The Genes for Mouse Salivary Androgen-Binding Protein (ABP) Subunits Alpha and Gamma Are Located on Chromosome 7

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ABSTRACT

We demonstrate that the previously described gene *Androgen binding protein* (*Abp*; DLOUHY and KARN, 1984) codes for the Alpha subunit of ABP and rename the locus *Androgen binding protein alpha* (*Abpa*). A study of recombinant inbred strains demonstrates that *Abpa* is located on chromosome 7 near *Glucose phosphate isomerase-1* (*Gpi-1*). Biochemical and genetic evidence indicates the existence of another ABP subunit, Gamma, and its locus, *Androgen binding protein gamma* (*Abpg*), that is closely linked to *Abpa*. Although no polymorphism has ye. been found for the previously described Beta subunit of ABP (DLOUHY and KARN, 1983; 1984), we suggest that it represents a third locus, *Androgen binding protein beta* (*Abpb*). ABP subunits appear to dimerize randomly and a model is presented demonstrating the origin of six ABP dimers in the salivas of $Abpa^{a}Abpg^{a}/Abpa^{b}Abpg^{b}$ heterozygous mice. The results of cell-free translation studies in which the pre-ABP subunits are identified specifically by immunoprecipitation with anti-ABP antibody supports the idea that independent mRNAs code for the Alpha, Beta and Gamma subunits.

TWO mouse saliva polymorphisms, *Androgen binding protein* (*Abp*) and *Salivary protein variant-1* (*Sal-1*), were described independently at Indiana University and Jackson Laboratory, respectively (for a listing of both, see *Mouse News Letter*, No. 72, 1985). Our laboratories have collaborated and we have determined that *Abp* and *Sal-1* are the same by using an anti-ABP antibody to probe Western blots of gels used to type *Sal-1* (DLOUHY *et al.* 1986); Abp^{a} is the same as $Sal\text{-}1^{b}$ and Abp^{b} is the same as $Sal\text{-}1^{a}$. Because the term androgen binding protein (ABP) is more functionally descriptive for the protein involved in this polymorphism, that designation will be retained.

ABP is produced *de novo* in the submaxillary gland and has been shown to be composed of disulfide-bridged subunits, Alpha (*ca.* 12,000 daltons) and Beta (*ca.* 8,800 daltons) (DLOUHY and KARN 1983). A similar structure has been described for rat prostatic steroid binding protein (PBP), a rat prostate secretory protein that also binds androgens (HEYNS and DE MOOR 1977; PARKER, NEEDHAM and WHITE 1982). The electrophoretic mobility of mouse salivary ABP is determined by the *Abp* structural gene (DLOUHY and KARN 1984) and by the androgen-mediated action of another gene, *Sex-limited saliva pattern* (*Ssp*) (KARN *et al.*, 1982; DLOUHY and KARN 1983, 1984). Those studies showed that Ssp^{S} causes an increase in the apparent molecular weight of the ABP Alpha subunit through an unknown, androgen-mediated mechanism, but did not show which subunit was encoded by *Abp*.

A number of ABP-like protein bands have been observed in various electrophoretic analyses of mouse saliva. The most anodal forms, previously referred to as Abp a and Abp b, are the major bands and were used to describe the *Abp* polymorphism (DLOUHY and KARN 1984). Because the system has grown in complexity, those two major ABP bands are referred to as ABP region I proteins, specifically ABP Ia and ABP Ib (or, for brevity, Ia and Ib, respectively). An additional ABP protein, previously called Abp b′ (DLOUHY and KARN 1984), is now referred to as ABP region II (ABP II). ABP II binds androgen and is composed of subunits of sizes similar to that of ABP I (DLOUHY and KARN 1984). However, in those studies the origin of the ABP II band was not known and preliminary gel extraction studies suggested that ABP II might be simply an aggregate of ABP Ib.

In this report we demonstrate that the previously described gene *Abp* (DLOUHY and KARN 1984) is the ABP Alpha subunit gene and rename the locus *Androgen binding protein alpha* (*Abpa*). Our recombinant inbred strain studies show that *Abpa* is closely linked to *Glucose phosphate isomerase-1* (*Gpi-1*) on chromosome 7. We also describe another ABP protein region, referred to as ABP III, and provide evidence that ABP II and ABP III are not due to aggregation of ABP I proteins, but instead are the expression of a second polymorphism closely linked to the *Abpa* locus. Our genetic and biochemical data suggest that three

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distinct genes determine mouse submaxillary ABP subunits and we present a model to explain how the subunits interact to form the major ABP bands in regions I, II and III. We propose a nomenclature consistent with the guidelines developed by the Committee on Standardized Genetic Nomenclature for Mice (1973, 1985).

MATERIALS AND METHODS

Inbred mice and biological samples: Mouse strain C3H/St was obtained from the West Seneca Laboratory, Roswell Park Memorial Institute, West Seneca, New York. Other inbred strains were from the Jackson Laboratory, Bar Harbor, Maine. Animals were maintained and their salivas collected as previously described (KARN *et al.* 1982).

Electrophoresis and isoelectric focusing: Thin-layer polyacrylamide (typing gel) electrophoresis, isoelectric focusing methods and two-dimensional electrophoresis have been described (KARN *et al.* 1982; DLOUHY and KARN 1983; 1984).

For some experiments, purification of ABP proteins was accomplished by separation of mouse salivary proteins in a typing gel followed by light staining and excision of the protein bands. ABP proteins in the gel pieces were then reduced with 0.5 mg/ml dithioerythritol in 6 M guanidine 0.3 M Tris-HCl (pH 8.2) and the sulfhydryl groups alkylated with 1.0 mg/ml iodoacetic acid to prevent reassociation of subunits. Subunits were then separated by applying the gel pieces to a pH 3.5–11, 4 M urea focusing gel and isoelectric focusing conducted for 4–5 kV hr. Channels from the focusing gel were applied to an SDS gel for further separation of the subunits.

