ber of claims of cognitive and even conscious processes in animals are on the rise[16]. We do not intend to address this debate here, but suggest that only carefully designed novel experiments can provide a breakthrough.

With respect to experiments and evidence, there have been advocates of naturalistic observations on the one hand[17] and carefully controlled protocols with trained captive animals on the other[7]. The naturalistic approach suffers from problems such as being anecdotal or relying on convergence[7] whereas captive experiments are criticized on account of the problems associated with training and the artificiality and arbitrariness of tasks[17]. We do not claim our protocol to be immune to criticism or alternative explanations. It is however, a good combination of natural conditions and experimental manipulations. The task given to the birds is close to what they may be required to do naturally. At the same time the experimental situation is reproducible and there are adequate controls. The experiment also opens up the field for non-primate studies on theory of mind.

1. Premack, D. and Woodruff, G., *Behav. Brain Sci.*, 1978, **4**, 515–526.
2. Byrne, R., *The Thinking Ape: Evolutionary Origins of Intelligence*, Oxford University Press, Oxford, 1995.
3. Wimmer, H. and Perner, J., *Cognition*, 1983, **13**, 103–128.
4. Baron-Cohen, S., Leslie, A. M. and Frith, U., *Cognition*, 1985, **21**, 37–46.
5. Baron-Cohen, S., Leslie, A. M. and Frith, U., *Br. J. Dev. Psychol.*, 1986, **4**, 113–125.
6. Leslie, A. M., *Psychol. Rev.*, 1987, **94**, 412–426.
7. Heyes, C. M., *Behav. Brain Sci.*, 1998, **21**, 101–148.
8. Baron-Cohen, S., *J. Child Psychol. Psychiatry*, 1989, **30**, 285–297.
9. Baron-Cohen, S., *Psychiatr. Clin. North Am.*, 1991, **14**, 33–51.
10. Seyfarth, R. M. and Cheney, D. L., *Sci. Am.* 1992, **267**, 122–128.
11. Dharamkumarsinghji, R. S. and Lavkumar, K. S., *Sixty Indian Birds*, Pulication Division, Min. Information and Broadcasting, Government of India, 1972.
12. Shreedhar, S. and Praveen Karanth, K., *Curr. Sci.*, 1993, **25**, 489–490.
13. Wardlaw, A. C., *Practical Statistics for Experimental Biologists*, John Wiley & Sons, 1985.
14. Leavens, D. A., *Behav. Brain Sci.*, 1998, **21**, 123–124.
15. Baron-Cohen, S., Baldwin, D. A. and Crowson, M., *Child. Dev.*, 1997, **68**, 48–57.
16. Griffin, D. R., *Anim. Cogn.*, 1998, **1**, 3–16.
17. Matheson, M. D., Cooper, M., Thompson, R. and Fragaszy, D., *Behav. Brain Sci.*, 1998, **21**, 124–125.

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Sequences that facilitate high fidelity of pairing by RecA: A model

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Homologues of *E. coli* RecA in eucaryotes (Rad 51) are conserved during evolution in their structural and physical properties. They form structurally similar presynaptic filaments on single-stranded DNA. These proteins bind to certain sequences that are G- and T-rich with higher affinity. Hot-spots of recombination in *E. coli* are embedded in GT-rich stretches. The DNA bases in the presynaptic filament show a high degree of promiscuous pairing excepting the C residue, which is paired with a high degree of fidelity. A model is proposed in this study, suggesting that the binding preference and pairing fidelity are two separate parameters that might together ensure proper recombinational pairing in hot-spots.

RECOMBINATION hot-spots is well characterized in *E. coli* and *S. cerevisiae* at the genetic and molecular level[1,2]. In higher eukaryotes such as mammals and plants, a few candidate sequence motifs are described as recombination hot-spots[3–5]. In spite of a wealth of information on hot-spots in *E. coli* and *S. cerevisiae*, there is no obvious consensus at the DNA level as to what makes a region 'hot-spot' for recombination. In this paper, we try to focus on this issue and propose a molecular model for the same. This proposal is based on our work on *E. coli* RecA as well as that published from Stephen Kowalczykowski's lab[6,7]. A genetic hot-spot is characterized by extrinsic and intrinsic factors. The former includes accessibility to the recombination machinery and chromatin structure. Intrinsically, a 'hot-spot' should contain DNA sequences that might have higher affinity to RecA protein and thereby promote a relatively stable RecA nucleoprotein filament that initiates recombination at a higher frequency. It should also have DNA sequences that can pair well with homologous sequences. Recent work addresses the issue of RecA affinity[6] whereas our results provide an insight on the pairing preferences of RecA. In this communication, we have focused on the intrinsic factors that influence recombination.

