cDNA sequence and chromosomal localization of the mouse parvalbumin gene, Pva

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Summary
In the homozygous condition, the mutation adr (arrested development of righting response) of the mouse causes a myotonia and a drastic reduction of the Ca\textsuperscript{2+}-binding protein parvalbumin (PV) in fast muscles. Using a rat PV probe, a mouse cDNA clone was isolated from a λgt11 wild-type fast-muscle library and its nucleotide sequence was determined. The protein coding and the 3' non-translated regions of the mouse gene show extensive homology with the rat PV gene. The result of Southern blot hybridization is consistent with a single copy gene for parvalbumin. Restriction fragment length polymorphisms (RFLPs) between Mus musculus domesticus (e.g. C57BL/6) and Mus spretus (SPE) were detected with the enzymes Eco RI, Pst I, and Sst I. The restriction fragment patterns of DNA samples from 65 individual offspring of (C57BL/6 × SPE)F1 × C57BL/6 backcrosses were tested with the PV probe and matched, for linkage detection, to pre-existing patterns established with various RFLP probes on the same samples. A co-distribution of PV-RFLPs with Pvt-1 and Mlvi-2, which had been localized on chromosome 15, was detected. Thus, the structural gene for PV, designated Pva, maps to chromosome 15 of the mouse whereas the adr mutation shows no linkage with markers on this chromosome. Gene locus homology between chromosome 15 of the mouse and chromosome 22 of man (which carries the human PV gene) is discussed.

1. Introduction
Parvalbumin (PV), a high-affinity Ca\textsuperscript{2+}-binding protein, is synthesized in high amounts in fast contracting/relaxing muscles of small mammals (Heizmann et al. 1982). It is also expressed in certain non-muscle tissues such as brain. The expression of PV in muscle is neurally and developmentally regulated (Leberer & Pette, 1986; Müntener et al. 1987; Berchtold, 1988). Northern blot analysis in rat and mouse revealed two PV mRNA species of 1100 and 700 nt of which the smaller species is more abundant (Epstein et al. 1986; Kluxen et al. 1988).

The PV content of muscle is reduced in certain hereditary neuromuscular diseases of the mouse e.g. 'dystrophia muscularis', DY\textsuperscript{22} (Klug et al. 1985) and 'arrested development of righting response', ADR\textsuperscript{*} (Stuhlfauth et al. 1984; Jockusch et al. 1988a). The ADR syndrome has been characterized as a myotonia (Reinhaguen et al. 1988) caused by a hyperexcitability of muscle fibres (Mehrke et al. 1988). This condition can be phenotypically cured by tocainide (Reinhaguen et al. 1988), a drug that stabilizes the membrane potential. In tocainide treated animals the PV protein and mRNA levels are partially restored (Jockusch et al. 1988a; Kluxen et al. 1988). This indicates that the myotonic phenotype interferes with the regulation of PV synthesis.

The genomes of mouse and man are far more extensively characterized than those of other vertebrates. A number of mutations are known in mouse that may serve as models for hereditary diseases of man. In addition, regions of homology between human and mouse chromosomes have been identified (Peters, 1988; Kaplan et al. 1987). Therefore, based on the knowledge of the rat PV gene (Berchtold et al. 1987), we have begun to characterize the PV gene of the mouse.

Here we present the isolation of a parvalbumin cDNA clone from a mouse fast-muscle library, its nucleotide sequence and the deduced amino-acid sequence. By the identification and use of RFLPs for PV we were able to map this gene in the mouse
Agtl 1 (Young & Davis, 1983) and packaged genome. A preliminary report of part of this work has been given (Zühlke et al. 1988).

2. Material and methods

(i) Animals

In addition to standard laboratory mouse strains, wild-type (+/adr?) and homozygous mutant (adr/adr) individuals of the A2G inbred strain (Watkins & Watts, 1984) were used for DNA and RNA isolation. Genomic DNA was also prepared of the Mus spretus strains SPE (Guénet, 1986) and SEG (Watkins & Watts, 1984) for DNA and RNA isolation. The isolated fragment of the coding region was labelled with $[^{32}P]$dCTP by random deoxyoligonucleotide-priming (Feinberg & Vogelstein, 1983). Positive clones were purified by single plaque passages. Phage DNA was isolated (Maniatis et al. 1982) and subcloned for sequencing.

(ii) Isolation of mRNA

Fast muscles were obtained from adult A2G wild-type mice. The tissue was frozen in liquid nitrogen immediately after dissection and stored at $-70\, ^\circ\mathrm{C}$. For the preparation of total RNA, the tissue was homogenized in 4 m guanidinium thiocyanate buffer according to Chirgwin et al. (1979). The RNA was pelleted by centrifugation through a 3 ml, 5-7 m-CsCl cushion. Poly(dT) paper (Medac, Hamburg) affinity chromatography was used to obtain messenger RNA (Werner et al. 1984). A total of 1–5 µg of poly(A)$^+$ RNA was used for cDNA synthesis.