[^{14}C]Testosterone-labeled proteins were detected by electrophoresis and autoradiography as described previously (DLOUHY and KARN 1983; 1984) and fluorography of ^{3}H-labeled cell-free translation products was accomplished as described by LASKEY and MILLS (1975).

Electroblotting of gels and immunologic staining of filters: Following polyacrylamide gel electrophoresis, proteins were transferred (Western blotted) to nitrocellulose paper (Schleicher & Schuell) using a Bio-Rad Trans-Blot chamber. ABP-like proteins were detected immunologically as described previously (DLOUHY, NICHOLS and KARN 1986).

Column chromatography: Separation of mouse salivary proteins was achieved by gel filtration at 4° in a Sephadex G-100 column of 1.5-cm bed diameter and 85-cm bed height. A sample of 0.6 ml of mouse saliva was incubated with [^{14}C]testosterone as previously described (DLOUHY and KARN 1983) to bind the androgen to ABP. The sample was then applied to the top of the bed and the column eluted with 10 mM NH_4HCO_3 (pH 8.3). The proteolytic inhibitors phenylmethylsulfonyl fluoride (PMSF; 1 mM) and bovine pancreatic trypsin inhibitor (Trasylol; 1% v/v) were added to all buffers in the purification steps. Elution positions of specific proteins were ascertained by electrophoresis of aliquots of the fractions (see below) and bound testosterone was detected by autoradiography.

Submaxillary gland RNA isolation: RNA was extracted from fresh mouse submaxillary glands by the guanidinium thiocyanate method (CHIRGWIN *et al.* 1979), with an additional extraction with chloroform/butanol (4:1 v/v; HARDING and RUTTER 1978). Polyadenylated RNA was isolated by binding to oligo(dT)-cellulose (type 3, Collaborative Research) as described by AVIV and LEDER (1972).

Cell-free translation: Poly(A) mRNA, was translated in a commercial cell-free translation kit (Bethesda Research Laboratories) incorporating [^{3}H]leucine. Incorporation was monitored by scintillation counting of chromatographed portions of the reaction mixture and labeled products were visualized by urea gel isoelectric focusing, followed by fluorography and autoradiography.

Immunoprecipitation of cell-free translation products: ABP-like translation products were precipitated by a double antibody method. A 20–30-μl sample of translation reaction was mixed with 30 μl of 0.1 M Tris-HCl buffer (pH 7.5) containing 4% Triton and 4% deoxycholate (TTD buffer) and 8 μl primary rabbit anti-ABP antiserum. Binding of primary antibody with ligand was conducted for one hour at 37°. Following that, 80 μl of goat anti-rabbit antibody (in dH_2O) and 80 μl of TTD buffer were added and incubation continued for another hour at 37°. Precipitates were collected by centrifugation through a pad of 1 M sucrose in ½ × TTD buffer and washed twice with ½ × TTD buffer without sucrose. Final pellets were dissolved in 6 M urea prior to application to the focusing gel.

RESULTS

***Abp* codes for the Alpha subunit of androgen binding protein (ABP):** The isoelectric points of the subunits of ABP Ia and Ib were compared to determine whether the Alpha or Beta subunit is encoded by the previously described gene, *Abp*. This was accomplished using small amounts of material obtained by excision of the ABP Ia and Ib bands from a typing gel, dissociation of the subunits and separation of the Alpha and Beta monomers by urea isoelectric focusing and SDS electrophoresis (see MATERIALS AND METHODS). By this method (see Figure 1), it is possible to visualize the relative isoelectric points and sizes of the different ABP subunits in a two-dimensional gel.

ABP Ia and Ib have different Alpha subunits, Alpha^{a} and Alpha^{b}, respectively, but identical Beta subunits (Figure 1, panel B). Alpha^{a} has a more basic isoelectric point than Alpha^{b} as previously shown for the intact Ia and Ib dimers (DLOUHY and KARN, 1984). Thus, *Abp* determines a charge property of the Alpha subunit of ABP, leading to the conclusion that it is probably the Alpha subunit structural gene. In order to make the gene designation more specific, and to clarify the discussion below, we now refer to this gene as *Androgen binding protein alpha* (*Abpa*), with alleles *Abpa*^{a} and *Abpa*^{b}, or, for brevity, a^{a} and a^{b}, respectively.

Inbred strains are polymorphic at the *Abpa* locus: In a previous report (DLOUHY and KARN 1984), a survey of 15 inbred strains suggested that ABP Ia is more common than Ib. This also has been found to be true for 53 additional inbred strains studied at the Jackson Laboratory. Table 1 indicates the ABP I types for inbred strains studied thus far. Unique ABP phenotypes (not shown) were observed in the salivary proteins of strains IS/CamEi (inbred from wild *Mus musculus domesticus* trapped in Israel), and CAST/Ei (inbred *Mus musculus castaneus*).

