

## Two isoforms of the kidney androgen-regulated protein are encoded by two alleles of a single gene in OF1 mice

E. MELANITOU, D. TRONIK AND F. ROUGEON\*

Institut Pasteur, Unité de Génétique et Biochimie du Développement, Département d'Immunologie, 25 rue du Dr Roux, 75724 Paris Cedex 15, France

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### Summary

Two cDNA clones coding for two forms of the mouse kidney androgen-regulated protein (KAP) distinguished by their electrophoretic mobilities on SDS gel electrophoresis have been isolated from libraries prepared from strains of mice having one (BALB/c) or two (OF1) forms of the KAP protein. The corresponding mRNAs have identical sizes, as well as identical sequences in their 5' non-translated regions. The size difference observed between the two proteins is due to two point mutations in the coding region of the KAP mRNA, leading to two amino-acid changes one of which resulted in the substitution of a glycine for a glutamic acid. As shown by *in vitro* transcription/translation experiments, these two amino-acid differences are responsible for the shift in the apparent molecular weight of the protein on SDS gels. Both forms of the protein are more abundant in males than in females.

*In vitro* translation of kidney RNAs isolated from six different strains and species of mice revealed the presence of other forms of the KAP protein, characterized by small variations of their molecular weights. Southern blot analysis data are consistent with the presence of only one *kap* gene in the mouse genome. A restriction fragment length polymorphism has been observed, which does not correlate with the protein polymorphism, indicating the presence of another allele in the OF1 mouse genome.

### 1. Introduction

Genetic studies of a considerable number of loci in mouse subspecies have provided important information for the understanding of molecular mechanisms of gene evolution in higher organisms. A large number of inbred and wild-derived strains and species are available (Chapman *et al.* 1979; Callahan & Todaro, 1978; Ferris *et al.* 1983; Bonhomme *et al.* 1984; Bonhomme, 1986), representing a pool of variation that has accumulated during approximately 10 million years of evolution and now fixed in the homozygous state in different mouse subspecies. Such an example is the duplication of the renin gene in some mouse strains which correlates with variations in the expression of renin in the submaxillary gland (Dickinson *et al.* 1984; Panthier *et al.* 1982; Piccini *et al.* 1982). More subtle differences have been shown associated with strain dependent variations in gene expression as illustrated by the ornithine decarboxyl-

ase gene expressed in the mouse kidney (Melanitou *et al.* 1987). Variations in the length of transcripts, encoded by the RP2 gene which is also expressed in the mouse kidney (Berger *et al.* 1981) appear to be due to the presence or absence of a B1 repetitive element in the 3'-untranslated region of the gene (Elliot & Berger 1983; King *et al.* 1986).

The kidney androgen-regulated protein has been described previously as a 20 kDa peptide identified by *in vitro* translation of kidney mRNA (Toole *et al.* 1979). It is encoded by an abundant mRNA which represents 4% of the total mRNA and accumulates in response to androgens (Watson *et al.* 1984). It has been recently reported that the KAP mRNA is expressed in two distinct regions of the kidney under the control of different hormones. In the epithelial lining of the proximal tubules it is expressed under androgen control and after castration, it is only found in tubules in the outer stripe of the medulla (Meseguer *et al.* 1989).

We have used *in vitro* translation of mRNA to examine KAP protein in two different mouse strains

\*Corresponding author.

(OF1 and BALB/c) and have observed the presence of a second polypeptide of higher molecular weight in OF1 mice. This protein is present in higher concentrations in males than in females. In order to elucidate the origin of the second polypeptide which could have arisen by gene duplication, by alternative splicing of a single gene generating two different mRNAs or by point mutations in the coding region of this gene, we have cloned and sequenced the cDNAs corresponding to these two proteins.

We report here, the characterization of the two alleles of the *kap* gene: *Kap<sup>a</sup>* and *Kap<sup>b</sup>* and the structural analysis of their respective cDNAs.

## 2. Materials and methods

### (i) Animals

Male and female mice of inbred BALB/c, C3H, CBA/J, SJL/J and DDK strains and the outbred OF1 strain were purchased from Iffa Credo (Rhône, France). Two wild-derived strains, SPE/*Pas* from the *Mus spretus* progenitor (Granada, Spain), strictly inbred strain in Institut Pasteur since 1981 and the MAI, from a *M. m. musculus* progenitor derived from wild animals trapped at Illmitz (Austria). First propagated as a closed colony, then brother × sister mated at the Institut Pasteur, (Bonhomme & Guénet, 1989). The animals were used between 5 and 7 weeks of age, and have been killed by cervical dislocation.