(iii) cDNA synthesis and cloning

First strand synthesis starting from the 3' end of the poly(A)$^+$ RNA was primed by oligo(dT) and elongated by MMLV reverse transcriptase (Maniatis et al. 1982). The DNA/RNA hybrids were treated with RNase H, providing 3'OH primers for DNA polymerase I (second-strand synthesis according to Gubler & Hoffmann, 1983). Double-stranded cDNAs were protected from Eco RI restriction by methylation (Huynh et al. 1985). Eco RI linkers were added to the cDNA and cleaved with Eco RI to generate cohesive termini. An appropriate cDNA fraction was size selected by low-melting-temperature agarose gel electrophoresis (Maniatis et al. 1982). The purified cDNAs were ligated in Eco RI digested phage vector $\lambda$gt11 (Young & Davis, 1983b) and packaged in vitro according to Maniatis et al. 1982.

(iv) Screening of the $\lambda$gt11 library

Overnight (o.n.) cultures (100 µl) of E. coli Y1088 (Young & Davis, 1983a) were infected with $10^9$ p.f.u. of recombinant $\lambda$gt11, diluted with 3 ml of top-agar, plated on LB-plates and incubated o.n. at 37°C (Maniatis et al. 1982). Plaques were transferred onto nitrocellulose filters and processed according to Davis et al. 1980. Hybridization was performed with a rat parvalbumin cDNA probe (Berchtold & Means, 1985). The isolated fragment of the coding region was

(v) DNA sequencing

M13mp18 and M13mp19 were used as cloning vectors for DNA sequencing by the dideoxy chain termination method (Sanger et al. 1977). Single-stranded DNA was isolated from plaques suspended in 1 ml LB-medium and incubated with 10 µl of an o.n. culture of E. coli JM103 for 6 h at 37°C. Bacteria were pelleted in a microfuge, 250 µl 20% PEG was added to the supernatant, mixed and incubated for 15 min at room temperature. After centrifugation (5 min) the phage pellet was resuspended in 100 µl TES-buffer. The suspended virus particles were extracted twice with phenol and phenol-CHCl$_3$, the aqueous phase was ethanol precipitated and the viral DNA was dissolved in 20 µl TE-Buffer. The 17-mer universal primer (Boehringer, Mannheim), the 15-mer $\lambda$gt11 primer and the $\lambda$gt11 reverse primer (Clontec, Palo Alto) were used to prime the sequencing reactions. Deletions of the cDNA were produced by exonuclease III. Double stranded DNA was isolated and cut with Bam HI and Sph I. Exonuclease III was added and the reaction was stopped after different time intervals. After treatment with S1 nuclease the deleted fragments were blunt ended by the Klenow fragment of E. coli polymerase I and ligated to the vector by T4 DNA ligase. Competent JM103 cells were transformed with the ligation product and plated onto agar medium. Recombinant clones were analysed by digestion with Eco RI and HindIII. Single-stranded DNA of selected clones covering entirely both strands of the cDNA was prepared as described above and used for sequencing.

(vi) Preparation of mouse genomic DNA, Southern blot and hybridization

High-molecular-weight DNA was prepared from organs (lung, liver, heart, brain) of adult mice. The organs were frozen in liquid nitrogen, crushed and resuspended in 5 ml of 1% sarcosyl, 0.1 mM-EDTA. The mixture was incubated with proteinase K and RNase A. DNA was extracted with phenol and phenol-CHCl$_3$ and purified by dialysis (Weydert et al. 1983) and 8 µg DNA were digested with 20 units of restriction enzyme, run on 0.8% agarose/TBE gels and stained with ethidium bromide. The DNA was denatured, neutralized and transferred to nitrocellulose or nylon filters according to Southern (1975).

Radioactive probes were prepared by random priming of 100 ng of electrophoretically purified mouse cDNA fragment. Hybridization was carried out at 42°C in 50% formamide o.n. Blots were
### Parvalbumin gene

| ATG TCG | ATG TAC | GAC GTG | CTC AGC | GCT GAG | GAC AGC |
|---------|---------|---------|---------|---------|---------|
| Met Ser Met Thr Asp Val Leu Ser Ala Glu Asp |
| ATC AAG | AAG GGC | ATA GAA |
| Thr Lys Lys Ala Ile Gln |
| GCA TTT | GCT CTT |
| Ala Phe Ala |
| GCT +60 |

| GCT GCA | GAC TCC | TTC GAG | GTA AAT |
|---------|---------|---------|---------|
| Ala Ala Asp Ser Thr Lys Ala |
| GAA AAG | AGG ATT |
| Lys Glu Thr |
| AAA GAA |
| Lys Glu |
| CCG GAT | GAT GAG |
| Pro Asp Glu Val |
| GAG CTA |
| Glu Leu |
| GAA GAA |
| Lys Lys |
| AGG AAA |
| Gin Gin |
| GAC CAC |
| Thr His |
| ACT TCT |
| Thr Ala |
| GAG TTT |
| Glu Val |
| CAG AGG |
| Gin Gin |
| GGC CTT |
| Gly Pro |
| GAA GCC |
| Lys Phe |
| TTA ACT |
| Leu Ile |
| CAA TAA |
| Tyr Leu |
| CAA +546 |