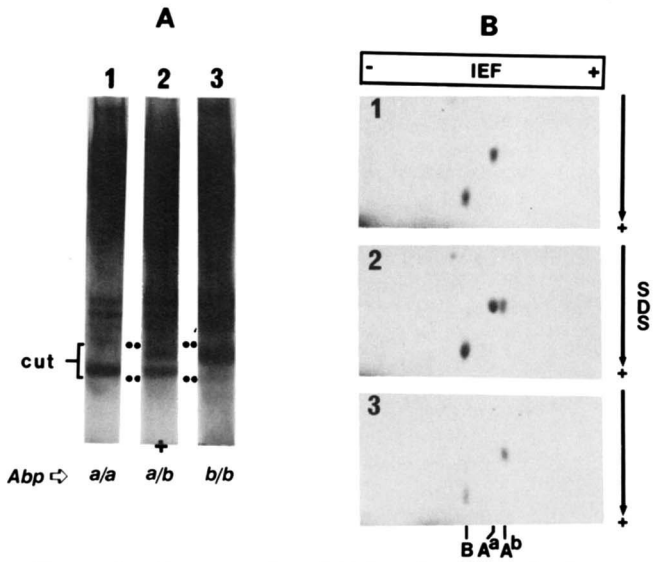

FIGURE 1.—Demonstration of Alpha subunit variation between ABP region Ia and region Ib proteins. Mouse salivary proteins were separated in a typing gel and the ABP region I bands were excised as shown (panel A). The ABP Alpha and Beta subunits from the Ia and Ib bands then were dissociated from each other and separated by urea isoelectric focusing and SDS second dimension electrophoresis (see MATERIALS AND METHODS and below). The SDS dimension allows identification of the Alpha and Beta subunits by their different apparent sizes. In panel B, the urea IEF gel orientation (*diagram at top*) was left (basic) to right (acidic); SDS electrophoresis (gels 1, 2 and 3) was conducted from top to bottom. Gels 1 and 3 show the ABP subunits resolved from region Ia and region Ib proteins, respectively. In each case there is a single Alpha and a single Beta subunit. The Alpha subunits have more acidic isoelectric points and are larger than the Beta subunits. Gel 2 shows the subunits resolved from a mixture of ABP region Ia and region Ib proteins. Only one type of Beta (B) subunit is observed but two Alpha types with differing isoelectric points are present. The Alpha with the more basic isoelectric point corresponds to the Alpha from ABP region Ia in Gel 1 and hence it is designated Alpha^a (A^a). The Alpha subunit with the more acidic isoelectric point corresponds to the Alpha from ABP region Ib and hence it is designated Alpha^b (A^b).

It is likely that the strains with a major band in the ABP Ia position have the Alpha^a subunit and hence are expressing *Abpa*^{a}, while those with a Ib band have the Alpha^b subunit and are expressing *Abpa*^{b}, although these inbred strains (except for C3H/St and DBA/2J) have not been tested by isoelectric focusing analysis of ABP subunits. The origins of the unique ABP phenotypes in IS/CamEi and CAST/Ei have not yet been determined.

***Abpa* is located on chromosome 7 near *Glucose phosphate isomerase-1* (*Gpi-1*):** The distribution of ABP types was determined in 53 BXD and AKXD recombinant inbred (RI) strains (Table 2). *Abpa* shows completely concordant inheritance with the chromosome 7 marker, *Glucose phosphate isomerase-1* (*Gpi-1*; DE LORENZO and RUDDLE 1969) in the 26 BXD RI strains, indicating close linkage. The AKXD RI strains are uninformative relative to the *Gpi-1* linkage since both progenitors carry the *Gpi-1*^{a} allele. However, significant linkage (four recombinants among 27 AKXD RI strains) is evident with *Akv-1*, the site of an AKR ecotropic murine leukemia virus provirus (JENKINS *et al.* 1981) that has been mapped near the centromere of chromosome 7, approximately 10 centimorgans (cM) proximal to *Gpi-1*. Four recombinants are evident between *Abpa* and *Tamase-1* (*Tam-1*), which is known to be approximately 11 cM distal to *Gpi-1*. Data from the BXD RI strains show that *Abpa* is bounded proximally by the *Coumarin hydroxylase* (*Coh*) locus (three recombinants among 24 typed RI strains; WOOD and TAYLOR 1979) and distally by the *Tam-1* locus (five recombinants among 26 RI strains; SKOW, 1978). The inheritance of *Abpa* relative to serum amyloid A (*Saa*; TAYLOR and ROWE 1984) is shown for NX129 RI strains (Table 3). There is one recombinant between *Abpa* and *Saa*.

In (DBA/2J × C57BL/6J)F_1 × C57BL/6J backcross mice (sexes combined), *Abpa* showed close linkage with *Gpi-1* (Table 4), confirming the RI data. Only two *Abpa-Gpi-1* crossovers were detected among 123 progeny for an estimated recombination frequency of 0.016 ± 0.011. Based on the segregation of the distal marker *Hbb*, the apparent gene order is *Abpa-Gpi-1-Hbb*. There were 37 recombinants between *Gpi-1* and *Hbb* for an estimated recombination frequency of 0.30 ± 0.04. This value is in good agreement with previously reported recombination frequencies between these markers.

Genetic determinants for ABP II and III are linked to *Abpa*: As mentioned in the introduction, mouse saliva contains other ABPs besides the ABP Ia and Ib proteins. Figure 2 demonstrates immunologic visualization of salivary ABPs on a Western blot of a typing gel of saliva from animals differing in *Abpa* genotypes. Three regions of ABP proteins are indicated: ABP I, ABP II and ABP III. While ABP I and II have been reported previously under various names, ABP III has not been described previously and cannot be identified in routine protein-stained typing gels of salivas. ABP I is present in (*Abp*) a^a/a^a, a^a/a^b and a^b/a^b mice, ABP II is present in a^a/a^b and a^b/a^b mice, and ABP III is present in a^a/a^a and a^a/a^b mice. Thus, ABP II is found in animals with the a^b allele and ABP III is found in animals with the a^a allele.

In RI strain studies, using protein stained typing gels, ABP II and a^b were inherited concordantly and allelic to a^a in all 53 BXD and AKXD RI strains reported in Table 2. That observation indicates that the determinant for ABP II is closely linked to a^b and agrees with previous backcross and intercross data using only the strains C3H/St and DBA/2J (DLOUHY and KARN, 1984). In studies of eight BXD RI strains using anti-ABP antibody to identify ABP III on Western blots, ABP III and a^a have been found to be inherited concordantly in BXD-1, 5, 8 and 9, while

TABLE 1

ABP types of inbred mouse strains

ABP Ia:	A/HeJ	A/J[a]	ABP/GnLe	AEJ/Gn
	AKR/J[a]	AU/SsJ	BALB/c[a]	BALB/cBy
	BALB/cByA[a]	BALB/cJ	BDP/J	BUB/BnJ
	CBA/BrA[a]	CBA/J	CBA/CaJ	CBA/H-*T6*J
	CE/J	C3H/HeJ	C3H/St[a]	C3HeB/FeJ
	C57BL/6By	C57BL/6J[a]	C56BL/6KsJ[a]	C57BL/10J
	C57BL/LiA[a]	C57BR/cdJ	C57L/J	C58/J
	DA/HuSn	FS/Ei	GRS/A[a]	HP/EiTy
	HRS/J	I/LnJ	LG/J	LP/J
	LT/Sv	MA/MyJ	MK/Re	MWT/Le
	P/J	PERU/AtteckEi	PL/J	PRO/Re
	RF/J	ROP/GnLe	RSV/Le	SEA/GnLe
	SEC/1ReJ	SF/CamEi	SJL/J	SK/CamEi
	ST/bJ	STX/Le	SWR/J[a]	STS/A[a]
	TSI/A[a]	WB/Re	129/J	129/Sv
ABP Ib:	DBA/1J	DBA/2J[a]	DW/J	MOLD/Rk[b]
	NZB[a]	NZB/BINJ	RIIIS/J	SM/J

[a] From DLOUHY and KARN (1984).
[b] Inbred *Mus musculus molussinus*.