### (ii) RNA isolation, blotting and in vitro translation

Total RNA was isolated from mouse kidneys as previously described (Tronik *et al.* 1987) except that 4 M guanidine thiocyanate was used instead of 6 M urea. Poly(A)<sup>+</sup> RNA was prepared as reported previously (Auffray & Rougeon, 1980).

*In vitro* translation of RNAs was performed with the mRNA-dependent reticulocyte lysate translation system (Pelham *et al.* 1976). The products were analysed by SDS/PAGE gel electrophoresis.

DNA-mRNA hybrid arrested cell-free translation experiments were carried out as previously described (Paterson *et al.* 1977).

### (iii) DNA analysis

DNA was prepared from individual OF1 mice. DNA of each animal was digested with the restriction enzymes *Hind* III, or *Bam*HI. The digests were separated by electrophoresis on 0.7% agarose gels and transferred to Hybond N nylon membranes (Amersham). The KAP probe used was the entire cDNA insert, labelled by the multi-prime DNA labelling method (Feinberg & Vogelstein, 1983). The filters were hybridized to the cDNA probe and washed under stringent conditions.

### (iv) Construction of cDNA libraries

Double-stranded cDNA was synthesized from poly(A)<sup>+</sup> RNA isolated from kidneys of 10 male OF1 and 10 male BALB/c mice, using the cDNA synthesis system from Amersham, as recommended by the manufacturer. The double stranded cDNA was methylated by *Eco*R I methylase, ligated to *Eco*R I polylinkers and after *Eco*R I digestion cloned into the corresponding site of pBluescript II phagemid vector (Stratagene GmbH, Heidelberg, FRG). Host XL-I Blue bacteria (Stratagene GmbH) were transformed and colonies were screened by hybridization with an oligonucleotide,

5'-GGCCTTGGCCCACTTGACCACGTGTAGAAG  
CTCT-3',

labelled at its 5' end with <sup>32</sup>P using T4 polynucleotide kinase. This oligonucleotide is complementary to a known partial sequence of KAP mRNA (Catterall *et al.* 1986). About 3000 recombinant clones of each library were screened on nitrocellulose filters.

### (v) DNA sequencing

Double-stranded DNA fragments cloned into the *Eco*R I site of the polylinker of the pBluescript vector have been denatured and sequenced by the Sequenase method (USB Corporation). SK and KS primers have been used for sequencing of both directions. The oligonucleotide used for the initial screening served as additional internal sequence primer. Moreover, additional sequence data have been obtained by purifying the *Eco*R I KAP cDNA fragments on agarose gels and subcloning into the bacteriophage M13 mp10 vector (Messing & Vieira, 1982). Single-stranded DNA templates were prepared as described previously (Sanger *et al.* 1980) and sequenced by the dideoxynucleotide chain termination method (Sanger *et al.* 1977). The search for sequence homologies between KAP and the proteins of the PSE QUIP library (Claverie & Bricault, 1986) was done with the FASTP program, according to Lipman & Pearson, (1985).

### (vi) Mapping of the 5' end of the KAP mRNA

The transcription start site was mapped by primer extension experiments using a 26mer oligonucleotide,

5'-ATTCTGACAGGGGGAAAGCCACAGTC-3'

complementary to a sequence located 43 nucleotides downstream the AUG initiation codon of the KAP mRNA (Fig. 3). The primer was labelled with <sup>32</sup>P at its 5' end using T4 polynucleotide kinase and was hybridized (30 000 cpm) with 1 μg of total RNA from male mouse kidneys or the same amount of mouse spleen RNA, in the presence of yeast t-RNA and extended as described (Dreyfus *et al.* 1987).

