomal vesicle was destroyed by Triton X-100, the protein was completely digested (Figure 2, lane 8). These results are consistent with those previously obtained for the human Ii-chain identified functionally by its oligomeric assembly with HLA-DR α and β chains (Kvist *et al.*, 1982b). They indicate that the murine Ii-chain, like its human counterpart, is a transmembrane protein with a cytoplasmic tail of ~2–3 kd in size. Thus, we are confident that the clone pIi-1 contains a cDNA sequence encoding the murine Ia-antigen-associated Ii-chain.

Isolation and sequence analysis of cDNA clones pIi-1 and pIi-5

Clone pIi-1 was digested with *Pst*I and a 920-bp fragment containing the entire insert was isolated and used in a colony hybridization experiment to probe ~20 000 clones representing the remainder of the cDNA library. Eight clones were identified and picked. Based on its restriction sites (Figure 3) one of these clones, pIi-5, had a significantly longer insert than the others, and was analysed further.

Partial restriction maps of clones pIi-1 and pIi-5 are shown in Figure 3. The inserts are ~920 and 1170 bp in length, respectively. Both are orientated in the same direction relative to the vector; both have reconstituted *Pst*I sites at each end,

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Gln Ala Thr Thr Ala Tyr Phe Leu Tyr Gln Gln Gln Gly Arg Leu Asp Lys Leu Thr Ile Thr Ser Gln Asn Leu
CAG GCC ACC ACT GCT TAC TTC CTG TAC CAG CAA CAG GGC CGC CTA GAC AAG CTG ACC ATC ACC TCC CAG AAC CTG      95

Gln Leu Glu Ser Leu Arg Met Lys Leu Pro Lys Ser Ala Lys Pro Val Ser Gln Met Arg Met Ala Thr Pro Leu
CAA CTG GAG AGC CTT CGC ATG AAG CTT CCG AAA TCT GCC AAA CCT GTG AGC CAG ATG CGG ATG GCT ACT CCC TTG     170

                                                  *                                   *
Leu Met Arg Pro Met Ser Met Asp Asn Met Leu Leu Gly Pro Val Lys Asn Val Thr Lys Tyr Gly Asn Met Thr
CTG ATG CGT CCA ATG TCC ATG GAT AAC ATG CTC CTT GGG CCT GTG AAG AAC GTT ACC AAG TAC GGC AAC ATG ACC     245

Gln Asp His Val Met His Leu Leu Thr Arg Ser Gly Pro Leu Glu Tyr Pro Gln Leu Lys Gly Thr Phe Pro Glu
CAG GAC CAT GTG ATG CAT CTG CTC ACG AGG TCT GGA CCC CTG GAG TAC CCG CAG CTG AAG GGG ACC TTC CCA GAG     320

Asn Leu Lys His Leu Lys Asn Ser Met Asp Gly Val Asn Trp Lys Ile Phe Glu Ser Trp Met Lys Gln Trp Leu
AAT CTG AAG CAT CTT AAG AAC TCC ATG GAT GGC GTG AAC TGG AAG ATC TTC GAG AGC TGG ATG AAG CAG TGG CTC     395

Leu Phe Glu Met Ser Lys Asn Ser Leu Glu Glu Lys Lys Pro Thr Glu Ala Pro Pro Lys Glu Pro Leu Asp Met
TTG TTT GAG ATG AGC AAG AAC TCC CTG GAG GAG AAG AAG CCC ACC GAG GCT CCA CCT AAA GAG CCA CTG GAC ATG     470

Glu Asp Leu Ser Ser Gly Leu Gly Val Thr Arg Gln Glu Leu Gly Gln Val Thr Leu
GAA GAC CTA TCT TCT GGC CTG GGA GTG ACC AGG CAG GAA CTG GGT CAA GTC ACC CTG TGA AGACAGAGGCCAGCTCTGCA     550