ABP II and a^b were inherited concordantly in BXD-2, 6, 14 and 15. Thus, the determinants for ABP II and III are closely linked to *Abpa* and appear to be allelic with one another.

ABP II and III are not due to aggregation of ABP I protein: We previously suggested that ABP II (then called b′) was an aggregate of ABP Ib (DLOUHY and KARN, 1984). That mechanism would provide a trivial explanation of the concordant inheritance of ABP II with *Abpa*^{b} and ABP III with *Abpa*^{a}. To explore potential aggregation, salivary proteins from *Abpa*^{a}/*Abpa*^{b} heterozygous mice were chromatographed on a calibrated Sephadex G-100 column to separate them by size. Saliva was treated with [^{14}C]testosterone prior to chromatography. The results of the chromatographic separation are shown in Figure 3. Except for a small amount in the breakthrough fraction, all ABPs eluted at fractions 60–69 as did the majority of the bound [^{14}C]testosterone. Our G-100 separations of mouse salivas reinforce the conclusion (DLOUHY, NICHOLS and KARN, 1986) that ABP is the 3S submaxillary gland androgen binding protein reported by VERHOEVEN and WILSON (1976).

An autoradiograph of the typing gel of G-100 column fractions (inset A in Figure 3) indicates four major ABPs: ABP Ia, Ib, II and III. The elution positions of the ABP II and III proteins indicate that they are not aggregates of ABP I. All the ABP proteins eluted corresponding to a molecular size of about 20,000 daltons, consistent with our previous molecular weight estimate of 17,000 daltons for ABP dimers based upon SDS electrophoresis (DLOUHY and KARN, 1983). The reason for a slight difference in elution position for Ia and Ib is not known.

Combining two observations: (1) that ABP II and III are not simple aggregates of ABP I and (2) that ABP II is inherited with *Abpa*^{b} and ABP III is inherited with *Abpa*^{a}, suggests that ABP II and III are due to the expression of alternative alleles of a gene closely linked to *Abpa*.

Origin of ABP II and III bands: When a typing gel is used to analyze saliva from an *Abpa*^{a}/*Abpa*^{b} (a^a/a^b) heterozygote, ABP II and III proteins each appear as a single band indistinguishable from the ABP II and III proteins of a^b/a^b and a^a/a^a homozygotes, respectively (Figures 2 and 3). However, when ABP proteins from a^a/a^b mice are subjected to two-dimensional electrophoresis using an isoelectric focusing gel as the first dimension and an SDS gel as the second dimension, ABP II and III are each resolved as doublets (Figure 4, panel A). In similar analyses, ABP II and III are singlets in saliva of a^b/a^b and a^a/a^a homozygotes, respectively (not shown).

As diagramed in Figure 4, panel B, and discussed below, the simplest explanation of these results is that ABP II and III are dimers resulting from the association of Alpha subunits (either A^a or A^b) with a subunit which we term Gamma. Two genetically determined forms of Gamma exist, Gamma^a (G^a) and Gamma^b (G^b), which represent expression of alternative alleles of the subunit locus *Androgen binding protein gamma* (*Abpg*). The linkage studies discussed above indicate that *Abpg* is closely linked to *Abpa* on chromosome 7. In the strains analyzed, *Abpg*^{a} (g^a) is in coupling with *Abpa*^{a} (a^a) while *Abpg*^{b} (g^b) is in coupling with *Abpa*^{b} (a^b). Because of this close linkage of the *Abpa* and *Abpg* genes, we will refer to *a g* haplotypes from this point on in this paper. Although no polymorphism has yet been detected for the Beta subunit, we tentatively designate the locus encoding it as *Androgen binding protein beta* (*Abpb*).

TABLE 2

Inheritance of ABP types in BXD and AKXD recombinant inbred strains

BXD[a]						AKXD[b]					
Strain	*Coh*		*Abp*	*Gpi-1*	*Tam-1*	Strain	*Akv-1*		*Abp*		*Tam-1*
1	B		B	B	B	1	A		A		A
2	D		D	D	D	2	D		D		D
5	B		B	B	×[c] D	3	A		A	×	D
6	D		D	D	D	4	A		A		
8	B		B	B	B	5	A		A		
9	B		B	B	B	6	A		A		A
11	B		B	B	B	7	D		D		D
12	D		D	D	D	8	A		A	×	D
13	B		B	B	B	9	D		D		D
14	D		D	D	D	10	A		A		A
15	B	×	D	D	× B	11	D		D		D
16	B		B	B	B	12	A		A	×	D
18	B		B	B	B	13	D	×	A		A
19	B		B	B	B	14	A		A		A
20	B		B	B	B	15	A		A		A
21	B		B	B	B	16	A		A		A
22	B		B	B	× D	17	D		D		D
23	B		B	B	B	18	A		A		A
24	B		B	B	B	20	D	×	A		A
25	B		B	B	× D	21	A		A		A
27	B	×	D	D	× B	22	A		A		A
28	B	×	D	D	D	23	A		A	×	D
29	B		B	B	B	24	A		A		A
30	B		B	B	B	25	A	×	D		
31			D	D	D	26	D	×	A		A
32			D	D	D	27	D		D		D
						28	D		D		D

[a] B and D are used as generic symbols for alleles inherited from C57BL/6J and DBA/2J, respectively. *Coh* types from WOOD and TAYLOR (1979); *Abpa* types, this paper; *Gpi-1* types this paper and WOOD and TAYLOR (1979); *Tam-1* types from SKOW (1978).