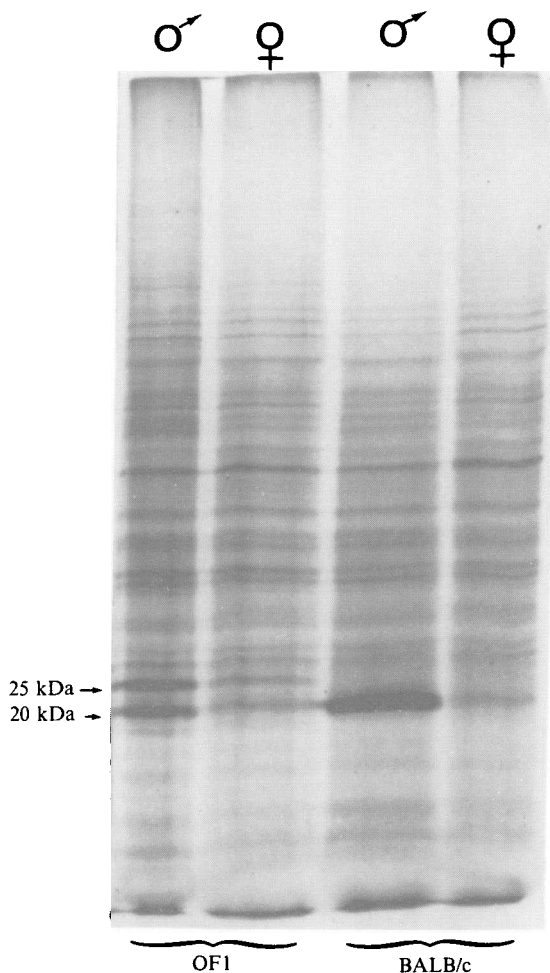


Fig. 1. *In vitro* translation analysis of total RNA isolated from kidneys of BALB/c and OF1 male and female mice. 5  $\mu$ g of total RNA was translated in a reticulocyte cell-free system in the presence of [ $^{35}$ S]methionine. *In vitro* translation products were separated on a 1.5% polyacrylamide gel and autoradiographed. Arrows indicate the two abundant peptides with their relative molecular weights.

(vii) *Polymerase chain reaction and in vitro transcription*

5  $\mu$ g of total RNA isolated from kidneys of DDK mice, were transcribed into cDNA in a total volume of 40  $\mu$ l using (dT)<sub>12-18</sub> as primer and avian myeloblastosis virus reverse transcriptase. After an incubation period of 1 hr at 37 °C, the PCR was carried out on a 5  $\mu$ l cDNA reaction mixture as a template, in a final reaction volume of 100  $\mu$ l, using the Ampli-Taq (Cetus, CA). The concentration of each of the two PCR primers was 100 pmol. These are oligonucleotide-1

5'-GCCTTAACCCTACTAAAGCATGATGC-3',

identical to nucleotides 25-51 of the 5' end of KAP cDNA and oligonucleotide-2

5'-GGGTAGAGCTTAGAGATGCCTGGCAC-3'

complementary to nucleotides 538-564 of the 3' non coding region of the KAP cDNA. Reactions were

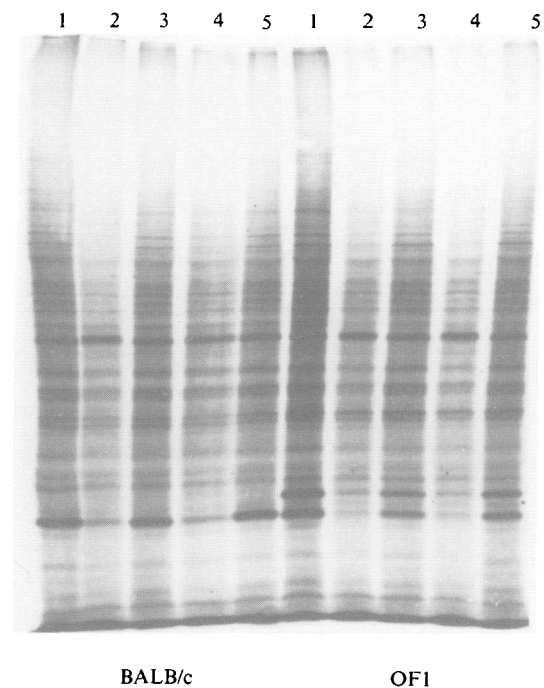


Fig. 2. Hybrid-arrested cell-free translation analysis of KAP cDNA clones. 10  $\mu$ g of total RNA prepared from kidneys of male BALB/c and OF1 mice were translated in a reticulocyte cell-free system alone (lane 1) or after hybridization with 600 ng of purified cDNA insert (lanes 2 and 4). Hybridization with the same amount of cDNA insert and further melting of the hybrid (lanes 3 and 5). Lanes 2 and 3 RNAs were incubated with the Ba-2 cDNA isolated from the BALB/c library. Lanes 4 and 5, RNAs were incubated with the Sw-66 cDNA isolated from the OF1 library. Lane 1: RNA transcribed in the absence of cDNA. The *in vitro* translation products were electrophoresed in a 1.5% SDS-PAGE, and autoradiographed.