An *in vitro* selection was performed in a random pool of 10^{14} oligos which were 70-mers and a pool of 10^{11} 18-mers to select sequences that have higher affinity for RecA binding[6]. Both selections were done with limiting concentrations of RecA. Several cycles (eight for the 70-mer pool and five for the 18-mer pool) of selection and PCR amplification yielded sequences that were substantially rich in G and T bases. The average base percentages of several such clones were: (from the 70-mer

pool) %G = 33.5; %A = 17.9; %T = 30.7; %C = 17.9; (from the 18-mer pool) %G = 38.3; %A = 12.7; %T = 37.3; %C = 11.2. An independent selection from a random pool of 18-mers yielded single strands of similar sequence bias when yeast Rad 51 was used instead of *E. coli* RecA which underscores the universality of such sequence biases[8]. So, it is clear that *E. coli* RecA and its eukaryotic counterparts are evolutionarily designed to bind G and T residues more strongly than C and A residues (G = T > A = C).

We have been interested in quantitating the intrinsic ability of A, G, C and T bases in RecA-filament to choose their complementary base from a milieu of mispairs during homology search. Two observations prompted us to study two-stranded complementary pairing of RecA to address this issue. (i) *E. coli* recombination ensues in spite of high sequence divergence between recombining partners (such as in conjugational mating between *E. coli* and *Salmonella typhimurium* when mismatch repair genes are mutated)[9] (ii) An *in vitro* counterpart of such a reaction that is also more tolerant to mismatches happens only when pairing leads to D-loop complexes[10–12]. D-loop complexes are essentially sustained by complementary pairing between the filament strand and its complement in the superhelical duplex. We monitored complementary pairing between an 83-mer oligo and a 33-mer (having equal preponderance of all the four bases) in an assay where we substituted a particular base for another at every position on the 33-mer. So all residues were changed to another at a time, thus giving rise to 12 different 33-mer tester sequences[13]. This leads to several specific mispairs on pairing with the 83-mer. In each case, RecA was coated on an 83-mer which is long enough to promote RecA binding in the presence of ATP. To elucidate the effects of specific sets of non-Watson–Crick base pairs, targeted recognition was monitored as a ligatable alignment between a tester and a reference tether immediately upstream of it[13]. The tether is a 25-mer, fully complementary to one end of the 83-mer. The tester is a 33-mer which carried base substitutions that reduced Watson–Crick complementarity between pairing substrates. In this way, we assessed the effects of all the 12 possible kinds of mispairs by quantitating the ligatable alignments on a denaturing polyacrylamide gel. To minimize the effects of blunt-ended ligations in the above assay, we used *E. coli* ligase in the present set of experiments as opposed to T4-ligase. The pairing hierarchy was expressed as the percentage of tester-oligo that was ligated due to targeted pairing (Figure 1, first base on the mispair is from the RecA-83-mer filament and the second base from the 33-mer tester). By and large, the hierarchy was similar to what was observed before and confirmed across two different sequence contexts[13]. However, *E. coli* ligase assay was more discriminatory than that of T4-ligase and was able to capture even small differences in steady state level of pairings across AG, AA, AC, GT and TG mispairs. This enabled us to detect the intrinsic fine hierarchy amongst this set which was earlier all clustered as equally *good*[13]. The overall hierarchy of ten out of twelve base mispairs measured by *E. coli* ligase assay was similar to that of T4-ligase assay, the two reversals being that of GG and CT. In *E. coli* ligase assay, GG was better than TT whereas CT was not as good as CA. However, this descrepancy has no bearing on our conclusions because the relative gradation of promiscuity among A, G, C and T bases is the main point of discussion here (see below). What is clear from this hierarchy is that A residues in the filament are the most promiscuous and C the least, while G and T fall in between. In other words, different bases in the filament have different degrees of pairing-promiscuity using which one could define a parameter called 'promiscuity index' (Table 1). The higher the promiscuity index, the higher is the tendency of that base to engage in biologically unproductive mispairing that would eventually get eliminated by mismatch repair proteins[14]. A filament that is richer with bases of high promiscuity index is more prone to get 'bogged' down with unproductive pairings. On the other hand, a filament of bases with low promiscuity index has higher chances of encountering the right base (complementary and productive pairing). Despite minor differences in the hierarchy-status of GG and CT in T4-ligase assay earlier[13] *vis-à-vis* that by *E. coli* ligase assay here, the relative grading of promiscuity index of A, G, T and C bases in the filament remains the same in both assays.