[b] A and D are used as generic terms for alleles inherited from AKR/J and DBA/2J, respectively. *Akv-1* types from JENKINS *et al.* (1981); *Tam-1* types, this paper.

[c] An "×" is used to denote a region of recombination.

TABLE 3

Inheritance of salivary protein markers in the NX129 RI strains

	NX129 RI Strains					
Locus	1	2	5	7	10	12
Abpa	N[a]	N	N	N	N	9 ×[b]
Saa	N	N	N	N	N	N

[a] N and 9 are used as generic symbols for alleles inherited from NZB/B1NJ and 129/J, respectively.

[b] The "×" denotes a region of recombination.

According to our model, a^ag^a/a^bg^b heterozygotes contain five different, genetically determined ABP subunits, A^a, A^b, G^a, G^b and B. Random association of either G or B subunits with A subunits allows formation of six different ABP dimers: two ABP I proteins (A^a:B and A^b:B), two ABP II proteins (A^a:G^b and A^b:G^b) and two ABP III proteins (A^a:G^a and A^b:G^a). Four of the six ABPs are the same as those found in

TABLE 4

Segregation of *Abpa*, *Gpi-1*, and *Hbb* Alleles in backcross (DBA/2J × C57BL/6J)F_1 × C57BL/6J[a]

Region of recombination	Genetic locus *Abpa*	*Gpi-1*	*Hbb*	No. of mice
Parental gametes	*a*	*b*	*s*	31
	b	*a*	*d*	53
(*Abpa*, *Gpi-1*)—*Hbb* recombinants	*a*	*b*	*d*	14
	b	*a*	*s*	23
Abpa—(*Gpi-1*, *Hbb*) recombinants	*b*	*b*	*s*	1
	a	*a*	*d*	1
Total				123

[a] The parental genotypes for *Abpa*, *Gpi-1* and *Hbb*, respectively, are:

DBA/2J: $Abpa^b$ $Gpi\text{-}1^a$ $Hbb^d/Abpa^b$ $Gpi\text{-}1^a$ Hbb^d;
C57BL/6J: $Abpa^a$ $Gpi\text{-}1^b$ $Hbb^s/Abpa^a$ $Gpi\text{-}1^b$ Hbb^s.

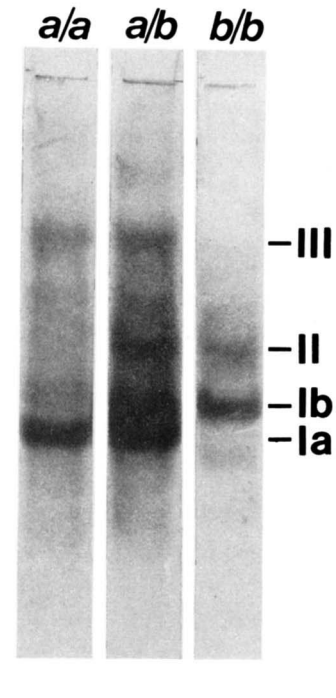

FIGURE 2.—ABP proteins visualized immunologically on a Western blot of a typing gel. Channels 1, 2 and 3 represent ABP proteins from animals of *Abpa* genotypes a^a/a^a, a^a/a^b and a^b/a^b, respectively. Four staining regions labeled Ia, Ib, II and III, are identified.

a^ag^a/a^ag^a and a^bg^b/a^bg^b homozygotes while two are novel ABP dimers. The two ABP I proteins (A^a:B and A^b:B dimers) are the same as those found in the respective homozygotes. ABP II from heterozygotes contains the A^b:G^b dimer present in a^bg^b/a^bg^b homozygotes and a new protein whose position corresponds to that expected for an A^a:G^b dimer. ABP III from heterozygotes contains the A^a:G^a protein present in a^ag^a/a^ag^a homozygotes and a new protein whose position corresponds to that expected for an A^b:G^a dimer. The existence of the novel dimers A^a:G^b and

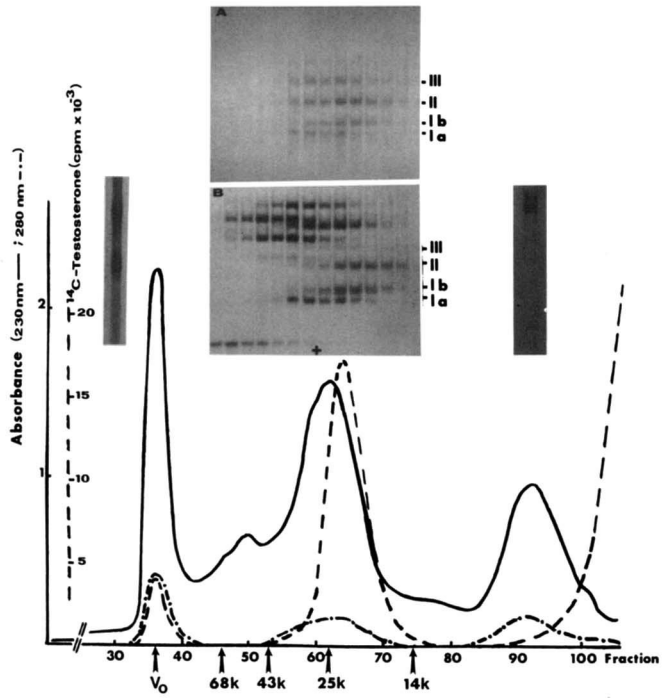

FIGURE 3.—Elution of ABP region I, II and III proteins from a G-100 gel filtration column. Salivas from a^a/a^b heterozygotes were pooled (0.6 ml total), treated with [^{14}C]testosterone and applied to the column after two previous calibration runs with the molecular size standards bovine serum albumin (68k), ovalbumin (43k), α-chymotrypsinogen (25k) and RNase (14k) (k = kilodaltons). The column profile shows the elution positions of protein standards (arrows), and mouse salivary proteins measured as absorbance at 230 nm and 280 nm. [^{14}C]Testosterone is shown as a dashed line. Inset B, a protein-stained typing gel showing the proteins found in the even-numbered fractions from 48 to 72 where the majority of protein-bound steroid elutes. Inset A, an autoradiograph (6-month exposure) of the typing gel (shown in inset B) which shows ABP region I, II and III proteins. Proteins in the two other main peaks, the void peak and the amylase peak, are shown in the insets on the left and right of the middle insets, respectively.