started at 95 °C and performed for 40 cycles (95 °C, 50 s; 50 °C, 1:30 min; 72 °C, 2 min) in a thermocycler (HYBAID). The amplified product was 539 nucleotides long and contained the entire coding region of the KAP cDNA. The amplified DDK cDNA has been isolated by standard methods (Maniatis *et al.* 1982), phosphorylated by T4 kinase and cloned in the *Sma* I site of the pBluscript vector. Four different clones have been chosen for sequence analysis using the T7 and reverse primers, (Stratagene GmbH, Heidelberg), to sequence both strands.

The Ba-2, Sw-66 and DDK-5 cDNA clones (described in the text), have been subcloned in the *Hind* III/*Bam*H I sites of the SP64 polylinker. *In vitro* transcription has been carried out using the Sp6 polymerase, in the presence of rNTPs and ribonuclease inhibitor (Melton *et al.* 1984).

### 3. Results

(i) *Two abundant androgen-regulated in vitro translation products are present in OF1 mice*

*In vitro* translation analysis of kidney RNA isolated from BALB/c inbred mice revealed a typical pattern

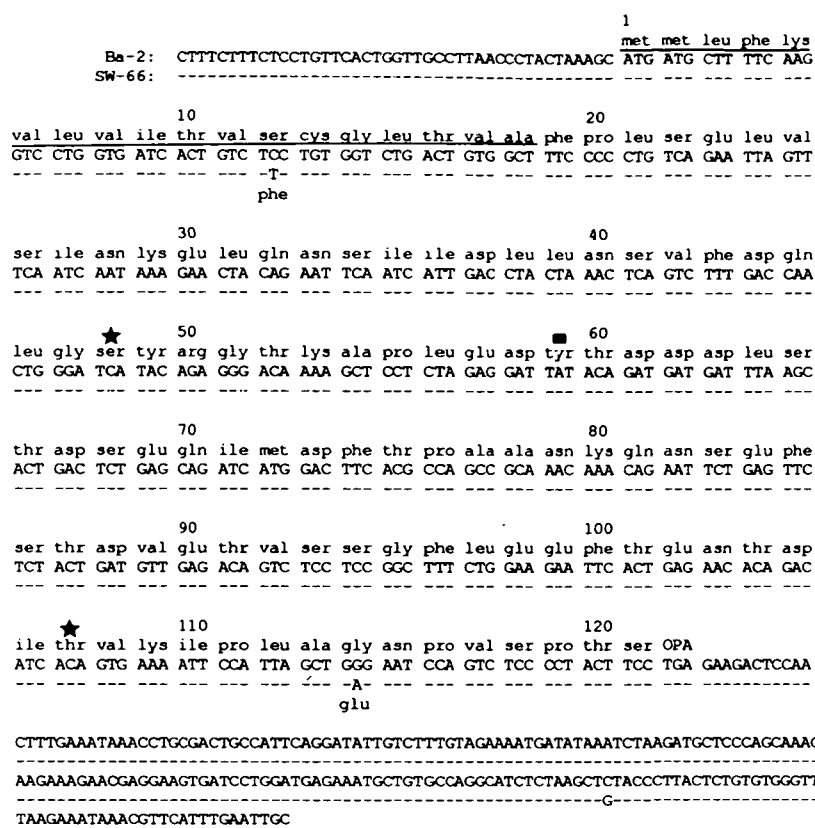


Fig. 3. Comparison of the complete nucleotide and amino-acid sequences of two KAP cDNA clones isolated from BALB/c (Ba-2) and OF1 (Sw66) libraries and corresponding to *Kap<sup>a</sup>* and *Kap<sup>b</sup>* alleles respectively. Underlined is the signal peptide. The point mutations and the aminoacid changes as well as the potential protein kinase C phosphorylation sites (★) and tyrosine sulphation site (■) are noted.

