The EMBO Journal Vol.1 No.4 pp.467–471, 1982

Identification of an H-2K^d gene using a specific cDNA probe

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Communicated by B. Dobberstein
Received on 8 April 1982

A cDNA clone known to code for a mouse histocompatibility (class I) antigen was found to contain a sequence specific for a subpopulation of H-2 genes. This unique sequence is located in the 3′ non-coding region close to the stretch of poly(A) nucleotides. A subclone containing this fragment (pH-2^d-5) has been used to select hybridizing mRNA. Translation of the mRNA *in vitro* shows that H-2K^d mRNA is selected. Southern blot analysis of DNA from congenic recombinant mice show that at least one gene containing this sequence is located at the K locus (region) of the major histocompatibility complex. This gene contains a 3.7-kb *Bgl*II and a 13-kb *Eco*RI restriction endonuclease fragment. This gene has been isolated from a genomic DNA library.

Key words: histocompatibility genes/hybrid-selected translation/gene mapping/3′ non-coding region/Southern blot analysis

Introduction

Genes located in the major histocompatibility complex (MHC) play a fundamental role in several aspects of the cellular immune response (Klein, 1975). They code for three classes of proteins: class I, the transplantation antigens H-2K, D, L, and R (here called H-2 antigens); class II, the immune-response-associated antigens, Ia; and class III, complement components (Nathenson and Cullen, 1974; Klein, 1979; Ploegh *et al.*, 1981).

Genes coding for lymphoid differentiation antigens, Qa and Tla are located at the telomeric side of the MHC (Flaherty, 1980). They are structurally similar to the H-2 antigens which are membrane glycoproteins with a mol. wt. of 43–47 kd and are associated with β_2-microglobulin (Coligan *et al.*, 1981; Michaelson *et al.*, 1977; Soloski *et al.*, 1981).

A detailed molecular analysis of genes coding for H-2, Qa, and Tla antigens has become possible with the isolation of cDNA probes in several laboratories (Ploegh *et al.*, 1980; Kvist *et al.*, 1981; Sood *et al.*, 1981). Their sequence analysis confirmed the high degree of homology postulated for H-2 antigens encoded by different loci (Bregegere *et al.*, 1981; Steinmetz *et al.*, 1981a). Southern blot analysis using these cDNA probes revealed that genes coding for H-2 antigens constitute a large multigene family (Steinmetz *et al.*, 1981a, 1981b; Cami *et al.*, 1981). One gene located in the Qa region has been isolated and characterized by sequence analysis (Steinmetz *et al.*, 1981b). From this analysis it was concluded that genes coding for H-2 and Qa antigens show a high degree of homology, and cDNA coding for H-2 antigens cross-hybridizes efficiently with that coding for Qa or Tla antigens (Steinmetz *et al.*, 1981b). Therefore, to define H-2 antigens it is necessary to obtain specific DNA probes which characterize a subgroup of genes in this multigene family.

In this paper we report on such a DNA probe derived from the extreme 3′ end of the non-coding region of a cDNA clone coding for an H-2 antigen. Using this probe in a Southern blot analysis, we mapped a gene located in the K locus of the MHC.

Results

Selection of a cDNA clone hybridizing to mRNA coding for an H-2K^d antigen

H-2-like antigens coded by genes located at different loci of the MHC show extensive homology; cDNAs containing coding regions cross-hybridize (Coligan *et al.*, 1981; Kvist *et al.*, 1981; Steinmetz *et al.*, 1981a). In order to identify and characterize genes from a particular region or locus it is necessary to obtain DNA probes of higher specificity.

We tested cDNA clones coding for various parts of H-2 antigens for their ability to hybridize selectively to H-2 mRNAs. Clones containing extended regions common to all H-2, and possibly Qa and Tla, antigens should hybridize to mRNA coding for all H-2-like antigens. Those containing unique sequences should only hybridize to a subgroup of mRNA which possesses such sequences.