CAGCAGCAGCGCCCCCTGCTCTCCTGTGCCTCAGCCCTTCTTATGTTCCCTGATGTCACACCCCACTTCCCGTCTCCCTGCACCCTGGGGCTTGAGACTG     650

GTGTCTGTTTCATCGTCCCAGGACACGGCAAATGAAGTCAGAACAGAAGGAGGACGCTGGAGGGCCTTGCTGGCACCGCATCTAAAGGGAACCCCCATTT     750

CTGACCCATTAGTAGTCTTGAATGTGGGGCTCTGAGATAAAGGCCCGCAGACAGGGACAAGGGATGCCCTACCCTTAACCTAGGCTGGACACATTTGCTG     850

CCTTCTCCTCAAGGAAGAAGAACCCAA GCCCCTCCTCCCAGTAACCCCTCCTCACATCCTGCCACCCCCCCTCAAGCCCCACCCCCTTTCAGGTTCCTTG     950

CTCAGCCAAGCTTGTCAGCAGCCTGTAGGATCATGGTTCAAGTGACAATAAAGGAAGAAAGTAGA ≈130 C ≈15 TGCA
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Fig. 4. Nucleotide and deduced amino acid sequence of clone pIi-5. The nucleotide sequence reveals one open reading frame encoding 169 amino acid residues at the 5′ end followed by 487 bases of 3′ non-coding region and the poly(A) and oligo(C) tracks. Asterisks indicate the two potential glycosylation sites.

no internal *Pst*I sites, and identical cleavage sites for the indicated restriction enzymes in the overlapping region. The nucleotide sequence was determined from both clones as indicated in Figure 3. The 3′ non-coding region lies to the left in the restriction map, with the start of the poly(A) tract positioned as indicated (Figure 3).

The complete coding region sequence of pIi-5 and the consensus non-coding sequence are shown in Figure 4. All regions comparable between the two clones were identical. The only open reading frame defines a region (to the right on the restriction map) encoding a sequence of 169 amino acid residues. Judging from the apparent 25 K mol. wt. of the *in vitro* synthesized, unprocessed Ii chain, the complete polypeptide chain should comprise ~220 amino acid residues. Thus, clone pIi-5 appears to lack the sequence corresponding to the amino-terminal 50 amino acid residues of the complete Ii chain.

In the amino acid sequence predicted from clone pIi-5, 13 residues are methionine (Figure 5), an unusually high amount, but consistent with the known methionine-rich nature of the Ii chain (Charron *et al.*, 1983; McMillan *et al.*, 1981). In addition, two sites for N-linked glycosylation (Asn66, Asn73) are clustered within a stretch of seven amino acid residues near the middle of the polypeptide chain.

A surprising feature of the Ii chain sequence from pIi-5 is the lack of hydrophobic, potential membrane-spanning segment near the carboxy-terminal end. Instead, there is a cluster of charged residues present in this region (Figure 5). Protease digestion of membrane-integrated human (Kvist *et al.*, 1982b) and murine Ii-chain (Lipp *et al.*, in preparation, and see

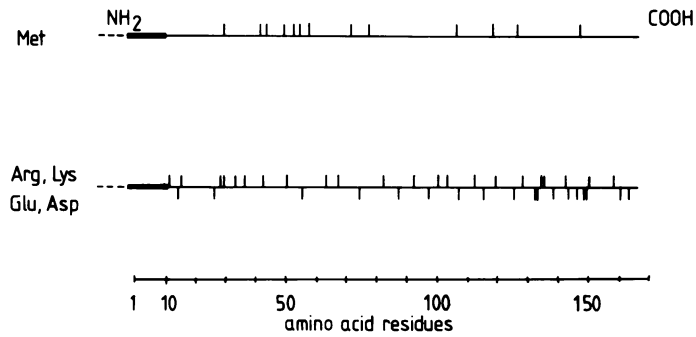

Fig. 5. Distribution of methionine and charged amino acid residues in Ii chain deduced from cDNA clone pIi-5. Positions of methionine, arginine and lysine residues are indicated by a bar above the line, and glutamic acid and aspartic acid residues by a bar below the line. The solid bar indicates a stretch of uncharged amino acid residues.

Figure 2, lane 7) suggested that the chain is indeed a membrane-spanning protein with 20–30 amino acid residues exposed on the cytoplasmic side of the ER. Such a sequence can only be located close to the amino-terminal end. In the amino acid sequence predicted from pIi-5 there is an unbroken stretch of 13 uncharged residues at the amino-terminal end (Figure 5), which might extend further to provide the usual stretch of 20–30 uncharged residues characteristic of membrane-spanning segments (Warren, 1980). Indeed, sequence data (not shown) from an Ii chain genomic clone extending upstream from clone pIi-5 do indicate an unbroken stretch of 17 additional uncharged residues. This finding suggests that the Ii chain spans the membrane close to its

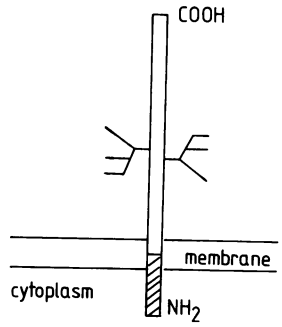

Fig. 6. Proposed structure and membrane orientation of Ii chain. Our data suggest that the Ii antigen spans the membrane close to the NH_2-terminal end, exposing this end to the cytoplasm. It contains two carbohydrate side chains linked to asparagine, six amino acid residues apart. Sequence data are lacking from the region marked with diagonal lines.

amino-terminal end, exposing this end on the cytoplasmic side.