giving one abundant protein of apparent  $M_r$  20000 Da which was present at a higher concentration in male than female mice (Fig. 1). This protein, called 'kidney androgen-regulated protein' (KAP) has been described previously (Toole *et al.* 1979). The level of this polypeptide showed a sexual dimorphism in OF1 mice. Unexpectedly, a second abundant translation product of apparent  $M_r$  25000 Da was obtained from RNA of OF1 mice and it also showed androgen-dependent expression. The presence of this second polypeptide has not been reported before and prompted us to determine its origin. To this end we synthesized double-stranded cDNAs from poly(A)<sup>+</sup> RNA prepared from kidneys of male BALB/c and OF1 mice and were used to construct libraries in the Bluescript plasmid vector. Three thousand recombinants of each library were screened with a <sup>32</sup>P-end labelled oligonucleotide, complementary to a known KAP partial RNA sequence (Catterall *et al.* 1986). Twenty positive clones of the OF1 library and five clones of the BALB/c library were chosen for further study. Enzymatic restriction analysis revealed a similar pattern for all these clones (data not shown), suggesting that they contained similar sequences.

The positive clones were characterized by DNA-mRNA hybrid-arrested cell-free translation experiments (Fig. 2). The cDNA clones derived from both BALB/c and OF1 libraries were able to inhibit the *in*

*vitro* synthesis of the two abundant polypeptides in OF1 mice. These data indicate that the two protein forms observed in OF1 mice derive from related mRNAs.

#### (ii) Comparison of BALB/c and OF1 cDNA sequences

As expected, analysis of the cDNAs isolated from the BALB/c library revealed an identical nucleotide sequence for all the clones studied. In contrast, the KAP cDNA clones, obtained from the OF1 library were of two types: group 1 contained those with sequences identical to the BALB/c KAP cDNA. Group 2 clones differed from the BALB/c KAP cDNAs, by three point mutations, one, at position 78 (C → T), a second, at nucleotide 384 (G → A) and the third, at nucleotide 559 (C → G) in the 3' non-coding region. The two mutations of the OF1 KAP cDNA located in the coding region lead to amino-acid substitutions: the first in codon 1 replaces the Ser (BALB/c cDNA) by a Phe (OF1 cDNA). The second, at amino-acid 114 (GGG codon), results in the substitution of a Gly (BALB/c cDNA) by a Glu (OF1 cDNA). The sequences of the KAP cDNAs of two representative clones, Ba-2 and Sw-66, derived from the BALB/c and the OF1 libraries respectively and their deduced aminoacid sequences are shown in

Figure 3. The  $M_r$  of the proteins deduced from these nucleotide sequences is 13 kDa.

By primer extension analysis using an oligonucleotide complementary to a sequence located 43 nucleotides downstream of the first ATG codon, we established that the entire mRNA sequence was contained in our cDNA clones (data not shown).

The KAP mRNA is 607 nucleotides long, not including the polyA tail. The 5' non-coding region is 44 nucleotides long and the 3' non-coding region 198. The polyadenylation signal AATAAA (Proodfoot & Brownlee, 1976) is located 22 nucleotides upstream of the polyA tail.

Although experimental evidence concerning post-translational modifications of the KAP protein is missing at present, potential sites of post-translational modifications, protein kinase C phosphorylation, *O*-glycosylation and tyrosine sulphation are found in four positions of the deduced KAP amino-acid sequence (Fig. 3).

(iii) *Two point mutations in the KAP mRNA sequence are responsible for the second form of the KAP protein*

In order to investigate whether the amino-acid substitutions found in the OF1 KAP mRNA are responsible for the differences in electrophoretic mobility of the protein on SDS-PAGE, we carried out *in vitro* transcription/translation experiments for the two cDNA clones Ba-2 and Sw-66. As shown in Fig. 4, the protein coded by the Sw-66 cDNA (lane 1) corresponds to the higher molecular weight form (25 kDa) found in the OF1 mice, the Ba-2 clone (lane 4) giving rise to the smaller (20 kDa) KAP protein.

