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RecA protein mediates homologous recognition via non-Watson–Crick bonds in base triplets

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SUMMARY

E. coli RecA protein, the prototype of a class, forms a helical nucleoprotein filament on single-stranded DNA that recognizes homology in duplex DNA, and initiates the exchange of strands in homologous recombination. The discovery of this reaction some years ago posed a quandary on how a third strand recognizes homology in duplex DNA, whose Watson–Crick bonds face inward in a hydrophobic core of stacked bases. Recent studies have shown that RecA protein promotes homologous recognition via non-Watson–Crick bonds in base triplets. The intermediates in the RecA reaction differ distinctly from triplex DNA that forms non-enzymically. The biological significance of the novel set of DNA interactions by which RecA protein effects homologous recognition is indicated by the importance of this protein in recombination, and the widespread distribution of homologous proteins in prokaryotes and eukaryotes.

1. AN OVERVIEW OF THE RecA REACTION

E. coli RecA protein, the prototype of a class that is widespread in both prokaryotes and eukaryotes, plays essential roles in recombination and repair (Ogawa *et al.* 1993; Shinohara *et al.* 1993; Story *et al.* 1993; for reviews, see Kowalczykowski *et al.* 1994; Radding 1991; West 1992). *In vitro*, RecA protein forms a right-handed helical nucleoprotein filament by head-to-tail polymerization on single-stranded DNA. This nucleoprotein filament recognizes homology in duplex DNA, which it quickly assimilates to form a three-stranded nucleoprotein filament that can be kilobases long (Stasiak *et al.* 1984; G. Reddy, B. Burnett & C. M. Radding, unpublished observations). The filament effects a switch of base pairs, creating heteroduplex DNA and a third strand which it displaces by a process that is fuelled by ATP hydrolysis (figure 1) (Menetski *et al.* 1990; Roca & Cox 1990; Rosselli & Stasiak 1990; Radding 1991; Burnett *et al.* 1994; Cox 1994).

2. RECOGNITION OF HOMOLOGY

Biologists have long known that in homologous genetic recombination, DNA molecules of different parentage are ultimately joined by Watson–Crick pairing of complementary strands in a molecular splice, called a heteroduplex joint; but the way in which homologous molecules initially recognize one another has remained mysterious.

The actions of RecA protein, as discovered fifteen years ago, brought to light a reaction in which a single strand recognizes homology in duplex DNA (figure 2) (McEntee *et al.* 1979; Shibata *et al.* 1979). Although three strands are involved, rather than two, recognition might nonetheless entail the Watson–Crick pairing of complementary strands, provided that RecA protein were to cause at least a local unpairing of strands in the duplex partner (figure 3). A non-enzymic precedent exists for such a mechanism, namely the thermally induced formation of D-loops by single strands and homologous superhelical DNA (Holloman *et al.* 1975). Characterization of the non-enzymic reaction, in which superhelicity plays an essential role, showed that it is based on the Watson–Crick pairing of an incoming single strand with its complement in a locally unwound region of duplex DNA (Beattie *et al.* 1977; Wiegand *et al.* 1977).

According to an alternative model, base triplets might mediate the recognition of homology in duplex DNA: an additional set of bonds would form between a third strand and duplex DNA while the latter temporarily maintains its Watson–Crick connections (figure 3) (Hsieh *et al.* 1990; Rao *et al.* 1993). A non-enzymic precedent also exists for this *base triplet* model, namely the spontaneous formation in solution of triplex DNA involving Hoogsteen and other non-Watson–Crick bonds which bind a third strand in the wide groove of duplex DNA. The formation of such triplex DNA, however, requires special sequences, runs of purines and pyrimidines, and produces a structure in which the third strand is antiparallel rather than parallel to its homologue (see figure 3) (Felsenfeld *et al.* 1957; Moser & Dervan 1987; Cooney *et al.* 1988;

