Genet. Res., Camb. (1968), **12**, *pp.* 117–124
Printed in Great Britain

The comparison of closely related groups of rodents by DNA/DNA annealing

BY ANNE McLAREN
A.R.C. Unit of Animal Genetics, Institute of Animal Genetics, Edinburgh
AND P. M. B. WALKER
Department of Zoology, Edinburgh University

(*Received* 18 *January* 1968)

1. INTRODUCTION

The investigation of DNA sequence homologies by the use of molecular annealing techniques should provide a powerful method for probing genetic relatedness, not only between species but also between individuals within a species. In its original form, however, the DNA-agar technique was rather insensitive to differences in base sequence, and could not distinguish even between such distinct species as the Rhesus monkey and the green monkey (Hoyer, McCarthy & Bolton, 1964).

Using DNA from mice and rats (*Mus musculus* and *Rattus norvegicus*), we have tried to develop methods of accentuating differences in annealing between the two species. When labelled fragments of each are incubated at 60 °C with high molecular weight DNA of the same or different species trapped in agar, the percentage of fragments bound averages 30% less in the between-species than in the within-species situation. By re-incubating the fragments which have failed to bind with DNA of the other species, this discrimination index can be increased, after three successive incubations, to 70% (McLaren & Walker, 1965). A similar increase in specificity is found if the conditions for stable duplex formation are made more rigorous by raising the incubation temperature to 70 °C (McLaren & Walker, 1966).

An alternative approach involves the fractionation of DNA fragments on a hydroxyapatite column (Miyazawa & Thomas, 1965). Elution at constant temperture and increasing phosphate concentration ('phosphate fractionation') separates the fragments according to their secondary structure, i.e. according to whether they are single- or double-stranded; conversely, elution at constant salt concentration and increasing temperature ('thermal fractionation') separates the fragments according to their base composition.

When denatured mouse DNA is subjected to phosphate fractionation on a hydroxyapatite column, only the 10% of rapidly renaturing material elutes in the double-stranded position. This fraction, termed 'stable' by Walker & McLaren (1965*a*) and subsequently identified with the DNA satellite found on caesium chloride density gradient centrifugation, shows remarkable powers of discrimination, in that it binds 80% less on rat than on mouse DNA-agar. When fragments were fractionated according to base composition on a hydroxyapatite thermal

https://doi.org/10.1017/S0016672300011733 Published online by Cambridge University Press

chromatogram, the AT-rich fraction gave the best discrimination (McLaren & Walker, 1966). Martin & Hoyer (1967) have also reported that an increased temperature of incubation (68 °C) and selection of AT-rich fragments improves discrimination among primate DNAs in competitive annealing experiments.

So far, only sequence homologies between DNA from animals belonging to different genera have been investigated by DNA annealing techniques. The present study was designed to see whether the degree of refinement achieved in our mouse–rat system would suffice to detect genetic differences between species belonging to the same genus, as well as differences within the species *Mus musculus*.

2. MATERIAL AND METHODS

DNA was obtained from the following sources:

Apodemus flavicollis
Apodemus sylvaticus
 Mainland form.
 Hebridean form.
Peromyscus maniculatus bairdii
Peromyscus polionotus polionotus
Mus musculus
 Wild mice caught on a farm in Fife.
 New Zealand Black (NZB) inbred strain.
 L-cell cultures (an established cell line originally derived from mice of the C3H inbred strain).

^{32}P-labelled DNA from *A. flavicollis*, *A. sylvaticus* (mainland), *P. polionotus* and Fife and NZB *Mus musculus*, was obtained from primary cell cultures grown from embryonic tissue; unlabelled DNA was extracted from liver and other internal organs. Labelled and unlabelled L-cell DNA was obtained from cultures. The design of the experiments was limited by the number of animals available. The preparation of DNA and the techniques used in DNA/DNA annealing were as described previously (Walker & McLaren, 1965*a*).