These results show that the point mutations giving amino-acid changes found in the Sw-66 cDNA clone confer the observed shift in the electrophoretic mobility of the protein. Indeed, the second point mutation, responsible for the substitution of a Gly by a Glu, adds an extra negative charge to the KAP protein, which could alter its electrophoretic mobility on SDS-polyacrylamide gels. The difference observed between the  $M_r$  deduced from the cDNA sequence (13 kDa) and the apparent  $M_r$  obtained by SDS-gel electrophoresis (20 kDa) is probably due to the fact that the KAP protein is very acidic. Such a discrepancy between the molecular weight predicted from sequence analysis and that determined from the electrophoretic mobility on SDS gels in the *in vitro* translation products has been described for proteins in the submaxillary gland of rat (Mirels *et al.* 1987; Chupin-Rosinski *et al.* 1988) and mice (Rougeon *et al.* 1981).

(iv) *Several alleles of the kidney androgen-regulated protein gene are present in mice*

Two hypotheses could account for the genetic origin of the polymorphism observed between the two strains

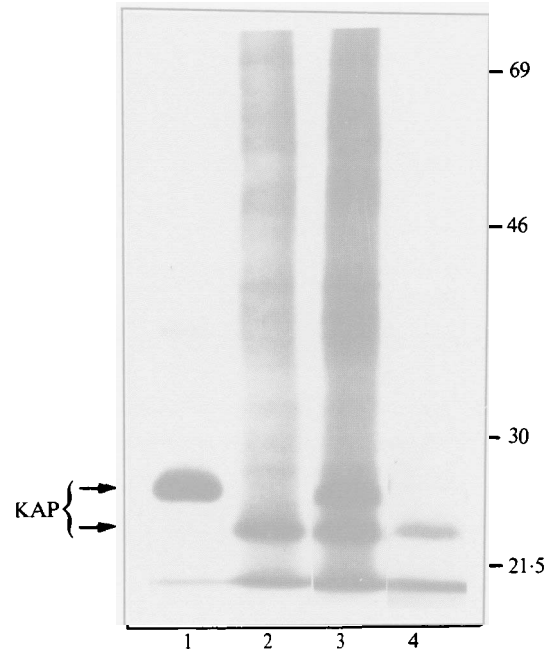


Fig. 4. Analysis of the *in vitro* translation products of the SP6 directed KAP transcription. *In vitro* transcription/translation of Sw-66 cDNA clone isolated from the OF1 library (lane 1), of Ba-2 cDNA clone isolated from the BALB/c library (lane 4), and analysis of the *in vitro* translation products of total kidney RNA isolated from BALB/c mice (lane 2) and OF1 mice (lane 3). The two forms of KAP are indicated by arrows.  $M_r$  markers are as indicated (kDa).

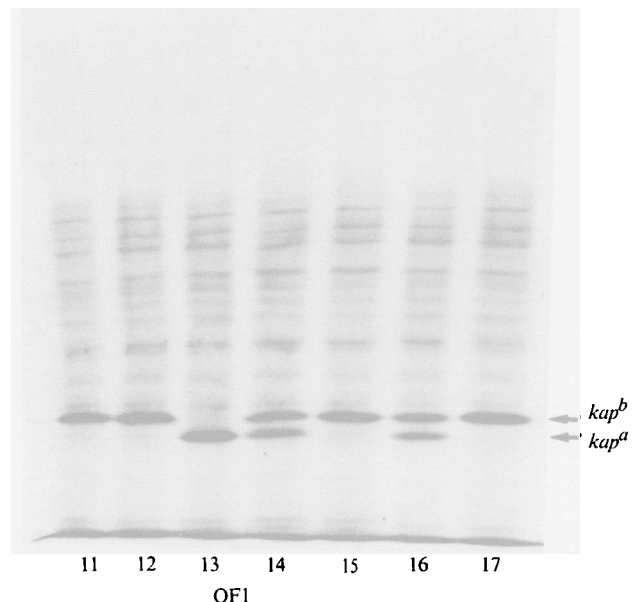


Fig. 5. *In vitro* translation analysis of total RNA isolated from male kidneys of 7 individual OF1 mice (11–17) was performed as described in the legend of Fig. 1. Arrows indicate the forms of the KAP protein, coded by the two alleles ( $Kap^a$  and  $kap^b$ ).

of mice: the presence of two recently duplicated genes or the existence of two alleles of one single gene. To that end, we prepared kidney RNA from seventeen individual male OF1 mice. Figures 5 and 6a show the *in vitro* translation patterns of these RNAs. We did