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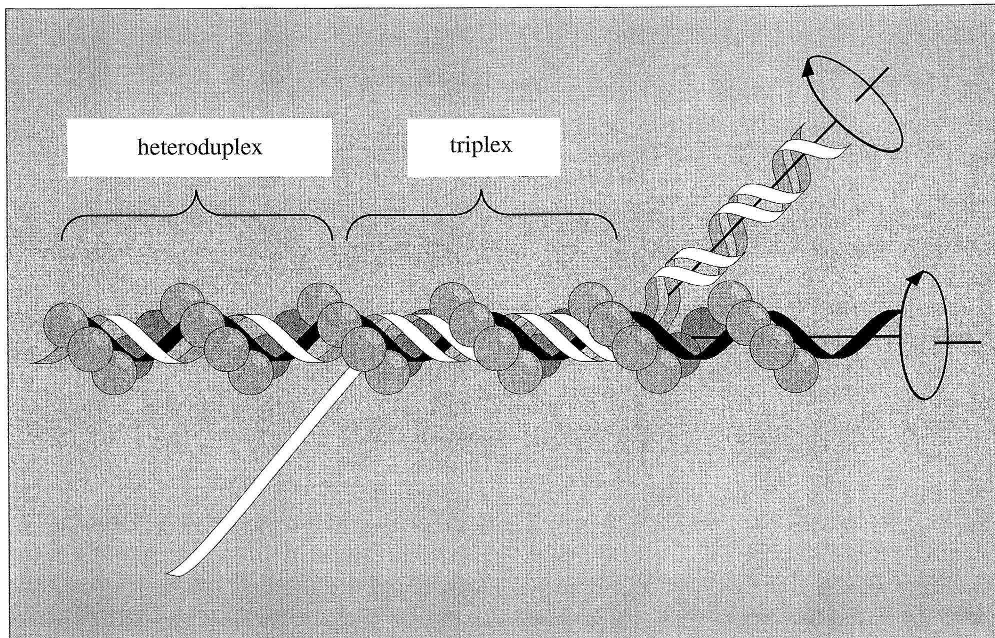

Figure 1. A model of strand exchange promoted by RecA protein (Howard-Flanders *et al.* 1984; Radding 1991). *In vitro*, the RecA nucleoprotein filament and duplex DNA meld to form a triplex intermediate, leading to exchange of base pairs and the separation of one strand from the original duplex.

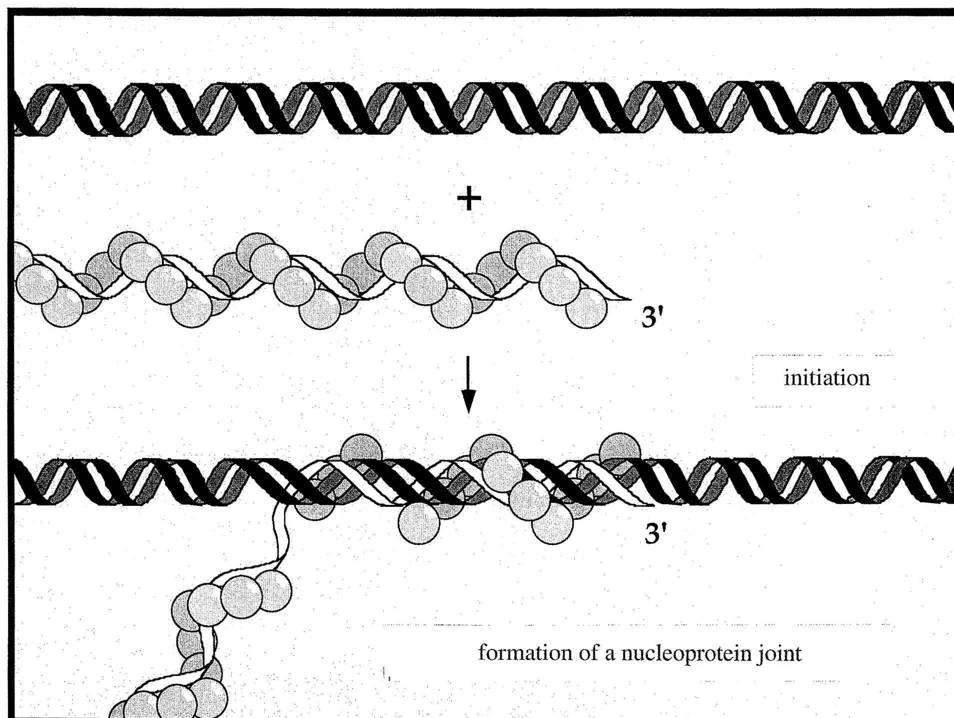

Figure 2. Recognition of homology in duplex DNA by the RecA nucleoprotein filament.

Rajagopal & Feigon 1989; Radhakrishnan *et al.* 1991). Neither the special sequences nor the antiparallel orientation pertains to the RecA reaction, which requires homologous rather than specific sequences, and which requires parallel homologues that undergo stepwise directional exchange that extends over kilobase lengths (cf. figures 1 and 3).