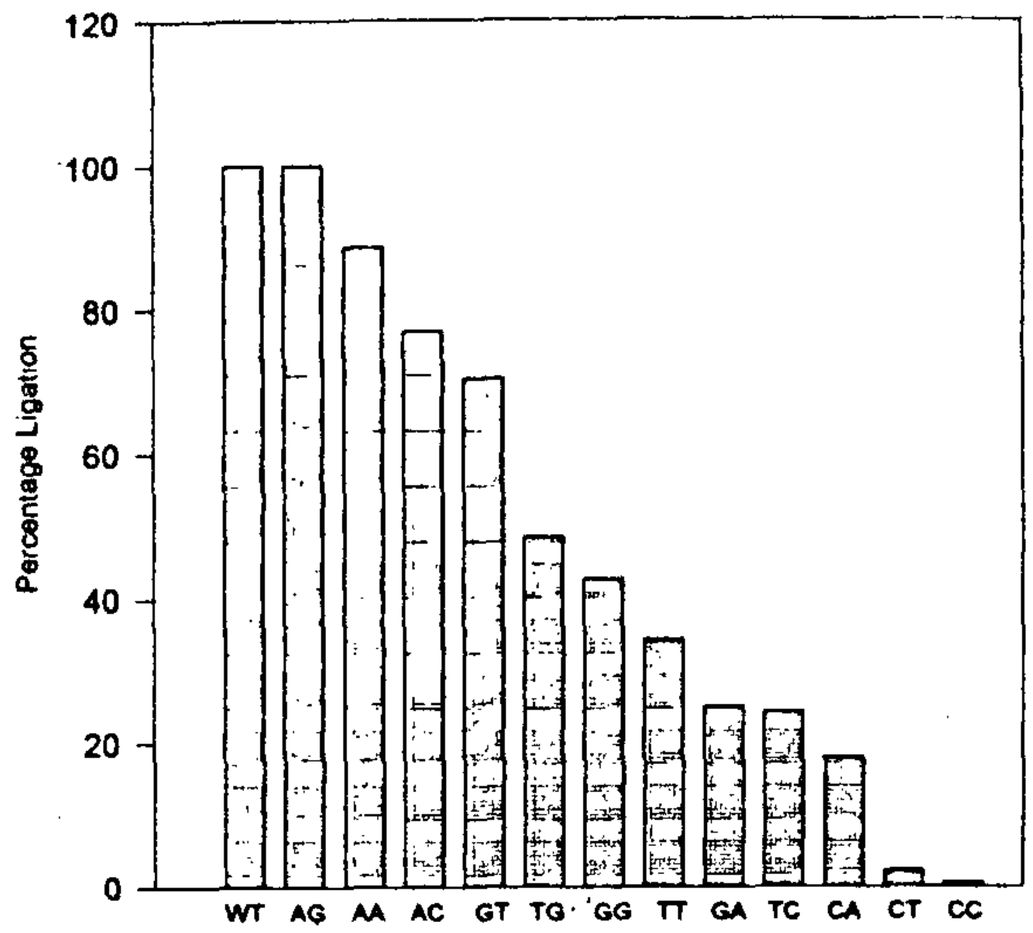

Figure 1. PhosphorImager analysis of targeted pairings in single changes as described[13]. Ligations were done for 30 min with *E. coli* DNA-ligase. WT = wildtype. The first base on the mispair is on the RecA presynaptic filament.

Table 1. Proximiscuity-index for a particular base is expressed as the ratio of the total percentage of promiscuous pairings involving that base to its wild type complementary pairing (as measured in the Targeted Ligation Assay[13], Figure 1)

Filament base	Promiscuity index
$A = \frac{AG + AA + AC}{3 \times WT}$	0.89
$G = \frac{GT + GA + GG}{3 \times WT}$	0.46
$T = \frac{TG + TC + TT}{3 \times WT}$	0.36
$C = \frac{CA + CT + CC}{3 \times WT}$	0.07

We hypothesize that *E. coli* recombination hot-spots could perhaps face two types of evolutionary pressures: one for better binding by RecA and the other for better chances of finding complementary pairs (those with a lower promiscuity index). RecA binds to some bases better, but has a higher fidelity of pairing with other bases. G, T richness in a hot-spot confers a much better binding affinity for RecA while C richness confers much better pairing success rate. A residues are selected out on both counts, namely poor binding as well as poor pairing ability.