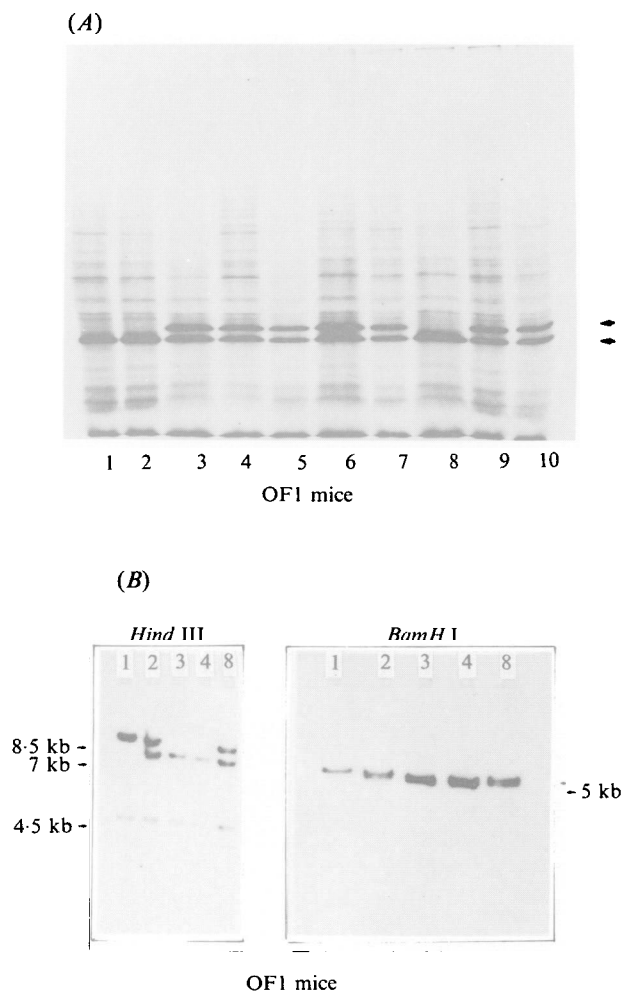


Fig. 6. (A) *In vitro* translation analysis of total RNA isolated from kidneys of individual OF1 mice (1–10). Experimental conditions were as described in legend of figure 1. Arrows indicate the two KAP forms coded by alleles *Kap<sup>a</sup>* and *Kap<sup>b</sup>*. (B) Southern blot analysis of the *kap* gene in OF1 individual mice. Numbers (1, 2, 3, 4, 8), correspond to the same mice as in (A). DNA was digested with *Hind* III and *Bam*HI, subjected to a 0.7% agarose gel electrophoresis and blotted onto nylon membrane and hybridized to the KAP cDNA probe as described in Materials and methods. *Hind* III-digested/ $\lambda$ DNA fragments served as size markers. Sizes of fragments are indicated (in kb).

indeed observe the existence of individuals homozygous for the *Kap<sup>a</sup>* or *Kap<sup>b</sup>* allele as well as individuals heterozygous for each of these two alleles (Fig. 5).

A restriction fragment length polymorphism of the *kap* gene has been previously described and permitted this gene to be mapped on mouse chromosome 6 (Melanitou *et al.* 1991). In order to examine if the presence of the two alleles correlated with a restriction pattern polymorphism, Southern blot analysis was carried out using DNAs prepared from individual OF1 mice (Fig. 6B), homozygotes for the *Kap<sup>a</sup>* allele (mice 1, 2 and 8, Fig. 6A) or heterozygotes for the *Kap<sup>a</sup>/Kap<sup>b</sup>* alleles (mice 3 and 4, Fig. 6A). Individual OF1 mice carrying only the *Kap<sup>a</sup>* allele showed two different restriction patterns (Lanes 1 and 2, Fig. 6B),

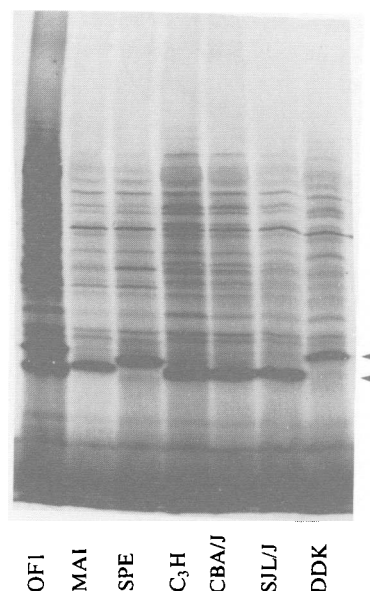


Fig. 7. *In vitro* translation analysis of total RNA isolated from male kidneys of 7 different mouse strains, was performed as described in legend of Fig. 1. Strains are mentioned under each lane. Arrows indicate the KAP protein coded by *Kap<sup>a</sup>* and *Kap<sup>b</sup>* alleles.

suggesting the existence of an additional polymorphism except the one found on the protein level.