3. HOMOLOGOUS RECOGNITION BY NON-WATSON–CRICK INTERACTIONS OF TWO STRANDS

Evidence of a triplet interaction that precedes switching of base pairs has come from observations on the interactions of single-stranded oligonucleotides

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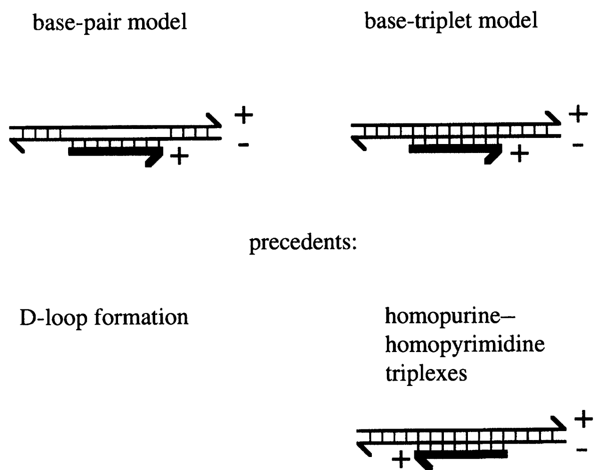

Figure 3. Alternative models for the recognition of homology in duplex DNA.

with DNA, either single-stranded or double-stranded, in the RecA filament (Rao & Radding 1993, 1994).

With a single strand in the RecA filament, homologous complexes were formed when a complementary oligonucleotide was added, as had been observed previously. Surprisingly, however, a single strand in the RecA filament recognized not only its complement, but also recognized an oligonucleotide of identical sequence (figure 4).

The pairing of identical strands, called *self-recognition*, required RecA protein, Mg^{2+}, and ATP or ATPγS. Pairing was observed with several homologous sequences, but not with heterologous sequences. A computer search at various levels of partial matches revealed no fortuitous complementary sequences that could account for the different behaviour of homologous versus heterologous oligonucleotides (Rao & Radding 1993).

Self-recognition promoted by RecA protein is not to be confused with the non-enzymic formation of parallel strand DNA (Van de Sande *et al.* 1988; Rippe *et al.* 1992; Rippe & Jovin 1992), which, like the non-enzymic formation of triplex DNA, requires specific sequences, and in some cases, acidic pH (Robinson *et al.* 1992; Robinson & Wang 1993). Moreover, unlike parallel strand DNA, which is a stable DNA structure in solution, the self-recognition complexes formed by RecA protein do not survive deproteinization; they are not stable DNA structures, but rather are transient intermediates in homologous pairing and strand exchange.

4. HOMOLOGOUS RECOGNITION INVOLVING THREE STRANDS OF DNA IN RecA FILAMENTS

The experiments described above revealed that the RecA filament is capable of using non-Watson–Crick bonds to recognize homology. Are these the bonds that mediate recognition of homology in duplex DNA, and what does the pairing of two identical strands have to do with the prototypic RecA reaction in which three strands are involved? When the pairing reaction was further explored, not with a single strand in the RecA