TGAGCCATCTGAGAATCTGAGAAGAAAAATAGGGCGTACCACTCCGACAC

**Fig. 1. (a) cDNA and deduced amino-acid sequence of a mouse parvalbumin cDNA clone in 5' - 3' orientation.**

### 3. Results and discussion

#### (i) Isolation and sequencing of a mouse parvalbumin cDNA clone

A cDNA library was constructed in λgt11 using as template poly(A)⁺ RNA from fast muscles, tibialis anterior and vastus, of the A2G wild-type mouse (genotype +/+adrl). Putative PV clones were identified by several rounds of screening with a 240 bp *Pst I*/*Eco RI* fragment of a rat parvalbumin cDNA (Berchtold & Means, 1985). One clone containing an insert of 0.5 kb, termed *mpv*, was chosen for further characterization.

The identity of clone *mpv* as a PV cDNA was demonstrated by hybridization to electrophoretically separated mRNAs from wild-type and myotonic mouse muscle. Similar hybridization patterns were detected for PV mRNAs (700 and 1000 nt) using the mouse (data not shown) or the rat probe (Kluxen et al. 1988).

The parvalbumin cDNA fragment was subcloned and sequenced in both orientations. A stretch of 524 bp containing the coding sequence and a part of the 3' non-coding region was determined (Fig. 1a). Although the 5' non-coding region is lacking, the comparison with the rat cDNA sequence (Epstein et al. 1986) suggests the ATG (+1) as the initiation codon. Strong homologies of nucleotide (96%) and amino-acid (94.5%) sequences were found between mouse and rat. The 3' non-coding region of the mRNA sequences is also conserved and putative polyadenylation signals for the short mRNAs are located at identical positions (+498 to +504). In rat, one poly(A)⁺ site was found 10 nt downstream of the sequence AATAAA. The mouse cDNA clone lacks the poly(A) tail of the mRNA although it contains a stretch of 20 nt beyond a putative polyadenylation signal. It is possible that this clone represents a shortened fragment derived from the longer, less abundant mRNA species.

Comparing the amino-acid sequences of three mammalian species homologies of 85% to 95% were found (Fig. 1b). Out of 110 amino acid residues, 8 are exchanged between mouse and rat, 18 between mouse and rabbit (Enfield et al. 1975) and 16 between rat and rabbit. Five residues are identical in rat and rabbit, but differ in mouse PV. This finding suggests a relationship between rodents and lagomorphs. Between carp (Coffee & Bradshaw, 1973) and mammals about 60% of the amino acids are identical. None of the amino-acid residues involved in calcium binding of carp III parvalbumin (Kretsinger & Nockolds, 1973) are exchanged, indicating the strong evolutionary conservation of these Ca²⁺-binding domains.

#### (ii) Identification of RFLPs and mapping of the mouse parvalbumin gene Pva

Genomic DNA of *Mus spretus, Mus musculus domesticus A2G (+/+adrl) and adrl/adrl* and from five additional laboratory strains was isolated and digested with *Eco RI, Hin dIII, Bam HI, Kpn I, Pst I, Pvu II, Ssr I and Tag I*. After gel electrophoresis the DNA was blotted and hybridized with the mouse cDNA probe. No differences in the hybridization patterns between the laboratory mouse strains could be detected (data not shown). The results of Southern-
|       | Mouse | Rat  | Rabbit | Carp  |
|-------|-------|------|--------|-------|
| 1     | M S M T D V L S A E D I K K A I G A F A A A D S F D H K |   |   |   |
| 2     | K F F Q M V G L K K K N P D E V K K V F H I L D K D K S |   |   |   |
| 3     | G F I E E D E L G S I L K G F S S D A R D L S A K E T K |   |   |   |
| 4     | T L L A A G D K D G D G K I G V E E F S T L V A E T |   |   |   |
| 5     |   | M   | A D   | S S   |
| 6     | F K S  |   |   |   |

Fig. 1. (b) Comparison of the amino-acid sequences (using the one-letter code) of parvalbumins from four vertebrate species. Only residues differing from those of the mouse are depicted for the other species. Residues marked by asterisks correspond to those involved in calcium binding of carp III parvalbumin (Kretsinger & Nockolds, 1973), missing residues are marked by —.

blot hybridization are compatible with the existence of a single parvalbumin gene (Pva) per haploid genome, although a tandem duplication of this chromosomal region with conservation of restriction sites cannot be excluded. The existence of a single copy PV gene has been shown for the rat (Berchtold & Means, 1985).