In a recent paper, Kowalczykowski and his coworkers have analysed sequences flanking all 1009 *chi* sites (5′-GCTGGTGG-3′) (*E. coli* hot-spots) and searched for statistically significant sequence bias around these sites[7]. The deviations from the genomic mean of A, C, G and T residues at 50 positions surrounding the 1009 aligned *chi* sites revealed a striking pattern: A residues are highly under-represented whereas G and T residues are highly over-represented in the entire vicinity of *chi*-tracts. C residues are distributed fairly equally excepting in a few positions in the immediate vicinity of *chi*.

Several enzymes play a role in initiating the recombination at *chi* sequences. The enzyme complex RecBCD initiates the process of recombination by processively degrading double-stranded DNA till it encounters a *chi* sequence where it attenuates its 3′-5′ exonuclease[15]. The enzyme continues to degrade the other strand, now leaving a 3′-single stranded tail to which RecA binds[16]. It is also known that the RecBCD enzyme stimulates the preferential loading of *chi* and its adjoining sequences (3′ to 5′) by RecA protein in preference to *E. coli* SSB as well as to any other non-*chi* related sequences[17]. Such a facilitated loading of RecA on *chi*-sequences depends upon the simultaneous action of both RecA and RecBCD proteins and demonstrates a new level of coordination during the initiation of recombination[17]. At the DNA sequence level, there is a distinct under representation of C in a short stretch immediately following *chi*. This might provide a cue for the attenuation of the 3′-5′ exonuclease and accentuation of 5′-3′ exonuclease of the RecBCD enzyme. RecA coats the single strand forming a presynaptic complex and begins the process of homology search. However, the stretch of DNA flanking *chi* is over-represented with G and T residues in a triplet pattern of GGT[7]. This DNA is a good substrate for binding to RecA. However, the triplet arrangement poses a problem for homology search as several frames would exist with which this can pair in a complementary manner. It is here that the random and average distribution of C residues plays a role. C is the base with the lowest promiscuity index or in other words pairs with the highest fidelity. This would ensure that the right frame of alignment is fixed by the C residue amidst a sea of GGT triplets. Indeed, recent *in vitro* experiments involving RecA pairing with dinucleotide repeats has borne out this notion[18]. RecA-ss-DNA filaments encompassing continuous repeats of either GT or CA exhibit poorer efficiencies of stable joints and strand exchange products than that of mixed sequence controls. This happens in spite of the ability of repeat stretches to bind RecA measurably better than mixed sequence control[18]. A simple explanation for such an effect could be that among pure repeat sequences, RecA reaction cannot decide on the right frame of alignment leading to shorter joints that are unstable to deproteinization and are slow in strand exchange[18]. This result strongly underscores the need of interspersed bases as *frame-fixers* even in those sequences that bind RecA very well, to aid RecA-pairing in the right (productive) frame of alignment. And C residues, being the least promiscuous in pairing serve this function well in the milieu of GGT-repeats of *chi-islands* in *E. coli*[7].

What are the implications of this argument? In simple organisms such as *E. coli*, molecular determinants that might have shaped hot-spots are much more recombinase-based (RecA, in this case). Extrinsic and higher order structural elements such as chromatin accessibility or nuclear matrix anchorages, etc. may play a much smaller role. In eukaryotes, where regulation is much more complex, extrinsic components become more important. Nevertheless, it is interesting to note that the *E. coli* RecA homologue of yeast, Rad51, has binding preference of DNA bases very similar to that of RecA[8] and perhaps has similar pairing preferences too.

1. Lam, S. T., Stahl, M. M. McMilin, K. D. and Stahl, F. W., *Genetics*, 1974, **77**, 425–433.
2. Nicolas, A., Treco, D., Schultes, N. P. and Szostak, J. W. *Nature*, 1989, **338**, 35–39.
3. Steinmetz, M., Stephan, D. and Lindahl, K. F., *Cell*, 1986, **44**, 895–904.
4. Nachman, M. W. and Churchill, G. A., *Genetics*, 1996, **142**, 537–548.
5. Patterson, G. I., Kubo, K. M., Shroyer, T. and Chandler, V. L. *Genetics*, 1995, **140**, 1389–1406.
6. Tracy, R. B. and Kowalczykowski, S. C., *Genes Dev.*, 1996, **10**, 1890–1903.