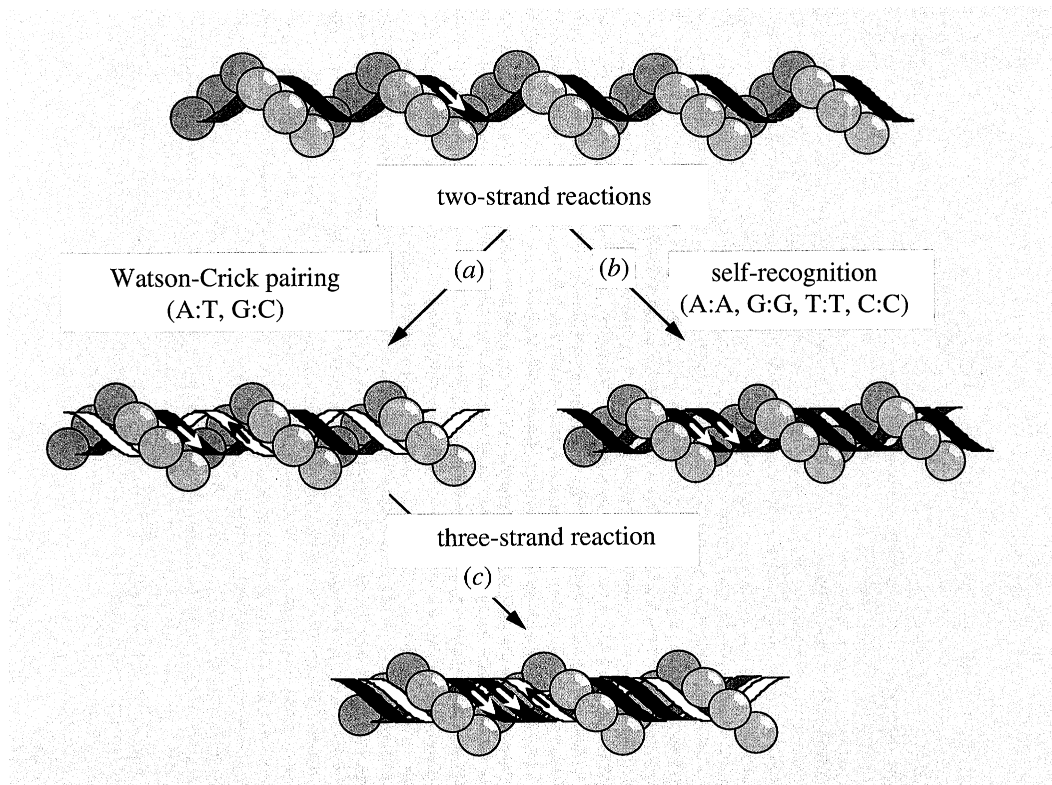

Figure 4. Experimental models of homologous recognition in the RecA filament. (*a*) Watson–Crick pairing of a complementary oligonucleotide with a single strand in the RecA filament; (*b*) Self-recognition of an oligonucleotide of identical sequence; (*c*) Recognition of a third strand by a RecA filament that contains a pair of complementary strands.

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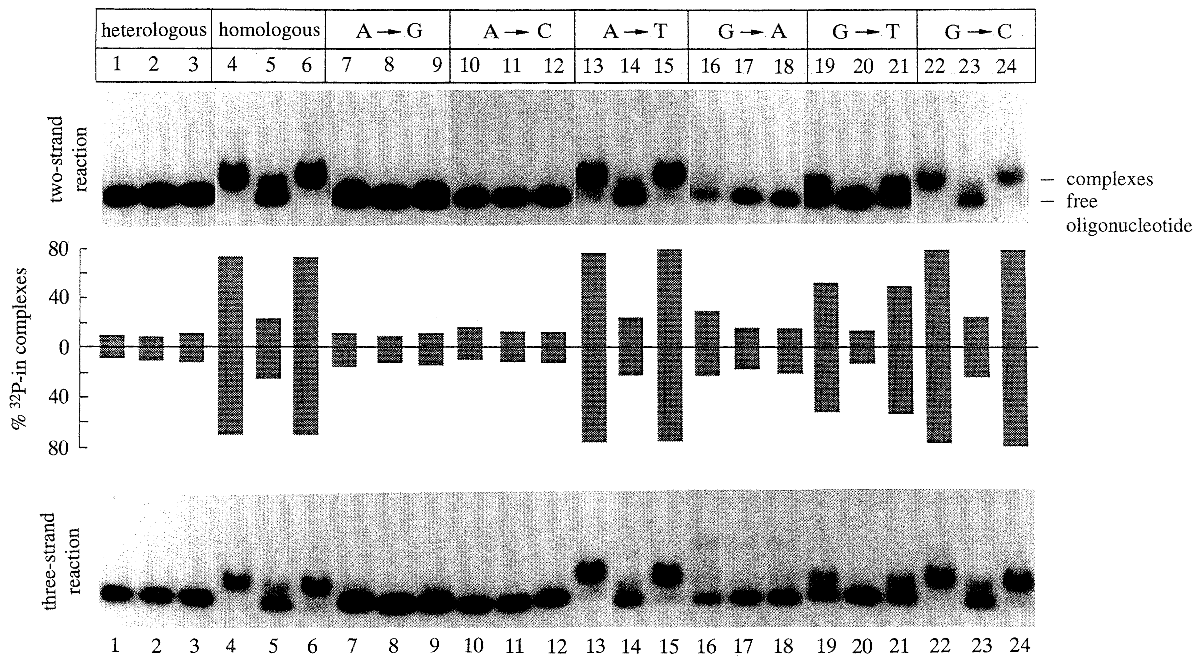