Discussion

A cDNA clone coding for the murine Ii chain has been identified by the following criteria: (i) mRNA hybridized to clone pIi-1 promoted translation of a 25-kd protein which could be quantitatively precipitated by mAb In-1. This antibody has previously been shown by Koch *et al.* (1982) to be specific for Ii chain. (ii) Microsomes from dog pancreas converted this 25-kd form of Ii chain into a 31-kd glycosylated protein, identical in mol. wt. with the mature Ii chain. (iii) The 31-kd protein spans the membrane and exposes a 2-kd segment on the cytoplasmic side. The same structural feature has previously been found for human Ii chain (Kvist *et al.*, 1982b) and recently also for mouse Ii chain (Lipp *et al.*, 1984). (iv) The deduced amino acid sequence from clone pIi-5 reveals that the encoded protein is rich in methionine. At least eight methionine-labeled tryptic peptides have been predicted for authentic Ii chain (McMillan *et al.*, 1981). We can identify 10 such tryptic peptides in the deduced sequence (Figure 4). (v) Two potential N-linked carbohydrate binding sites are found in the sequence and this is consistent with the number determined for authentic Ii chains (Charron *et al.*, 1983; Swiedler *et al.*, 1983). Taken together these facts strongly suggest that pIi-5 codes for the Ia-associated Ii chain. Complementary DNA coding for the human invariant chain has recently been isolated by Long *et al.* (1983), however, no sequence data are yet available.

Most membrane proteins thus far characterized span the membrane close to the COOH-terminal end exposing this end on the cytoplasmic side. For the following reasons we suggest that Ii chain spans the membrane in the opposite orientation, exposing the NH_2-terminal end on the cytoplasmic side. (i) The cluster of uncharged amino acid residues, typical for a membrane-spanning segment, is not found close to the COOH-terminal end (Warren, 1980). (ii) Our protection experiments (Figure 2, lane 7) however, show that a segment of ~20 amino acid residues is exposed on the cytoplasmic side. (iii) A stretch of 30 uncharged amino acid residues is found close to the NH_2-terminal end. Thirteen of these residues have been identified from clone pIi-5 and 17 from a genomic clone (Lauer *et al.*, unpublished observation). (iv) Assuming a length of ~220 amino acid residues for the Ii chain, the stretch of uncharged residues would be located 20 residues away from the NH_2 terminus.

The only consistent conclusion from these data is that the NH_2 terminus of the Ii chain, comprising ~20 residues, is exposed on the cytoplasmic side and is accessible in microsomal vesicles to protease. A similar orientation has also been found for the chicken hepatic lectin and the band 3 protein from human erythrocyte (Drickamer, 1978, 1980).

The association of the Ii chain with Ia molecules has led to the speculation that these chains might also be related structurally. Finn *et al.* (1983) observed that a monoclonal antibody recognizing human Ia β chains showed some reactivity with Ii chain. As the sequences for several Ia α and β chain genes or cDNAs are available (Benoist *et al.*, 1983; Choi *et al.*, 1983; Larhammar *et al.*, 1983; Malissen *et al.*, 1983), we used the program ALIGN to compare them with the sequence of the Ii chain (Needleman and Wunsch, 1970). No statistically significant homology could be detected. This is not surprising, as many features of the Ii chain are very different from those of Ia α and β chains: opposite orientation in the membrane, as well as the accumulation of charged and methionine residues, and the lack of cysteine residues in the portion exposed on the luminal side of the ER. Since, however, Ii chain can form disulfide-linked homodimers, a cysteine residue must exist on the cytoplasmic side (Koch and Hämmerling, 1982). In Ia α and β chains intramolecular disulfide bridges are found on the luminal side of the molecule.

The sequence characterization and the availability of a cDNA clone coding for the Ii chain should help to elucidate factors which determine the orientation of a protein in the membrane and its final destination in an intracellular organelle. They should also help to understand the role of Ii chain in its association with Ia α and β chains.