Because of the non-specific variation between batches of DNA-agar (McLaren & Walker, 1965), any attempt to detect differences in sequence homology between groups by the agar technique has to be based on comparisons using the same DNA-agar preparation. When labelled DNA fragments from both groups are available, this presents no difficulties. When, however, cell lines for isotopic labelling can only be grown from one of the two groups under comparison, as in some of the present work, it becomes necessary to use different DNA fractions, separated by such methods as successive incubation, or chromatography on a hydroxyapatite column, in order to make allowance for the variation in DNA-agar. The techniques of hydroxyapatite fractionation have been described by Walker & McLaren (1965*b*) and McCallum & Walker (1967), and the techniques of successive incubation and recovery by Walker & McLaren (1965*a*).

Where appropriate, the method of analysis of variance was applied to the data, after angular transformation of the percentage binding values.

https://doi.org/10.1017/S0016672300011733 Published online by Cambridge University Press

3. RESULTS

Apodemus

The five experiments in which unfractionated *A. flavicollis* or *A. sylvaticus* (mainland) fragments were incubated with DNA-agar from the two species are listed in Table 1. Within-group variance was small. The *flavicollis* fragments showed little variation between experiments, but a clear tendency for more fragments to be bound in within- than in between-species incubations ($P < 0 \cdot 01$). This cannot have been due to any non-specific inferiority of the *sylvaticus* DNA-agar, since within-species incubations gave consistently higher values for *sylvaticus* than for *flavicollis*. The *sylvaticus* fragments showed marked heterogeneity between

Table 1. *The percentage of labelled fragments of* Apodemus flavicollis *and* A. sylvaticus *DNA bound during overnight incubation at* 60 °C *with DNA-agar from* A. flavicollis *or* A. sylvaticus. *The standard deviation, calculated from the pooled within-group variance, is* 0·77 *and* 1·01 *for the* flavicollis *and* sylvaticus *fragments respectively*

Experiment	Source of DNA fragments	Source of DNA-agar: *flavicollis*	Source of DNA-agar: *sylvaticus*
1	*flavicollis*	18·4, 18·2	15·4
2	*flavicollis*	15·8, 15·8	12·8
3	*flavicollis*	18·8, 16·2, 16·0	13·5, 12·6, 12·1
	sylvaticus	18·6, 18·4, 17·6	19·3, 17·4, 16·6
4	*sylvaticus*	29·9, 28·4	27·6, 26·5
5	*sylvaticus*	30·3, 27·2	35·9, 31·7
Means for *flavicollis*		17·0	13·3
Means for *sylvaticus*		24·3	25·0

Table 2. *The percentage of labelled DNA fragments from the mainland form of* Apodemus sylvaticus *bound during overnight incubation at* 60 °C *with DNA-agar from the mainland or hebridean form. In cycles* 2 *and* 3, *the 'control' fraction consisted of fragments unbound after one and two successive incubations with homologous (mainland) DNA-agar; the 'discriminant' fraction similarly consisted of fragments unbound after incubation with heterologous (hebridean) DNA-agar*

Cycle	Fraction	Source of DNA-agar: Mainland	Source of DNA-agar: Hebridean	'Discrimination'* (%)
1	Total	30·0 (4)†	28·0 (4)	7
2	'Control'	11·2 (1)	9·6 (1)	14
	'Discriminant'	12·2 (2)	10·1 (2)	17
3	'Control'	7·9 (2)	6·0 (2)	24
	'Discriminant'	11·5 (2)	9·3 (1)	19

* Here and elsewhere, 'discrimination' is calculated as the amount by which the between-group falls short of the within-group binding percentage, expressed as a percentage of the latter.

† Here and in the other tables, numbers in parentheses indicate the number of observations on which the mean is based.

https://doi.org/10.1017/S0016672300011733 Published online by Cambridge University Press

experiments, and little tendency to bind better in within- than in between-species incubations. The pooled mean values for both species give a discrimination factor of 10%.