Figure 5. Comparison of the effects of base substitutions in an oligonucleotide on self-recognition (figure 4*b*) versus the recognition of that same oligonucleotide by duplex DNA in the RecA filament (figure 4*c*) (Rao & Radding 1994). The top of each panel shows the effects of base substitutions on the two-strand reaction, the bottom on the three-strand reaction. For the two-strand reaction, a RecA filament was formed on an 83 mer oligonucleotide in the presence of ATPγS; for the three-strand reaction, the filament was formed on the same 83 mer to which a complementary 43 mer had been annealed. Pairing was initiated by adding 5′ labelled 33 mer homologous oligonucleotide, a homologous oligonucleotide carrying base substitutions, or a heterologous 33 mer oligonucleotide, in the presence of a 24-fold excess of heterologous unlabelled carrier oligonucleotide. Each pairing reaction was done in three ways: without any additional competitor (first lane in each set of three, e.g. lane 4), with a tenfold excess of unlabelled competitor of the same sequence as the labelled 33 mer (second lane in each set of three, e.g. lane 5), or with another heterologous competitor (third lane in each set of three, e.g. lane 6). Pairing was done for 6 min at 37 °C, the samples were analysed by a gel retardation assay and quantitated by scanning the gel (PhosphorImager, Molecular Dynamics) (Rao & Radding 1993). The quantitative data from the three-strand reactions are plotted as inverted bars for ease of comparison with the two-strand reactions. Additional controls included: omission of RecA protein, omission of ATPγS, and deproteinization of wild-type reactions with SDS and proteinase K as described (Rao & Radding 1993), all of which resulted in no detectable pairing. (Copyright permission from *Proc. natn. Acad. Sci. U.S.A.*, 1994.)

filament, but rather with a segment of homologous duplex DNA in the filament, observations were made that closely paralleled those made on self-recognition. The nucleoprotein filament containing duplex DNA recognized a single-stranded oligonucleotide that had the sequence of either the Watson or the Crick strand, and all the requirements and stereochemical properties of two-strand and three-strand reactions were the same (figure 4, and see (§7) below).

5. IDENTICAL EFFECTS OF BASE SUBSTITUTIONS ON REACTIONS INVOLVING TWO VERSUS THREE STRANDS OF DNA

A comparison of the effects of base substitutions on self-recognition versus the recognition of homology in duplex DNA revealed that these two reactions are either the same or nearly so: Base substitutions that affected the two-strand reaction had the same quantitative effect on the three-strand reaction, and, likewise, those substitutions that did not affect one reaction did not affect the other (figure 5) (for information on other substitutions, see Rao & Radding 1994). As the mutual recognition of two identical sequences occurs by non-Watson–Crick bonds, it follows that recognition of homology involving three strands must occur via the same or similar bonds, and hence that base triplets rather than Watson–Crick base pairs mediate the initial recognition in the three-strand reaction. Controls showed that similar base substitutions had no detectable effect on the pairing of complementary strands.

As illustrated in figure 6, the reaction of the RecA nucleoprotein filament with oligonucleotides can be written as a reversible reaction whose equilibrium lies to the right. In the experiments on the three-strand reaction cited here, we have studied the reverse reaction. To the extent that the reaction is freely reversible, the mechanisms of the reverse reaction should be the same as those of the forward reaction. Our interpretation that recognition in the reverse reaction reflects recognition in the forward reaction rests on the assumption of reversibility, which has been justified by preliminary experiments and is being studied in more detail (R. L. Bazemore and C. M. Radding, unpublished observations).

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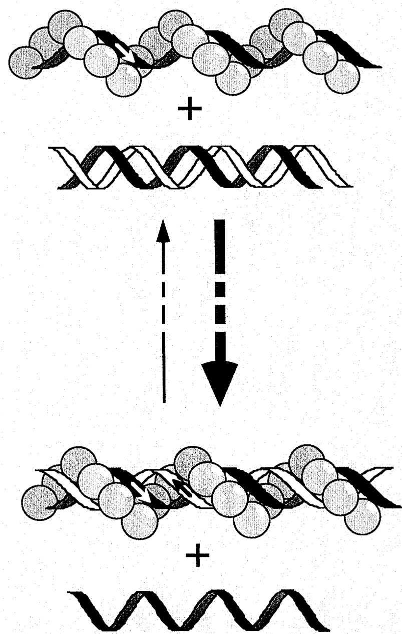

Figure 6. The reversibility of strand exchange when a duplex oligonucleotide is one of the substrates.