Plasmid DNA of clones pH-2^d-1, pH-2^d-4, pH-2^d-5 (Bregegere *et al.*, 1981; Lalanne *et al.*, 1982) and pBR322 was fixed onto nitrocellulose filters and tested by hybrid-selected translation using spleen cell mRNA from DBA/2 mice. Hybridized mRNA was eluted and translated in a reticulocyte

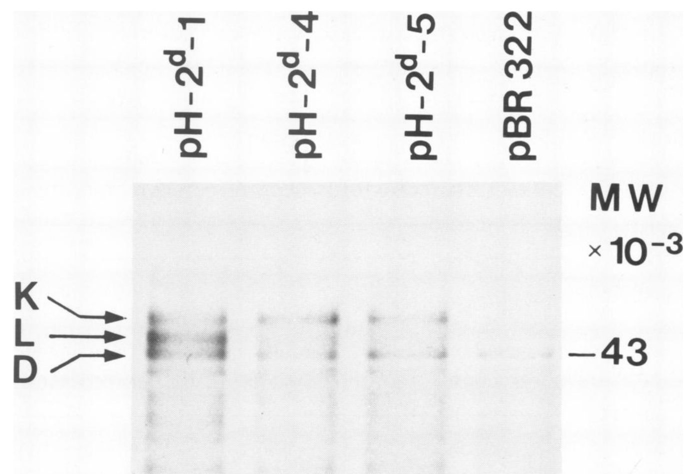

Fig. 1. Selective hybridization of H-2 mRNA to cDNA clone pH-2^d-5. Plasmid DNA of pH-2^d-1, pH-2^d-4, pH-2^d-5, and pBR322 was immobilized on nitrocellulose filters and hybridized with DBA/2 spleen mRNA. After elution, mRNA was translated in a reticulocyte cell-free system supplemented with dog pancreas microsomes. Antigens inserted into microsomes were characterized by SDS-PAGE and autoradiography. The positions of the H-2K^d, L^d, and D^d antigens were determined by co-running of *in vivo* 10-min labelled antigens precipitated with alloantisera (Robinson, 1982).

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cell-free system in the presence of dog pancreas microsomes (Kvist *et al.*, 1981). Clone pH-2^d-1 hybridized to mRNA coding for H-2D, L, and K antigens (Figure 1). Separation of these antigens by polyacrylamide gel electrophoresis (PAGE) has been demonstrated by Robinson (1982). The region of the H-2D^d antigen was obscured by endogenous proteins of the cell-free system. That the clone pH-2^d-1 also hybridizes to mRNA coding for H-2D^d antigens has been shown previously (Kvist *et al.*, 1981). Clone pH-2^d-4 preferentially hybridizes to mRNA coding for H-2K^d antigens and clone pH-2^d-5 nearly exclusively binds mRNA coding for H-2K^d antigen (Figure 1). We concluded that mRNA coding for H-2K^d antigens contains an unique region present in clones pH-2^d-4 and pH-2^d-5. Rabbit anti H-2 antibodies unfortunately could not be used in this analysis as they did not react efficiently with H-2K^d and L^d antigens synthesized in the cell-free system (Dobberstein *et al.*, 1979). We believe that this is because, in the cell-free system, β_2-microglobulin is not bound to the heavy chains.

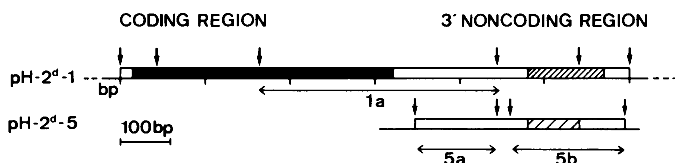

Fig. 2. Partial restriction maps of cDNA clones pH-2^d-1 and pH-2^d-5. The restriction maps were constructed as described (Kvist *et al.*, 1981). The vertical arrows indicate *Pst*I sites. The filled box shows the coding region and the hatched box in the 3′ non-coding region indicates the non-homologous portion of the two clones (see Figure 3). Subcloned fragments used for hybridization are outlined by the horizontal arrows and denoted 1a, 5a, and 5b.

Part of the 3′ non-coding region shows high diversity

The cDNA clone pH-2^d-5 was further characterized by restriction map analysis and Southern blot hybridization using subcloned *Pst*I fragments 5a and b (Figure 2) as probes. Fragment 5b inefficiently hybridized to pH-2^d-1 whereas 5a hybridized to it efficiently (data not shown). Thus, fragment 5b was identified as the unique region hybridizing specifically to mRNA coding for H-2K^d antigen. Sequence analysis of fragment 5b revealed a stretch of poly(A) residues at one end, and 37 residues at the other end, that were nearly identical to nucleotides 278–317 of the 3′ non-coding portion of pH-2^d-1 (Figure 3). pH-2^d-5 thus contained part of the 3′ non-coding region of an H-2 antigen. The sequence of the 3′ non-coding region in pH-2^d-1 was further compared with those of the corresponding regions in clone pH-2II and in Qa pseudogene 27.1 recently characterized by Steinmetz *et al.* (1981a, 1981b). Extensive homology was again found up to bp 317 (Figure 3). Following bp 317, pH-2^d-1, pH-2^d-5, and gene 27.1 diverge greatly whereas clones pH-2^d-1 and pH-2II show near identity.