We attempted also to distinguish between DNA from the mainland and Hebridean populations of *A. sylvaticus*. Since labelled fragments were only available from the mainland animals, no reciprocal incubations were possible, so we carried out a three-cycle experiment, comparing the discriminating ability of fragments unbound after one or two successive cycles of incubation with either mainland or hebridean DNA-agar (Table 2). The lower binding throughout on Hebridean DNA-agar does not necessarily indicate any true discrimination, as it may be due to non-specific inferiority of this batch of DNA-agar; and the crucial comparison, of the discriminating ability of fragments which had previously failed to bind with hebridean ('discriminant') versus those which had failed to bind with mainland DNA-agar ('control') showed no difference.

Table 3. *The percentage of* Peromyscus polionotus *DNA fragments bound by* P. polionotus *and* P. maniculatus *DNA-agar. Unfractionated fragments were compared with 'discriminant' and 'control' fractions isolated by a three-cycle incubation procedure (see text)*

	Source of DNA-agar		Discrimination
	polionotus	*maniculatus*	(%)
Unfractionated	24·4	18·0	26
'Control' fraction	38·0	28·4	25
'Discriminant' fraction	7·6 6·5	3·6 4·4	43

Peromyscus

Labelled fragments were only available from *P. polionotus*.

In a three-cycle serial incubation experiment, *P. polionotus* fragments were incubated with *P. maniculatus* DNA-agar and the unbound fragments recovered. These were again incubated with *P. maniculatus* DNA-agar, and both the bound and the unbound fragments recovered. In this experiment the bound fragments were used as a 'control' fraction for the unbound ('discriminant'), and both were subjected to a third incubation on DNA-agar of the two species, along with an unfractionated sample of fragments (Table 3). The generally lower level of binding on *P. maniculatus* DNA-agar may be due to non-specific inferiority of this batch of DNA-agar; the greater depression of heterologous binding with the 'discriminant' fragments certainly suggests true discrimination, but cannot be considered conclusive in the absence of replicate experiments.

Neither thermal nor phosphate fractionation of the fragments on hydroxyapatite was successful in separating out a more highly discriminating fraction. Of the six thermal fractions, that eluting at 87·5 °C showed the highest binding ability in within-species incubations on each of two experiments, with the percentage of fragments bound decreasing as more AT-rich or GC-rich fractions were taken.

https://doi.org/10.1017/S0016672300011733 Published online by Cambridge University Press

Mus musculus

Our within-species results are given in Tables 4 (Fife *v.* L-cell and NZB) and 5 (L-cell *v.* NZB). The high molecular weight Fife DNA trapped in agar turned out to give higher binding values than either the L-cell or NZB DNA, whatever the source of DNA fragments. Reciprocal experiments were therefore averaged to give

Table 4. *The percentage of labelled fragments bound on DNA-agar during reciprocal incubations of Fife, L-cell and NZB DNA*

Origin of DNA		Conditions	Percentage of fragments bound				Mean % of fragments bound	
a, A	b, B	of binding	a/A	b/A	a/B	b/B*	Within strains	Between strains
Fife	L-cell	70° C	10·9	9·1	10·3	8·5	9·7 (4)	9·7 (4)
Fife	NZB	60° C (1st cycle)	22·2	22·8	20·9	18·6	20·4 (2)	21·9 (2)
Fife	NZB	60° C (2nd cycle)	7·5	7·3	4·7	4·6	6·1 (2)	6·0 (2)
Fife	NZB	60° C (3rd cycle)	41·8	29·0	23·8	22·6	32·2 (4)	26·9 (4)
Fife	NZB	70° C	14·5	13·1	11·4	11·5	13·0 (6)	12·3 (6)

* Terminology is as in McLaren & Walker (1965), with lower case letters representing labelled DNA fragments and upper case, high molecular weight DNA trapped in agar. Thus in the top line, b/A represents L-cell fragments incubated with Fife DNA-agar.