#### (v) *In vitro* translation analysis of RNAs isolated from 6 different strains of mice

Given the second form of the KAP protein and the polymorphism of the *kap* gene found in the OF1 mice we looked at the protein patterns of other inbred and wild-derived strains. *In vitro* translation of RNAs isolated from the kidneys of 6 different strains was carried out and interestingly, the DDK strain showed a single abundant *in vitro* translation product having an electrophoretic mobility similar to that of the second KAP form of OF1 mice (Fig. 7). Curiously, the proteins of the wild derived inbred strains, MAI and *Mus spretus* (SPE) showed intermediate electrophoretic mobilities (Fig. 7).

By PCR amplification we isolated a KAP cDNA from the DDK mice. Sequence analysis showed that it contains a phenylalanine at amino-acid position 1, and a glutamic acid at amino-acid position 114, corresponding to the *Kap<sup>b</sup>* allele as expected by its apparent  $M_r$ .

#### 4. Discussion

We report here the existence of several alleles of the kidney androgen-regulated protein gene, in mice. We have characterized two cDNAs from OF1 mice, corresponding to two forms of the KAP protein, encoded by two alleles. Specifically, these two cDNAs differ by three point mutations two of which are located in the coding region of the KAP mRNA.

We have identified two additional forms, present in two wild-type *Mus* species: MAI and *Mus spretus* (Fig. 7). These genes have not yet been sequenced, but code for intermediate forms of the KAP protein. Another cDNA sequence corresponding to the KAP mRNA isolated from A/J mice has been reported (Meseguer *et al.* 1989). It is of interest to note that the BALB/c KAP cDNA is different from the reported A/J sequence at two positions. The first nucleotide in our sequence is a C instead of an A for the A/J sequence and at position 78 there is a C instead of an A in the reported A/J sequence. These data taken together suggest the presence of several alleles for the *kap* gene in different strains of mice.

In addition to the strain dependent size variation of the protein on SDS gels, Southern blot analysis has shown a restriction fragment length polymorphism in OF1 individuals. Therefore, one additional allele is present in the OF1 mouse genome.

No evidence has been found for the presence of two *kap* genes in the mouse genome. Indeed, the chromosomal localization of the *kap* gene, the existence of inbred strains homozygous for the *a* or *b* allele, as well as the similar amounts of protein detected by *in vitro* translation of heterozygous individuals, suggest that a single *kap* gene exists per haploid genome and that several alleles are present in different strains of mice. Additional evidence for the presence of a single *kap* gene has been also reported by the recent cloning of this gene (Niu *et al.* 1991). Although, the possibility of the existence of two closely linked genes coding for the kidney androgen-regulated protein cannot be completely excluded, it seems unlikely in the light of our results.

Structural polymorphisms have been correlated with variations in the level of gene expression concerning the  $\alpha$ 1-antitrypsin gene in the kidney (Berger & Bauman, 1985), the major urinary proteins in the liver (Sampsel & Held, 1985), and the lysozyme in the small intestine (Hammer & Wilson, 1987), indicating extensive interspecies diversity within the genus. In the case of the *kap* gene there are no variations of gene expression associated with protein polymorphism.

We did not detect KAP mRNA when RNAs isolated from kidneys of rat (genus *Rattus*) or the *Nannomys minutoides* species have been used for Northern blot analysis (Melanitou, unpublished observations). This suggests that the appearance of the KAP mRNA is a recent acquisition in the evolution of the *Mus* species, since mice have diverged from the rat approximately 10 million years ago, and the *Nannomys* species is placed between rat and mouse in the phylogeny map (Bonhomme & Guenet, 1989). The study of the *kap* gene expression, among related species, could be useful in order to understand the origin of the high level of expression found in mice.

The biological role of the KAP protein remains unresolved. The observation that only one gene codes

for this protein makes it a good candidate for gene disruption studies by homologous recombination.

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