Fragment 5b hybridizes exclusively to 17S mRNA

Portions of the 3′ non-coding region of H-2 mRNAs might be shared by unrelated mRNAs of different sizes. To test this, we compared probe 5b and coding region 1a in a Northern blot hybridization on total mRNA from DBA/2 mouse spleen. Under the conditions used, both probes exclusively hybridized to 17S mRNA (Figure 4). The value obtained here for the H-2 mRNA agrees well with that obtained earlier by sucrose gradient centrifugation (Dobberstein *et al.*, 1979).

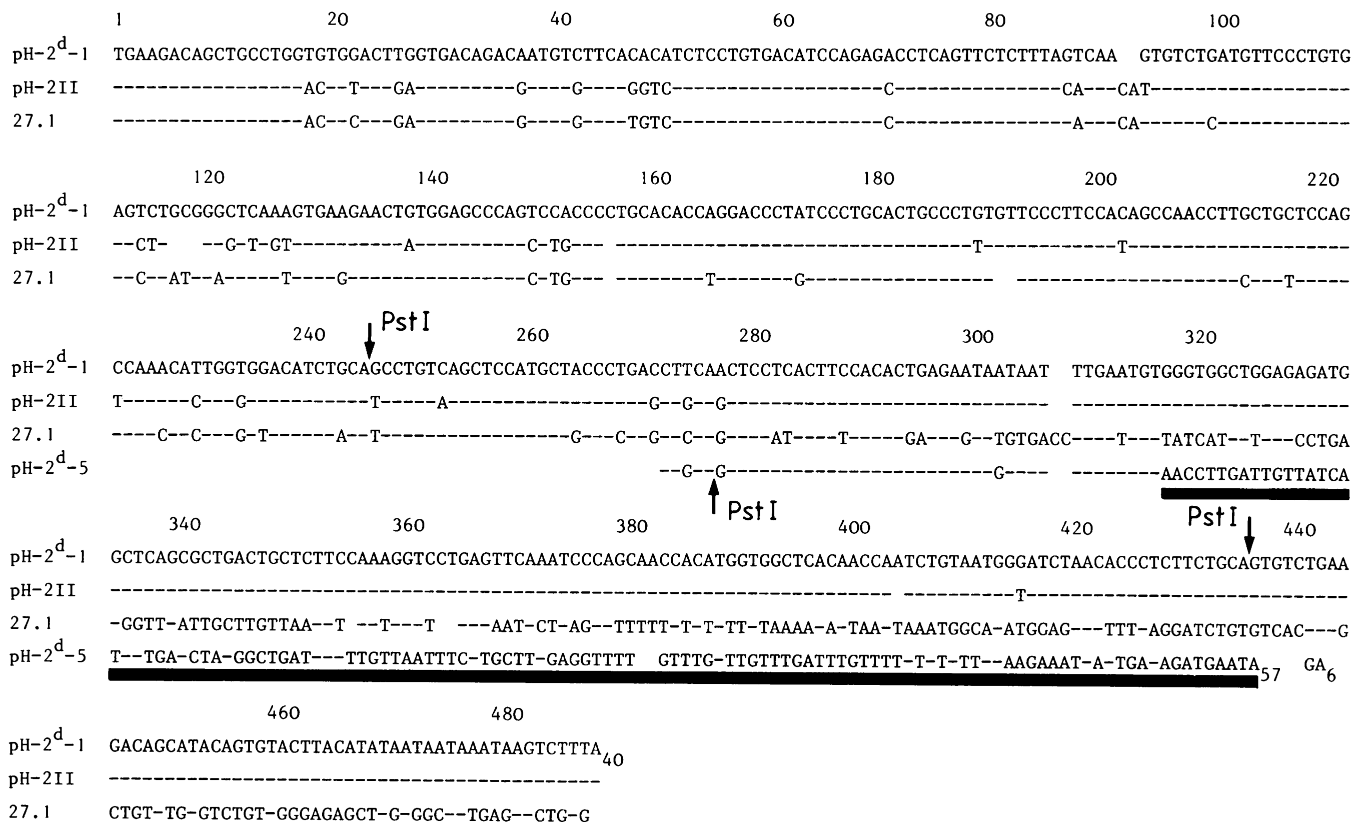

Fig. 3. Comparison of DNA sequences in the non-coding portions of cDNA clones pH-2^d-1, pH-2II (Steinmetz *et al.*, 1981b), and genomic clone 27.1 bp 5023–5504 (see Steinmetz *et al.*, 1981b) and pH-2^d-5b. The non-homologous region between the clones is underlined.