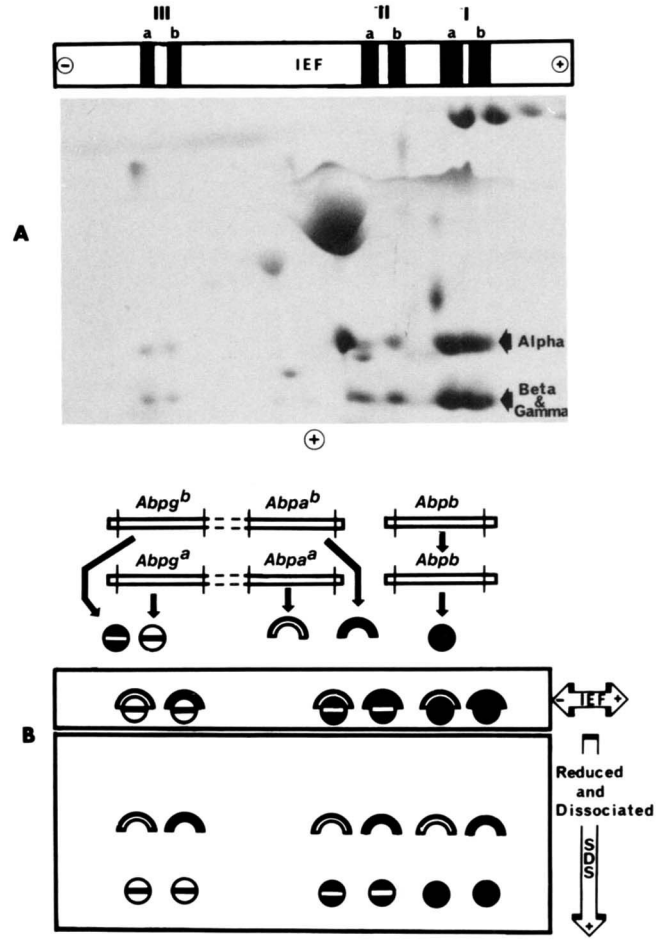

FIGURE 4.—Subunit composition of ABP proteins from an a^a/a^b heterozygote as resolved by IEF/SDS two dimensional electrophoresis. Panel A, protein stained two-dimensional gel of mouse salivary proteins separated by IEF and treated with BME prior to electrophoresis in the SDS dimension; thus, ABP subunits are dissociated. ABP regions I, II and III are indicated above the diagram of the IEF gel. Six Alpha subunits spots are present, two in region I, two in region II and two in region III. Two Beta subunit spots are present in region I and two Gamma subunit spots are present in regions II and III. Panel B, diagramatic representation of the relationship of *Abp* subunit genes and the formation of the various ABP dimers in an a^ag^a/a^bg^b heterozygote. All dimers contain a large subunit, Alpha, which is either an Alpha^a or Alpha^b subunit depending upon which allele of the *Abpa* locus is being expressed. Each dimer also contains a smaller subunit which can be a Beta (B), a Gamma^a (G^a) or a Gamma^b (G^b), the latter two representing the allelic alternatives of the *Abpg* gene. Random association of two different kinds of α subunits with the Beta and two Gamma subunits allows the formation of six different ABP dimers, two each for the three regions. The ABP II A^a:G^b dimer and ABP III A^b:G^a dimer are only produced in a^ag^a/a^bg^b heterozygotes.

A^b:G^a indicates that G and B subunits associate randomly with A subunits.

The two-dimensional IEF/SDS gels are valuable for these studies because they demonstrate similarity of subunit sizes for the six major ABP bands. This is important because minor ABP-like bands with different sized subunits appear during IEF analysis particularly when salivas of female mice are analyzed. The minor bands cross react with ABP antibody and are capable of binding androgen (not shown). The IEF demonstration of the double band nature of ABP II and III in heterozygotes indicates that ABP II and III consist of comigrating proteins in the typing gel.

Supporting evidence for a three-gene model: Using the method of reduction/alkylation separation of subunits described above, it is possible to demonstrate that ABP II does indeed contain a novel subunit, Gamma^b (Figure 5). As expected, under these conditions Gamma^b has a different isoelectric point than Beta although both have similar molecular weights. Due to difficulties detecting ABP III on protein stained typing gels of salivas, this method has not been useful for visualizing Gamma^a.

The random dimerization model discussed above suggests that the Alpha, Beta and Gamma subunits are freely diffusible prior to disulfide bridge formation and probably are produced as distinct translation products. To investigate this, RNA was extracted from the submaxillary glands of a^ag^a/a^ag^a homozy-

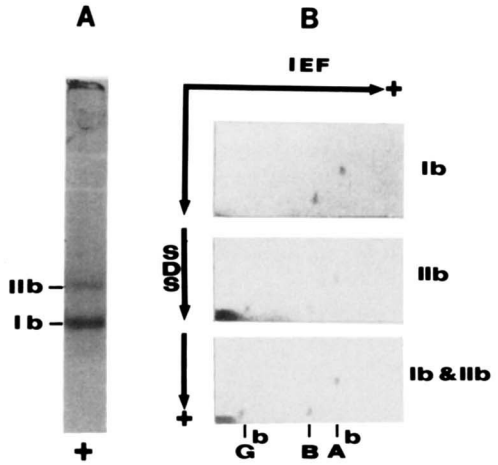

FIGURE 5.—Analysis of ABP II subunit structure. The ABP I and ABP II bands from saliva of a a^bg^b/a^bg^b homozygote were excised from a typing gel and the subunit structures analyzed by reduction/alkylation as done for ABP I in Figure 1. ABP II contains a small subunit, Gamma^b, which is not found in ABP I (panels A and B). Small amounts of Beta are sometimes found in excised ABP II, probably due to gel cutting artifact. It is apparent in panel C that the difference between ABP I and ABP II is not due to Alpha subunit variation.