6. RECOGNITION VIA NOVEL TRIPLETS

On the basis of studies of deproteinized products of the RecA reaction, Rao & Radding proposed a model for homologous recognition via base triplets that was similar to several earlier proposals (figure 7) (Lacks 1966; Umlauf *et al.* 1990; Chiu *et al.* 1993; Rao *et al.* 1993). Such models, however, fail to account satisfactorily for self-recognition if the third complementary strand (C(−) in figure 7) is simply removed from the triplet. Recently, Zhurkin and colleagues pointed out that in such triplet schemes, there is a complementary pattern of partial charges, including the well-known charges that produce hydrogen bonds, and weaker charges as well (Zhurkin *et al.* 1994) (see figure 7); they specifically suggested that this pattern of complementary charges might constitute an electrostatic code for recognition. That complementary pattern is precisely the same for a two-strand versus a three-strand reaction, although major rotations are required to bring pairs of complementary charges into proximity (see figure 7). Thus in principle, the electrostatic code rationalizes the observed identity of the two- and three-strand reactions: but further chemical and physical studies are required to identify the bonds that mediate the formation of these base triplets and to determine the structure of the intermediate.

Three sets of observations now support the view that base triplets and non-Watson–Crick bonds mediate homologous recognition: These are the similar requirements and similar stereochemistry of the two-strand versus three-strand reactions (Rao & Radding 1993), the indistinguishable effects of base substitutions on both reactions (Rao & Radding 1994), and studies of a RecA mutant that promotes Watson–Crick pairing normally, but is partly defective both in self-recognition and recognition of homology in duplex DNA (Ogawa *et al.* 1992; Kurumizaka *et al.* 1994*a*, *b*).

7. THE PERMISSIVE NATURE OF RECOGNITION

Two properties that are common to both non-Watson–Crick self-recognition and the recognition of homology in duplex DNA reveal that the initial stage of recognition is not fastidious. The stereochemical specificity of these reactions was explored by pairing the usual RecA filaments with oligonucleotides in which the linear array of bases was the same, but the 5 to 3 orientation of the phosphodiester backbone was reversed. Surprisingly, this major stereochemical alteration had virtually no effect on homologous recognition, although it did completely eliminate any strand exchange (Rao & Radding 1993).

When, as described above, the specificity of the pairing reactions was examined by base substitutions, homologous sequences were distinguished from heterologous sequences, but the number of base substitutions required to eliminate the recognition of an otherwise homologous sequence was surprisingly large. In a 33 mer oligonucleotide, out of nine G residues, more than six had to be changed to A before homologous recognition was abolished (Rao & Radding 1994). At present, we can only speculate about the significance of the lack of stringency of homologous recognition. Perhaps reduced stringency permits a more rapid search, facilitating the location of the right sequence out of many. Previous observations have shown that strand exchange promoted by RecA protein is also very permissive (DasGupta & Radding 1982*a*, *b*, Livneh & Lehman 1982; Bianchi & Radding 1983; Hahn *et al.* 1988). Further studies with oligonucleotides are in progress to determine if strand exchange plays a separate role in discriminating homologous from heterologous sequences.

8. IMPLICATIONS FOR INTERCHROMOSOMAL COMMUNICATION

Several groups demonstrated that a segment of the RecA nucleoprotein filament containing duplex DNA can recognize homology in a completely duplex molecule (Conley & West 1990; Lindsley & Cox 1990; Chiu *et al.* 1990). In light of the experiments described above, it seems likely that non-Watson–Crick interactions also mediate such four-strand reactions.

In certain lower eukaryotes, there are striking instances of interchromosomal communication. In *S. cereviseae*, homologous genes that are located at non-allelic positions on the same or non-homologous chromosomes undergo gene conversion at rates that are similar to conversion between genes at allelic sites (Petes & Hill 1988; Haber *et al.* 1991). In *Neurospora crassa* and *Ascobolus immersus* repeated sequences, which may also be at ectopic sites, are inactivated prior to meiosis by extensive methylation or mutation (Faugeron *et al.* 1990; Selker 1990). In each of these cases, the data indicate that recognition occurs by