a 'within-strain' and a 'between-strain' binding value, as in McLaren & Walker (1965). For the Fife *v.* L-cell comparison, a 70 °C incubation was carried out (Table 4, top line). There was no difference between the amount of binding occurring within and between strains. For the Fife *v.* NZB comparison, three successive cycles of incubation at 60 °C were performed. On the first incubation, between-strain binding was if anything slightly higher than within-strain. Unbound fragments were recovered from the between-strain incubations, and re-incubated. On the second cycle the two binding values were closely similar. Bound fragments were then recovered from the within-strain incubations, in an attempt to concentrate the discriminant fraction, and a third cycle of incubation was performed, for which the mean between-strain binding value was 16 % lower than that within strains. A 70 °C incubation was also carried out; the difference was again in the expected direction, but was not significant.

For the NZB *v.* L-cell comparison, fragments of labelled L-cell DNA were subjected to hydroxyapatite fractionation at 70 °C with increasing phosphate concentrations, to give a 0·12M ('labile') and a 0·3M ('stable') fraction (Walker & McLaren, 1965*b*). These two fractions were then incubated with NZB and L-cell DNA-agar at 60 °C., using the relatvely undiscriminating 'labile' material as a control to indicate the non-specific binding properties of the DNA-agar. Table 5 shows that in this respect the NZB material was superior to the L-cell, giving a higher between- than within-strain binding value for the 'labile' L-cell fragments.

https://doi.org/10.1017/S0016672300011733 Published online by Cambridge University Press

On the other hand the discriminating 'stable' fraction bound nearly 20% better in the homologous within-strain incubations than in the between-strain ones. The interaction factor in the analysis of variance was highly significant ($P < 0{\cdot}002$).

Table 5. *The percentage of labelled L-cell DNA fragments bound on L-cell and NZB DNA-agar, after fractionation on a hydroxyapatite column. Incubations with 'labile' and 'stable' fractions were carried out in duplicate and triplicate respectively*

Hydroxyapatite fraction	% of L-cell fragments bound on DNA-agar		Mean % of fragments bound:	
	L-cell	NZB	Within strain	Between strain
'Labile'	22·1 21·5	26·1 25·6	21·8	25·9
'Stable'	52·8 51·6 49·4	45·4 42·8 40·6	51·3	42·9

4. DISCUSSION

Apodemus flavicollis and *A. sylvaticus* are closely related species, and indeed were at one time classified as subspecies. However, they do not interbreed, either in the wild or under laboratory conditions (Jewell & Fullagar, 1965). The discrimination factor of just over 10% observed in reciprocal incubation experiments may be compared with the factor of 30% which we obtained for the less closely related species, mouse and rat (McLaren & Walker, 1965). The asymmetry observed in the interactions between the two species, with *A. flavicollis* but not *A. sylvaticus* fragments showing significant discrimination, is reminiscent of the situation with mouse and rat (McLaren & Walker, 1965, 1966), where most of the discriminating ability is found among the mouse DNA fragments.

The population of *A. sylvaticus* from the Hebrides is phenotypically distinct from the mainland population, but according to Berry, Evans & Sennitt (1967) has been separated relatively recently, perhaps only in the last few hundred years, and should not be regarded as a separate subspecies. *Peromyscus maniculatus* and *P. polionotus* are classified as separate species, but will give viable and fertile hybrids in the laboratory, provided *P. maniculatus* females are crossed with *P. polionotus* males (McLaren, unpublished). *P. polionotus* DNA fragments gave suggestive but not conclusive evidence of discrimination between the two *Peromyscus* species, while *A. sylvaticus* DNA fragments from the mainland population failed to show any discrimination between mainland and Hebridean *A. sylvaticus*. However, these experiments were neither very extensive nor very sensitive, as in each case only a limited amount of labelled fragments was available, from one of the two DNA sources only.

Within the species *Mus musculus*, our results strongly suggest that sequence differences between NZB DNA and the other two mouse DNAs tested are large enough to be detected by the agar technique. The NZB inbred strain was bred in

https://doi.org/10.1017/S0016672300011733 Published online by Cambridge University Press

New Zealand, and is not directly related to any of the older established inbred strains. Popp (1967) found that the alpha chain of haemoglobin in NZB mice differed from that of the other strains examined by two to three peptides.