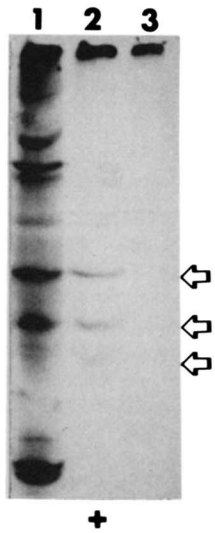

FIGURE 6.—Fluorograph of a urea isoelectric focusing gel demonstrating the existence of three different ABP-like proteins in a cell-free translation of submaxillary gland mRNA. Poly(A) RNA was extracted from the submaxillary glands of C3H/St inbred mice (a^ag^a/a^ag^a homozygotes) and translated in a rabbit reticulocyte cell-free translation system incorporating [^{3}H]leucine. Channel 1, translation without immunoprecipitation; channel 2, proteins immunoprecipitated with anti-ABP antiserum and channel 3, absence of ABP-like proteins when control (preimmune) serum was used to form precipitates.

gotes and the pooled RNA subjected to cell-free translation (see MATERIALS AND METHODS). Following cell-free translation, the mixture was analyzed for the presence of ABP-like subunits by double antibody immunoprecipitation and isoelectric focusing (Figure 6). When anti-ABP was used as the primary antibody, three bands of ABP-like protein were precipitated (Figure 6, channel 2). The bands were not found when preimmune serum was used to prepare precipitates (Figure 6, channel 3). These results suggest the existence of three ABP mRNAs.

DISCUSSION

In this paper we present evidence for the existence of three, genetically distinct subunits of mouse salivary androgen binding protein (ABP): Alpha, Beta and Gamma. The genes for the Alpha and Gamma subunits, *Androgen binding protein alpha* (*Abpa*) and *Androgen binding protein gamma* (*Abpg*), respectively, are polymorphic and are closely linked to each other and to the gene *Glucose phosphate isomerase-1* (*Gpi-1*) on mouse chromosome 7. Although no polymorphism has yet been detected for the Beta subunit, we tentatively designate its structural locus, *Androgen binding protein beta* (*Abpb*). The existence of three subunit loci is supported by the immunoprecipitation of three different ABP-like pre-proteins from cell-free translation products of submaxillary gland RNA from an inbred strain.

Close linkage of subunit genes has been described for a number of polymeric proteins such as the hemoglobins (ANTONARAKIS, KAZAZIAN and ORKIN, 1985). Linkage of genes encoding structurally and/or functionally related proteins is often interpreted as evidence that the genes are evolutionarily related. It is likely that the Beta subunit locus (*Abpb*) also is part of the *Abpa-Abpg* linkage group but that conclusion must await discovery of a Beta variant or development of molecular probes. The identification of this gene complex in *M. musculus* provokes the question of its phylogenetic signficance. Examination of other murine species and subspecies will help determine if *Abp* linkage groups are a universal feature of the mouse genome.

The subunit structure of mouse salivary ABP (DLOUHY and KARN 1983) is similar to that of rat prostatic steroid binding protein (PBP; PARKER, NEEDHAM and WHITE 1982; PARKER *et al.* 1983). Both are secreted proteins capable of binding androgen. The physiologic significance of the mouse ABP/rat PBP group of small molecular weight steroid binding proteins is poorly understood. Rat PBP is known to bind a variety of steroids and may be responsible for the accumulation of related compounds in the prostate. For example, it has been suggested that a human prostate protein cross-reactive with anti-rat PBP antibody is responsible for binding estramustine, a compound sometimes used in chemotherapy of prostate cancer (BJORK *et al.* 1982). The physiologic function of rat PBP remains unknown although it may modulate the action of the androgen receptor at the chromatin level (CHEN, HIIPAKKA and LIAO 1979, JUDGE, SALTZMAN and LIAO 1983). The physiologic significance of mouse salivary ABP also has not yet been

established, but it is clear that it has a relatively high capacity for androgen binding (VERHOEVEN and WILSON 1976; DLOUHY and KARN 1983).

It has been reported that an anti-rat PBP antibody reacts with both mouse prostate and submaxillary gland extracts (POUSETTE *et al.* 1969). We have developed an antibody to mouse salivary ABP and, while we have not yet found cross-reactive material (CRM) in extracts of rat prostate (S. R. DLOUHY and KARN unpublished data), we did find CRM in extracts of some mouse prostates (DLOUHY, NICHOLS and KARN 1986). Our current results (this report) and a previous analysis of Western blots (DLOUHY, NICHOLS and KARN 1986) suggest (1) that *Abpa* is not expressed in mouse prostate and (2) that the minimal immunoreactivity of ABP II, compared to ABP I, in crossed immunoelectrophoresis is due to presence of the Gamma subunit in ABP II rather than the Beta subunit found in ABP I.

Three subunits are involved in the formation of rat PBP and molecular evidence indicates that at least two of the three genes encoding those subunits may be the result of gene duplication (PARKER, NEEDHAM and WHITE 1982; PARKER *et al.* 1983). Thus, considering our recent linkage studies and documentation of three ABP subunits, the many similarities of the mouse salivary ABP and rat PBP systems are intriguing. To our knowledge, no classical genetic analysis of the rat PBP system has been conducted.

Our finding of three cell-free translation products does not necessitate the conclusion that there are three completely distinct coding sequences for the ABP subunits we see. The origin of the three mRNA species whose products we immunoprecipitated from cell-free translation may represent transcripts of independent loci or, alternatively, could be the products of variously processed exons from one or two coding sequences. Numerous examples of such alternative products resulting from "exon shuffling" have been reported (*e.g.*, KING and PIATIGORSKY 1983; NABESHIMA *et al.* 1984). The only precedent for the concept of individual genes for the three ABP subunits is the demonstration of three independent genes for the C1, C2 and C3 subunits of rat PBP. In any event, the system described here provides fertile ground for molecular genetic studies and further genetic studies of an interesting mouse gene complex.