Restriction fragment length polymorphisms (RFLPs) for Eco R1 (about 20 kb, the hybridizing fragment in SPE is slightly smaller than in A2G), Psi I (A2G: 3 kb, SPE: 8/2-8/1-5 kb) and Sst I (A2G: 5/2-5 kb, SPE: 4/5-2/5 kb) were identified between Mus spretus strain SEG from Freiburg and Mus musculus domesticus A2G (Fig. 2 a) and verified for Mus spretus strain SPE from Paris.

Because the genetic polymorphism among laboratory mouse strains is limited, and Mus spretus shows the greatest degree of allelic variation compared with other mouse species (Avner et al. 1988) the DNA of the offspring of inter-specific crosses between Mus spretus and laboratory inbred strains were used for gene localization (for review see Guenet, 1986). DNAs were isolated from individuals of backcrosses between the F1 of an interspecific cross: (C57BL/6 x SPE) x C57BL/6. The restriction fragment length distribution of 65 individual DNA samples was tested using the PV probe (Fig. 2 b). For linkage detection the PV hybridization pattern was compared to pre-existing patterns of the same samples generated by various mapped RFLP probes. A co-distribution of the RFLPs for Pva with Pot-1 (Banerjee et al. 1985) and Mlvi-2 (Kozak et al. 1985) was detected. Out of 63 tested individual DNAs, 57 were concordant for Pva/Pot-1. The concordance for Pva/Mlvi-2 was 33 out of 56 tested animals and 41 out of 59 samples indicated localization of Pva to the same linkage group, chromosome 15, as Pot-1 and Mlvi-2.

No linkage has been found between markers on chromosome 15 and ‘myotonia’, adr (Heller et al. 1982) an allele of adr (Jockusch et al. 1988b). The tissue specific reduction of PV in the myotonic ADR mouse is probably due to a secondary effect of the myotonia as indicated by pharmacological experiments (Reininghaus et al. 1988; Jockusch et al. 1988 a; Kluxen et al. 1988) and not by a mutation in the parvalbumin gene itself.

By in situ hybridization Mlvi-2 has been mapped to band 15A2 near the nucleolus organiser (Adolph et al. 1987) and Pot-1 to band 15D (Banerjee et al. 1985). From our linkage data it may be concluded that the Pva gene is located in the terminal chromosomal region (band 15E/F, Fig. 3), as are the genes for arylsulfatase A (As-2), diaphorase-1 (Dia-1) and the proto-oncogene Sis (band 15E, Adolph et al. 1987). In man, the structural gene for PV has been assigned to chromosome 22 (Berchtold et al. 1987) and the genes SIS, ARSA and DIA1 have been mapped to the distal part of the q arm of this chromosome. The human SIS

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Fig. 2. Restriction fragments of the parvalbumin gene *Pva* in mouse strains and intercrosses. (a) Southern blot hybridization of genomic DNA from *Mus musculus domesticus* wild-type (A2G, lanes 2), the myotonic mutant (ADR, lanes 1) and *Mus spretus* (SPE, lanes 3). Genomic DNA, 10 μg respectively, extracted from lungs, were digested with the restriction enzymes *Eco* RI, *Bam* HI, *Sst* I or *Pst* I and analysed by Southern blotting. A fragment of the mouse PV gene was labelled and used as a probe. (b) RFLPs for *Sst* I in eight individuals of interspecific backcrosses. The DNA from (C57BL/6 x SPE)F1 x C57BL/6 and from *Mus spretus* SPE was digested with *Sst* I, blotted and hybridized with the mouse cDNA probe. Animals homozygous and heterozygous for the *Pva* fragment are characterized by, respectively, 1 and 2 hybridizing fragments.
Fig. 3. Schematic representation of linkage and G-bandning patterns of mouse chromosome 15 and the G-bandning pattern of human chromosome 22. Linkage distances to the marker genes *Pvt-1* (plasmacytoma variant translocation, Banerjee *et al*. 1985) and *Mlv-2* (Moloney virus integration site 2, Kozak *et al*. 1985) are given in cM with *P* < 0.05. Chromosomal segments presumed homologous between mouse and man are indicated by dashed lines. The lengths of the mouse and the human chromosomes are not to scale.

Mammalian proto-oncogene is the most proximal gene in this syntenic group (Kaplan *et al*. 1987). The extent of this man–mouse synteny is further supported by the localization of the gene for cytochrome P450IID and a hemoglobin pseudogene on chromosome 15 of the mouse (Peters, 1988) and chromosome 22 of man (Kaplan & Carritt, 1987).

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