We feel that with this demonstration of within-species discrimination, the agar technique has been pushed to the limit of its usefulness. The marked differences in the level of binding between different experiments (see, for example, Table 1), together with the non-specific differences in binding power among different batches of DNA-agar (Walker & McLaren, 1965*a*), make it unsuitable for exploring individual differences in DNA within a species.

SUMMARY

Within the genus *Apodemus*, DNA from *A. sylvaticus* and *A. flavicollis* could be distinguished on the basis of reciprocal DNA/DNA annealing in agar, even when unfractionated DNA was used. In preliminary experiments, DNA from geographical races of *A. sylvaticus* could not be distinguished, nor could DNA from two closely related species of *Peromyscus*.

Within the species *Mus musculus*, the fast-renaturing fraction of L-cell DNA, separated on a hydroxyapatite column, was able to discriminate between L-cell (originating from the C3H inbred strain) and NZB (New Zealand Black) inbred strain DNA in agar.

We would like to thank Dr P. A. Jewell (Wellcome Institute of Comparative Physiology, Zoological Society of London) for providing *Apodemus*, and Dr R. J. Berry (Royal Free Hospital School of Medicine, London) for catching the wild *Mus musculus* in Fife. We are particularly indebted to Miss McCallum (Department of Zoology, Edinburgh University) for carrying out the DNA fractionation procedures on hydroxyapatite, and to Arthur Mitchell and David Richmond for able technical assistance. We are also indebted to the Nuffield Foundation for financial support, and to the Medical Research Council for providing special equipment.

REFERENCES

Berry, R. J., Evans, I. M. & Sennitt, B. F. C. (1967). The relationships and ecology of *Apodemus sylvaticus* from the Small Isles of the Inner Hebrides, Scotland. *J. Zool.* **152**, 333–346.

Hoyer, B. H., McCarthy, B. J. & Bolton, E. T. (1964). A molecular approach in the systematics of higher organisms. *Science* **144**, 959–967.

Jewell, P. A. & Fullagar, P. J. (1965). Fertility among races of the field mouse (*Apodemus sylvaticus*) and their failure to form hybrids with the yellow-necked mouse (*Apodemus flavicollis*). *Evolution* **19**, 175–181.

McLaren, A. & Walker, P. M. B. (1965). Genetic discrimination by means of DNA/DNA binding. *Genet. Res.* **6**, 230–247.

McLaren, A. & Walker, P. M. B. (1966). Discriminating power of rodent deoxyribonucleic acid on incubation in agar. *Nature, Lond.* **211**, 486–490.

Martin, M. A. & Hoyer, B. H. (1967). Adenine plus thymine and guanine plus cytosine enriched fractions of animal DNA's as indicators of polynucleotide homologies. *J. mol. Biol.* **27**, 113–129.

McCallum, M. & Walker, P. M. B. (1967). Hydroxyapatite fractionation procedures in the study of the mammalian genome. *Biochem. J.* **105**, 163–169.

Miyazawa, Y. & Thomas, C. A. (1965). The nucleotide composition of short segments of DNA molecules. *J. mol. Biol.* **11**, 223–237.

https://doi.org/10.1017/S0016672300011733 Published online by Cambridge University Press

POPP, R. A. (1967). Hemoglobins of mice: sequence and possible ambiguity at one position of the alpha chain. *J. mol. Biol.* **27**, 9.

WALKER, P. M. B. & McLAREN, A. (1965*a*) Specific duplex formation *in vitro* of mammalian DNA. *J. mol. Biol.* **12**, 394–409.

WALKER, P. M. B. & McLAREN, A. (1965*b*). Fractionation of mouse deoxyribonucleic acid on hydroxyapatite. *Nature, Lond.* **208**, 1175–1179.

https://doi.org/10.1017/S0016672300011733 Published online by Cambridge University Press