7. Tracy, R. B., Chedin, F. and Kowalczykowski, S. C., *Cell*, 1997, **90**, 205–206.
8. Tracy, R. B., Baumohl, J. K. and Kowalczykowski, S. C., *Genes Dev.*, 1997, **11**, 3423–3431.
9. Rayssiguier, C., Thaler, D. S. and Radman, M., *Nature*, 1989, **342**, 396–401.
10. Beattie, K. L., Wiegand, R. C. and Radding, C. M., *J. Mol. Biol.*, 1977, **116**, 783–803.
11. Hsieh, P., Camerini-Otero, C. S. and Camerini-Otero, D., *Proc. Natl. Acad. Sci.*, 1992, **89**, 6492–6496.
12. Adzuma, K., *Genes Dev.*, 1992, **6**, 1679–1694.
13. Karthikeyan, G., Wagle, M. D. and Rao, B. J., *FEBS Lett.*, 1998, **425**, 45–51.
14. Worth, Jr. L., Clark, S., Radman, M. and Modrich, P., *Proc. Natl. Acad. Sci.*, 1994, **91**, 3238–3241.
15. Dixon, D. A. and Kowalczykowski, S. C., *Cell*, 1991, **66**, 361–371.
16. Dixon, D. A. and Kowalczykowski, S. C., *Cell*, 1993, **72**, 87–96.
17. Anderson, D. G. and Kowalczykowski, S. C., *Cell*, 1997, **90**, 77–86.
18. Dutreix, M., *J. Mol. Biol.*, 1997, **273**, 105–113.

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Lability of sex differentiation in fish

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The processes of sex determination and differentiation are labile in teleosts and are amenable for manipulations by ploidy during fertilization, hormone during hatching, temperature during the juvenile stage and other environmental or surgical factors during the adult stage.

IN oviparous teleosts early embryonic events, namely insemination, second polar body extrusion and first mitotic cleavage are manipulable and render 37 different types of ploidy induction possible; such ploidy inductions during early embryonic stages result in the production of all-male, all-female or all-sterile population[1]. However, the scope for ploidy alterations to regulate sex determination is restricted to early embryonic stages alone. A large number of previous publications have attempted to precisely delineate the optimum (labile) period, during which it is possible to successfully induce ploidy[2,3]. The process of sex differentiation in teleosts is also labile[4], rendering hormonal induction of sex reversal possible in 37 gonochoristic species and 13 hermaphroditic species[5]; hormonal manipulations during the labile period result in the production of monosex population; again, the labile period is restricted mostly to just before and after hatching stages. Thus sex determination and differentiation in fish are labile and can be reversed by manipulating ploidy, and hormone during fertilization and hatching stages, respectively.

A series of publications by Strussmann *et al.*[6] and others[7] have recently documented thermal lability of sex determination in a number of teleosts; for instance, fish exposed to colder or warmer temperature from hatchling to juvenile stage lead to the production of all-female or all-male progenies[8,9]; hence, thermal induction may serve as a third technique to regulate the sex of teleosts. A number of others, such as social and surgical factors may also induce sex reversal in adults. In many coral fish and in the freshwater Chinese paradise fish *Macropodus opercularis*, hierarchy and aggressive behaviour have led to the formation of a definite social organization and any manipulation to alter the social structure lead to sex reversal[10–12]. Besides, it has long been known that gonadectomy induces sex reversal in a few teleosts[13]. For instance, female *Betta splendens* developed testes after ovariectomy and became functional male[14]. Therefore, the processes of sex determination and differentiation are labile in teleosts, rendering manipulations of ploidy during fertilization, hormone during hatching, temperature during juvenile, and surgical and social during adult stages. This communication reports the amenability of teleosts to sex regulation almost throughout life by manipulating ploidy, hormone, temperature and other selected environmental factors.

Table 1 lists selected representative species, in which sex reversal has been successfully accomplished by manipulating one or more of the following: ploidy, hormone, temperature and environmental factors. The scope for ploidy manipulation is indeed very strictly restricted to a limited period of few seconds and minutes during fertilization[1]; likewise, the duration of the labile period is also very much restricted to a few minutes just before and after hatching, when the immersion technique is chosen for hormonal induction[5], or a few days immediately following hatching in ornamental fish[15], or a few months in foodfish like carps[16] and salmon[17], when dietary administration is chosen for hormonal induction of sex reversal; rarely, *Poecilia reticulata* has been shown to be amenable for hormonal induction of sex reversal during embryogenesis, just before and after hatching and post-maturity stage[15,18–20]; yet, the optimum period for hormonal induction of sex reversal is mostly restricted to the hatchling stage. Likewise, the optimum stage for successful sex reversal by manipulating thermal, or any other environmental factor is now shown to be restricted to the juvenile or adult stage. In more than 60% of the selected representatives species, successful sex reversal has been induced by ploidy and/or hormonal manipulation(s) by different authors. In hermaphroditic species like *Monopterus albus*, sex is spontaneously reversed in adults[21]; such spontaneous sex reversal during adult stage is recorded in hermaphrodites characterized by polyandrous or polygynous[11] social system; a manipula-

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