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LITERATURE CITED

ANTONARAKIS, S. E., H. H. KAZAZIAN, JR. and S. H. ORKIN, 1985 DNA polymorphism and molecular pathology of the human globin gene clusters. Hum. Genet. **69:** 1–14.

AVIV, H. and P. LEDER, 1972 Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid cellulose. Proc. Natl. Acad. Sci. USA **69:** 1408–1412.

BJORK, P., FORSGREN, B., GUSTAFSSON, J., POUSETTE, A. and B. HOGBERG, 1982 Partial characterization and "quantitation" of a human prostatic estramustine-binding protein. Cancer Res. **42:** 1935–1942.

CHEN, C., HIIPAKKA, R. A. and S. LIAO, 1979 Prostate α-protein: subunit structure, polyamine binding, and inhibition of nuclear chromatin binding of androgen receptor complex. J. Steroid Biochem. **11:** 401–405.

CHIRGWIN, J. M., A. W. PRZYBYLA, R. J. MACDONALD and W. J. RUTTER, 1979 Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **11:** 5294–5299.

Committee on Standardized Genetic Nomenclature for Mice, 1973 Guidelines for nomenclature of genetically determined biochemical variants in the house mouse, *Mus musculus.* Biochem. Genet. **9:** 369–374.

Committee on Standardized Genetic Nomenclature for Mice, 1985 Rules and guidelines for gene nomenclature. Mouse News Lett. **72:** 2–12.

DE LORENZO, R. J. and F. H. RUDDLE, 1969 Genetic control of two electrophoretic variants of glucosephosphate isomerase in the mouse (*Mus musculus*). Biochem. Genet. **3:** 151–162.

DLOUHY, S. R. and R. C. KARN, 1983 The tissue source and cellular control of the apparent size of Androgen binding protein (Abp), a mouse salivary protein whose electrophoretic mobility is under the control of Sex-limited saliva pattern (Ssp). Biochem. Genet. **21:** 1057–1070.

DLOUHY, S. R. and R. C. KARN, 1984 Multiple gene action determining a mouse salivary protein phenotype: identification of the structural gene for Androgen binding protein (Abp). Biochem. Genet. **22:** 657–667.

DLOUHY, S. R., W. C. NICHOLS and R. C. KARN, 1986 Production of an antibody to mouse salivary androgen binding protein (ABP) and its use in identifying a prostate protein produced by a gene distinct from Abp. Biochem. Genet. **24:** 443–463.

DLOUHY, S. R., B. A. TAYLOR, L. ROWE and R. C. KARN, 1986 Two genes coding for mouse salivary androgen binding protein (ABP) subunits are located on chromosome 7. Genetics **113** (Suppl): s60.

HARDING, J. D. and W. J. RUTTER, 1978 Rat pancreatic amylase mRNA, tissue specificity and accumulation during embryonic development. J. Biol. Chem. **253:** 8736–8740.

HEYNS, W. and P. DE MOOR, 1977 Prostatic binding protein. Eur. J. Biochem. **78:** 221–230.

JENKINS, N. A., N. G. COPELAND, B. A. TAYLOR and B. K. LEE, 1981 Dilute (*d*) coat colour mutation of DBA/2J mice is associated with the site of integration of an ectropic MuLV genome. Nature **293:** 370–374.

JUDGE, S. M., SALTZMAN, A. G. and S. LIAO, 1983 Alpha-protein: a marker for androgen action in the rat ventral prostate. pp. 267–275. In: *Gene Regulation by Steroid Hormones II*, Edited by A. K. ROY and J. H. CLARK. Springer-Verlag, New York.

KARN, R. C., S. R. DLOUHY, K. R. SPRINGER, J. P. HJORTH and J. T. NIELSEN, 1982 Sex-limited genetic variation in a mouse salivary protein. Biochem. Genet. **20:** 493–504.

KING, C. R. and J. PIATIGORSKY, 1983 Alternative RNA splicing of the murine alphaA-crystallin gene: protein-coding information within an intron. Cell **32:** 707–712.

LASKEY, R. A. and A. D. MILLS, 1975 Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. **56:** 335–341.

NABESHIMA, Y-I., Y. FUJI-KURIYAMA, M. MURAMATSU and K. OGATA, 1984 Alternative transcription and two modes of splicing result in two myosin light chains from one gene. Nature **308:** 333–338.

PARKER, M., M. NEEDHAM and R. WHITE, 1982 Prostatic steroid binding protein: gene duplication and steroid binding. Nature **298:** 92–94.

PARKER, M. G., R. WHITE, H. HURST, M. NEEDHAM and R. TILLY, 1983 Prostatic steroid-binding protein. Isolation and characterization of C3 genes. J. Biol. Chem. **258:** 12–15.

POUSETTE, A., P. BJORK, K. CARLSTROM, B. FORSGREN, J-A. GUSTAFSSON and B. HOGBERG, 1979 On the presence of "prostatic secretion protein" in different species. Acta Chem. Scand. **34:** 155–156.

SKOW, L. C., 1978 Genetic variation at a locus (*TAM-1*) for submaxillary gland protease in the mouse and its location on chromosome 7. Genetics **90:** 713–724.

TAYLOR, B. A. and L. ROWE, 1984 Genes for serum amyloid A proteins map to chromosome 7 in the mouse. Mol. Gen. Genet. **195:** 491–499.

VERHOEVEN, G. and J. D. WILSON, 1976 Cytosol androgen binding in submandibular gland and kidney of the normal mouse and the mouse with testicular feminization. Endocrinology **99:** 79–92.

WOOD, A. W. and B. A. TAYLOR, 1979 Genetic regulation of coumarin hydroxylase activity in mice. J. Biol. Chem. **254:** 5647–5651.

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