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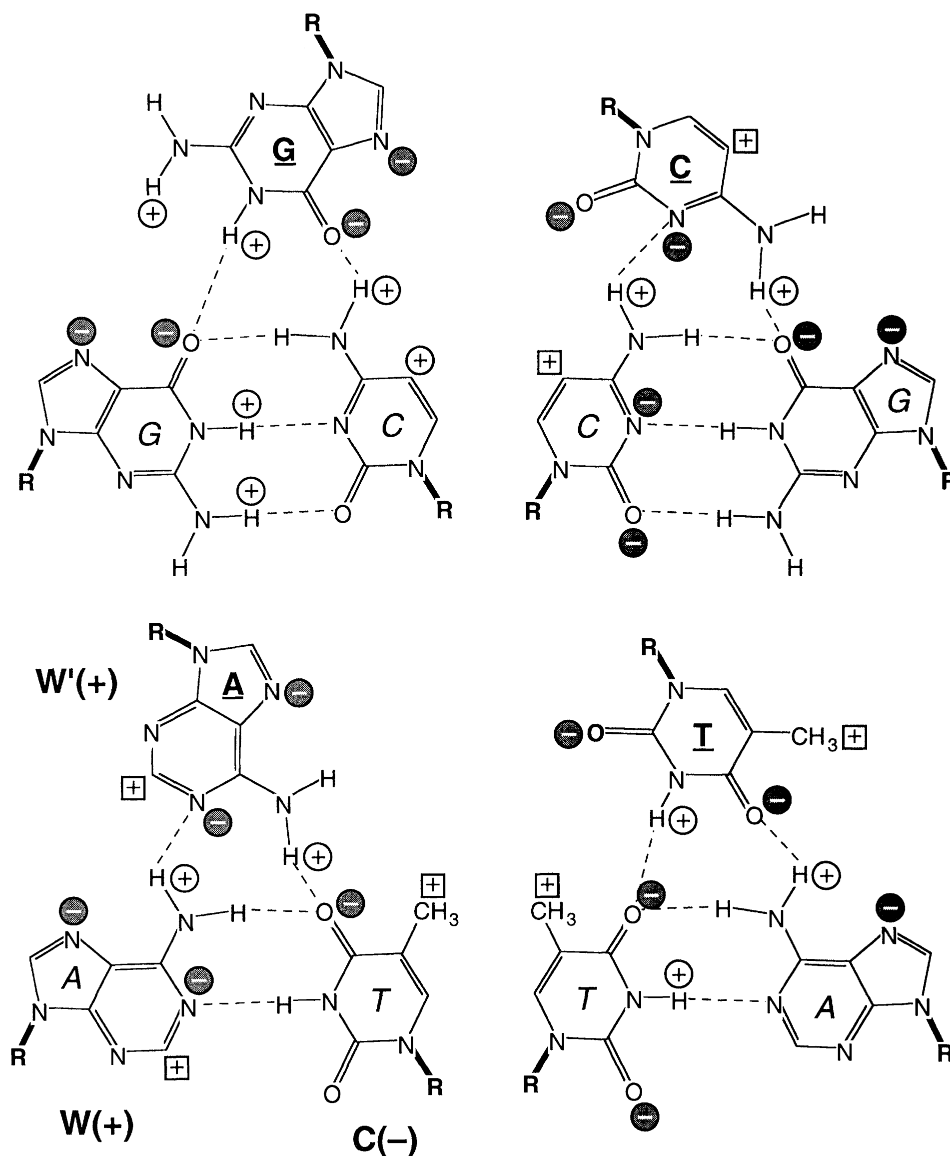

Figure 7. A base-triplet model for homologous recognition. W′(+) represents the base from incoming naked 33 mer oligonucleotide which is recognized by strands in the filament, either a single strand, W(+) (self-recognition in a two-strand reaction), or a pair of complementary strands, W(+):C(−) (triplet-recognition in a three-strand reaction). Charges in circles are stronger than those in squares (Renugopalakrishnan *et al.* 1971; Saenger 1984). This scheme was originally proposed to explain the effects of methylation of N-4 Cytosine and N-6 Adenine and the lack of effect of methylation of N-7 Guanine (Rao *et al.* 1993). The complementarity of partial charges suggested by Zhurkin *et al.* (1994) as an electrostatic recognition code, rationalizes the identity of two-strand and three-strand pairings as reported here. When the strand labelled C(−) is removed, the identical pattern of charge complementarity of a two-strand reaction with that of a three-strand reaction may be realized by either clockwise or counter-clockwise rotation of bases in W′(+). (Copyright permission from *Proc. natn. Acad. Sci. U.S.A.*, 1994.)

pairing of the interacting sequences even though they are not located near one another nor on homologous chromosomes, implying the existence of a rapid search that can scan the entire genome prior to the alignment of homologues later in meiosis. Recognition in these cases may involve non-Watson–Crick interactions of the kind that are promoted by RecA protein.