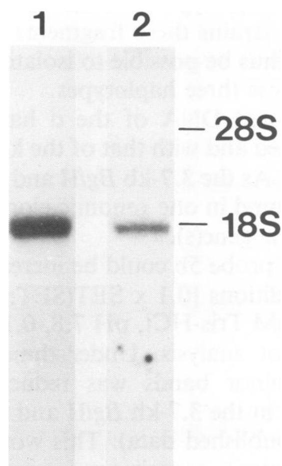

Fig. 4. Size of mRNA coding for H-2 like antigens. 10 μg mRNA from DBA/2 mouse spleens was separated on a 1% agarose gel, transferred to nitrocellulose filter, and hybridized to probe 1a, **(lane 1)** or probe 5b, **(lane 2)**. Mouse 18S and 28S rRNA was run in parallel tracks. Hybridization was in 50% formamide, 5 x SSC, 1 x Denhardt's (Gergen *et al.*, 1980) at 42°C for 12 h. The final wash was in 0.1 SET at 50°C.

Fragment 5b is a low copy H-2 gene probe specific for H-2K^d region gene

The number of H-2 genes in a genome can be roughly estimated by the number of hybridizing bands obtained in a Southern blot analysis using an H-2 cDNA probe comprising the coding region. In such an experiment >15 bands had been obtained, suggesting a large gene family (Steinmetz *et al.*, 1981b; Cami *et al.*, 1981 and Figure 5A). Using fragment 5b as a hybridization probe, we expected to select only a subgroup of the H-2 gene family. When *Bgl*II and *Eco*RI digested DNA from DBA/2 mice was probed with fragment 5b in a Southern blot analysis only two major bands were detected (Figures 5B and C). Thus, probe 5b is a low copy gene probe and could be used to map the corresponding gene(s) within the MHC complex. For this purpose congenic mouse strains of the d, b, and k haplotype and recombinants in different parts of the MHC complex were used (Klein *et al.*, 1978). Congenic mouse strains are identical except for a limited segment on one chromosome carrying a different allele. As hybrid-selected translation had suggested to us that 5b-specific sequences are contained in the H-2K^d molecule, we selected recombinant mouse strains in which the recom-

Fig. 5. Southern blot of mouse DNA using fragment 1a **(A)** or 5b **(B** and **C)** as hybridization probes. 10 μg of liver DNA from mice of different inbred H-2 haplotypes d (DBA/2; B10.D2), k (B10,BR), b (B10) and H-2 recombinants d/b (B10,D2[R103]) k/d (B10.A), k/b (B10.A[4R]) was cleaved to completion with *Bam*HI **(A)**, *Eco*RI **(A** and **C)** or *Bgl*II **(B)**. After separation on an 0.5% agarose gel, DNA was transferred to nitrocellulose filters. Hybridization was with 40 ng/ml of fragment 1a **(A)** for 20 h at 68°C in 1 x Denhardt, 4 x SET and the final wash in 0.1 x SET, 0.1% SDS at 68°C. In **B** and **C** hybridization was with 60 ng/ml of fragment 5b at 60°C and also the final wash at 60°C. The sizes of the marker DNA fragments are given in kilobases. The genomic fragments mapped to the K locus are indicated by arrows and their estimated sizes given in kilobases.

Table I. Mapping of the 3.7-kb *Bgl*II and 13-kb *Eco*RI fragment

Cell line	Locus		Hybridizing fragment	
	H-2K	H-2D	*Bgl*II	*Eco*RI
DBA/2	d	d	3.7 kb	13 kb
B10.D2	d	d	3.7 kb	13 kb
B10.D2(R103)	d	b	3.7 kb	13 kb
B10.A	k	d	3.2 kb	10.5 kb
B10.A(4R)	k	b	3.2 kb	10.5 kb
B10.BR	k	k	3.2 kb	10.5 kb
B10	b	b	2.5 kb	10.5 kb

binational event had occurred between the K and D locus. Using these mouse strains and restriction site polymorphism as a marker, the approximate (relative) location of genes within the MHC can be mapped. A similar approach had been successfully applied to the mapping of genes located in the Qa and Tla region (Steinmetz *et al.*, 1981b; Margulies *et al.*, 1982).