Materials and methods

The reticulocyte lysate was from New England Nuclear; guanidium thiocyanate from Fluka A.G.; oligo(dT)-cellulose from Collaborative Research; terminal transferase from P-L Biochemicals; restriction endonucleases from Boehringer or Bethesda Research Laboratory, and AMV reverse transcriptase from Life Sciences, Inc.

mRNA isolation

Total cellular RNA was isolated from the spleens of ~100 C57Bl/10 mice by the guanidinium thiocyanate method as described by Chirgwin *et al.* (1979). Poly(A)$^+$ RNA was selected by two passages over oligo(dT)-cellulose, and further fractionated on a 5–20% sucrose gradient. Fractions corresponding to 12–16S RNA were pooled and ethanol-precipitated.

Protein synthesis and antibody precipitation

mRNA was translated in a reticulocyte lysate system supplemented, where indicated, with dog pancreas microsomal membranes. Antigens were immunoprecipitated as described (Kvist *et al.*, 1982b). Antibodies used were mAb In-1 recognizing Ii chain (Koch *et al.*, 1982) and mAb 17/227 recognizing a determinant on the Ia-A chain (Lemke *et al.*, 1979). For metabolic labeling, spleen cells were incubated for 15 min with [^{35}S]methionine. Proteins were then solubilized with Triton X-100 and immunoprecipitated as described by Koch *et al.* (1982), separated on 10–15% SDS-polyacrylamide slab gels (PAGE), and visualized by fluorography using ENHANCE (New England Nuclear) (Laemmli, 1970).

cDNA synthesis and cloning

Complementary DNA synthesis was carried out as described by Wahli *et al.* (1978) using 10 μg of 12–16S poly(A)$^+$ RNA. Following S1-nuclease digestion, the ds cDNA (0.2 μg) was dC-tailed in a 100 μl reaction mixture containing 0.1 M K^+ cacodylate (pH 6.8), 1 mM $CoCl_2$, 0.2 mM dithiothreitol, 250 pmol unlabeled dCTP (~100-fold over 3′ ends), 15 μCi [^{32}P]dCTP (3000 Ci/nmol), and 100 units terminal transferase. After pre-warming the mixture (5 min, 37°C), enzyme was added and the reaction allowed to proceed for 1 min before being stopped by the addition of EDTA to 20 mM, followed by immediate phenol extraction. *Pst*I-cleaved pBR322 was dG-tailed by the same procedure, except that the 100 μl reaction mixture contained 6.5 μg DNA (5 pmol 3′-ends), 500 pmol (5.8 μCi) [^{3}H]dGTP, and the reaction was stopped after 30 s.

The dC-tailed ds cDNA was fractionated on a 5–20% sucrose gradient and the high mol. wt. fractions (total of 0.1 pmol cDNA) were passed over Sephadex G-50 equilibrated in TEN buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl). Annealing and transformation was done according to Roewekamp and Firtel (1980). Complementary DNA from the G50 column was combined with 0.25 μg (0.1 pmol) dG-tailed vector DNA in 0.75 ml TEN buffer, heated to 63°C for 3 min, and allowed to anneal at 42°C for 3 h followed by overnight incubation at room temperature. The annealing mixture was transferred to ice, and diluted to 5 ml with ice-cold 30 mM $CaCl_2$ before proceeding with the transformation protocol (Maniatis *et al.*, 1982). An aliquot (10%) of the final transformation culture was plated onto L-plates and the remainder diluted 4-fold with fresh L-broth and allowed to grow ~24 h. Plates and liquid medium contained tetracycline (15 μg/ml).

Plasmid DNA preparation

HB101 cells harboring recombinant plasmids were grown as described by Norgard *et al.* (1979), cleared lysate prepared (Bastia, 1978), and plasmid DNA isolated by banding in $CsCl_2$-EtBr density gradients. Hybrid-selected translation was done as described previously (Kvist *et al.*, 1982a).

Colony hybridization

Bacterial colonies were grown on nitrocellulose filters, fixed and hybridized to the nick-translated *Pst*I fragment of pIi-1 as described by Hanahan and Meselson (1980).

DNA sequencing

All sequencing was done by the chemical method of Maxam and Gilbert (1980). Subclones of clone pIi-1 were obtained for sequencing by the deletion subcloning method (Frischauf *et al.*, 1980).

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