As most congenic recombinant mouse lines are found on the B10 background, we determined whether DBA/2 and B10.D2 mice (both d haplotype) show identical bands in *Bgl*II and *Eco*RI digests of genomic DNA probed with fragment 5b in a Southern blot analysis. Figures 5B and C show that they indeed have both a major 3.7-kb *Bgl*II and a 13-kb *Eco*RI band. In DNA from B10.BR mice (k haplotype) the 3.7-kb *Bgl*II fragment was replaced by one of 3.2 kb, and in B10 mice (b haplotype) by one of 2.5 kb. Using the recombinant mice B10.D2 (R103), B10.A, and B10 (4R) the size of the *Bgl*II or *Eco*RI band followed the allelic form present at the K locus and not that at the D locus (Figure 5). This is summarized in Table I. As the recombinational event in B10A (4R) occurred between the A and B loci of the I region (Klein *et al.*, 1978), the 3.7-kb *Bgl*II fragment of the d haplotype, the 3.2-kb fragment in the k haplotype, and the 2.5-kb fragment in the b haplotype were mapped to the K locus. The 13-kb *Eco*RI fragment is replaced in DNA from k and b haplotype mice by a 10.5-kb fragment. As recombination in B10.A occurred between the E and C loci of the I region, the 13-kb fragment was also mapped to the K locus.

When fragment 5b was used to identify genes from a genomic phage library constructed from DBA/2 mouse DNA, at least three positive clones were detected per haploid genome. All three clones also hybridized to probe 1b. One of these clones contained a 3.7-kb *Bgl*II and a 13-kb *Eco*RI fragment hybridizing to probe 5b (Kvist *et al.*, in preparation).

Discussion

Using hybrid selected translation and Southern blot analysis, we identified a segment on a cDNA clone specific for H-2 gene(s) at the K locus. This segment is located in the 3′ non-coding region and comprises the 120 nucleotide residues upstream from the stretch of poly(A) in clone pH-2^d-5. It contains one polyadenylation site. An identical sequence is present in clone pH-2^d-4 although this clone extends at its 3′ end by a stretch of 20 nucleotides containing a second polyadenylation site (Lalanne *et al.*, 1982). It is thus likely that cDNA clones pH-2^d-4 and -5 are coded by the same gene but use different polyadenylation signals in their processing.

In a Southern blot analysis with *Bgl*II DNA from mice of the d haplotype, probe 5b hybridizes primarily to a 3.7-kb fragment, with k haplotype DNA to a 3.2-kb fragment, and with that of the b haplotype to a 2.5-kb fragment. Using recombinant mouse strains these fragments were mapped to the K locus. It will thus be possible to isolate and compare K locus genes from these three haplotypes.

With *Eco*RI-digested DNA of the d haplotype a 13-kb fragment was detected and with that of the k and b haplotype a 10.5-kb fragment. As the 3.7-kb *Bgl*II and the 13-kb *Eco*RI fragments can be found in one genomic clone, they probably characterize the same gene(s).

The selectivity of probe 5b could be increased by applying more stringent conditions [0.1 x SET(SET: 150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.8, 0.1% SDS), 65°C] in the Southern blot analysis. Under these conditions appearance of the minor bands was reduced, whereas no decrease was found in the 3.7-kb *Bgl*II and the 13-kb *Eco*RI fragment (Xin, unpublished data). This would indicate that only these two fragments contain sequences identical to that contained in fragment 5b. Under stringent conditions probe 5b is, therefore, K locus-specific. The number of genes at the K locus and whether all contain this segment in the 3′ non-coding region have still to be established.

The selectivity of fragment 5b and its property as a low copy probe is in contrast to the equivalent segment in clones pH-2^d-1 and pH-2II. Here, the portion close to the poly(A) tail is highly repetitive in the mouse genome (Steinmetz *et al.*, 1981a). Non-homologous regions in the 3′ non-coding region have also been described by Cosman *et al.* (1982) for SWR/J mice (q haplotype). As their observations are only based on cross-hybridization, and sequence analysis is not yet available, it remains unclear whether fragment 5b is homologous to one of the D fragments described by Cosman *et al.* (1982).

Taken together, the data presented here suggest that the 3′ non-coding region in mRNA coding for H-2 antigens has a bipartite composition. The first portion comprises the stretch of ~300 nucleotides following the stop codon TGA and the second the 120 – 160 nucleotides upstream from the stretch of poly(A). Extensive homology of ~80% is generally found in the first with no significant homology being found in the second portion [cf. pH-2^d-1, pH-2^d-5, and gene 27.1 (Steinmetz *et al.*, 1981a) in Figure 3]. The divergence in the second portion might indicate that this region has been joined to an H-2 gene in a recent recombinational event, probably during the event of gene duplication. The functional relevance of this portion remains unclear. The finding, however, that genes from different loci bear non-homologous portions in their 3′ non-coding region make these segments excellent tools in defining genes of different regions or loci.

Materials and methods

Restriction enzymes, Klenow DNA polymerase, and polynucleotide kinase were purchased from Biolabs (Bishop's Stortford, UK) or Boehringer (Mannheim, FRG). [α-^{32}P]dNTPs (10 mCi/ml, 3000 Ci/mmol) and [γ-^{32}P]ATP (10 mCi/ml, 5000 Ci/mmol) were from the Radiochemical Centre (Amersham, UK). Nitrocellulose filters were obtained from Schleicher and Schuell (Dassel, FRG), Oligo(dT)-cellulose (T3) from Collaborative Research (Waltham, MA). Rabbit reticulocyte lysate and [^{35}S]methionine were from New England Nuclear (Dreieich, FRG). Mice were obtained from J. Klein, Max Planck Institute (Tübingen, FRG), or from our own colony. Plasmid pUC8 was obtained from J. Messing, University of Minnesota.

Selection and isolation of cDNA clones pH-2^d-1 to -5 have been described earlier (Kvist *et al.*, 1981; Bregegere *et al.*, 1981; Lalanne *et al.*, 1982). Hybrid selected translation was performed according to Ricciardi *et al.* (1979) and selected mRNA translated in a reticulocyte lysate in the presence of dog pancreas microsomes (Kvist *et al.*, 1981).

mRNA isolation and Northern blot analysis

RNA was extracted from DBA/2 (H-2^d) mouse spleens by the guanidinium chloride method (Cox, 1968) as described by Deeley *et al.* (1977). The chloroform-butanol extraction was replaced by a phenol-chloroform-isoamylalcohol extraction. Poly(A)$^+$ mRNA was isolated on oligo(dT)-cellulose (Aviv and Leder, 1972) and separated on a 1% agarose gel containing 2.2 M formaldehyde (Rave *et al.*, 1979) and blotted onto nitrocellulose filter (Thomas, 1980).

DNA sequence analysis

cDNA clone pH-2^d-1 was subcloned by the method of Frischauf *et al.* (1980) into pBR322 and sequenced by the method of Maxam and Gilbert (1980). cDNA clone pH-2^d-5 was digested with *Pst*I, and fragments were cloned into pUC8. Inserts were characterized by hybridization to cDNA clones pH-2^d-1 and -5 (Thomas, 1980). One subclone, containing a 250-bp insert, which did not hybridize to pH-2^d-1 but to pH-2^d-5, was selected and named pH-2^d-5b. The plasmid was cut at the *Bam*HI site, labelled, recut with a *Eco*RI, and sequenced (Maxam and Gilbert, 1980).

Isolation of genomic DNA and blot hybridization

DNA was isolated from a single mouse liver essentially as described by Blin and Stafford (1976). Powdered tissue was mixed with 15 ml of the Sarkosyl-proteinase K solution and DNA extracted by gentle agitation. DNA was dialysed against water and concentrated by Aquacide. Restricted DNA was run on a 0.5% agarose gel and transferred to a nitrocellulose filter according to Southern (1975). DNA probes were labelled by nick-translation to a specific activity of $1-5 \times 10^8$ c.p.m./μg (Rigby *et al.*, 1977). Hybridization and washings of the filters were essentially as described by Gergen *et al.* (1980) and as specified in the figure legends.

Acknowledgements

We are especially grateful to J. Klein and P. Singer for advice on selecting the appropriate mouse strains and for providing them. We thank H. Lehrach for advice on using pUC8 as a cloning vehicle, Ms. W. Moses for typing the manuscript, and D. Meyer for critically reading it. This work was supported by a grant from the Deutsche Forschungsgesellschaft Do 199/4-1.

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