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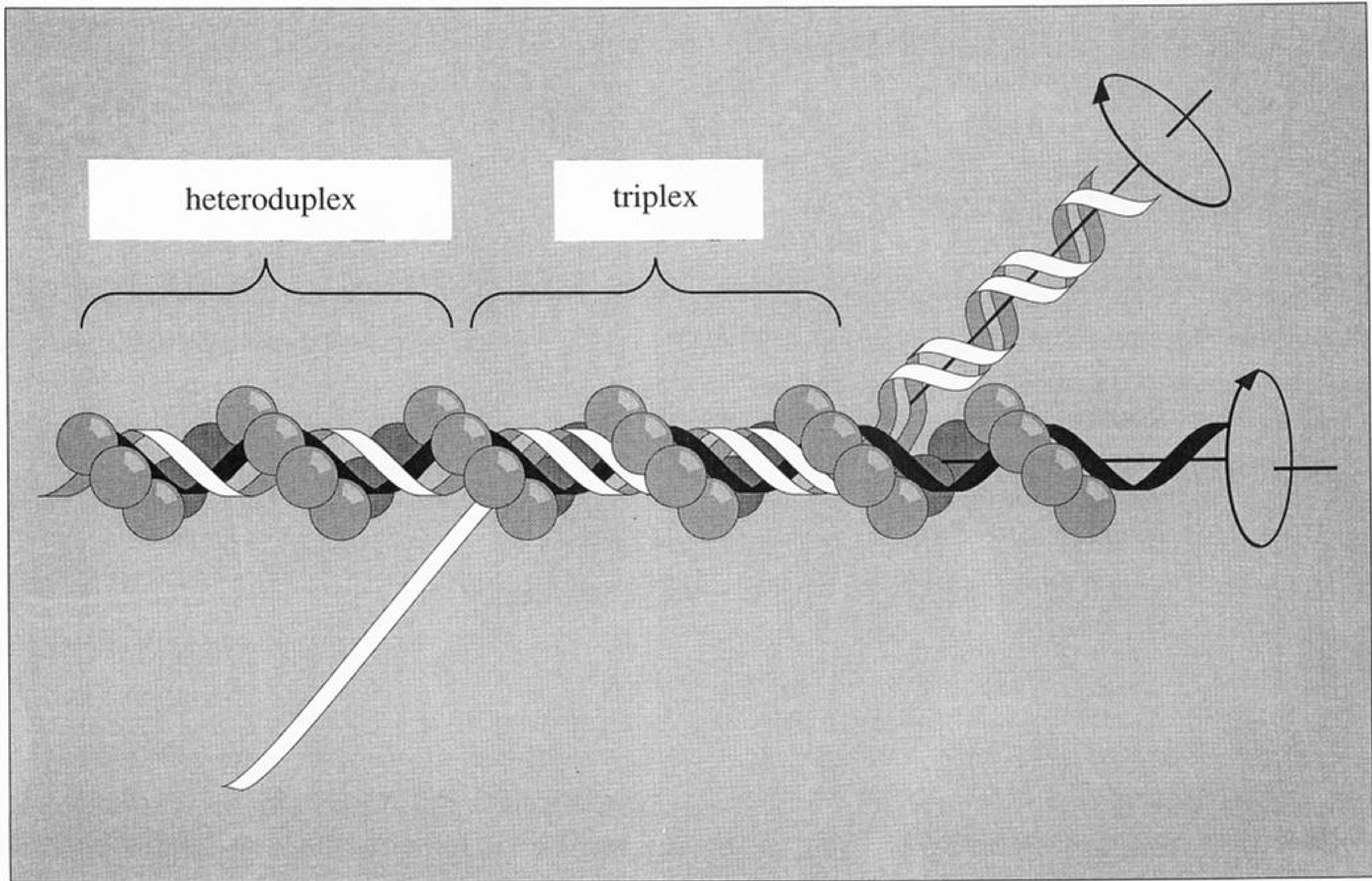

igure 1. A model of strand exchange promoted by RecA protein (Howard-Flanders *et al.* 1984; Radding 1991). *In tro*, the RecA nucleoprotein filament and duplex DNA meld to form a triplex intermediate, leading to exchange of ase pairs and the separation of one strand from the original duplex.

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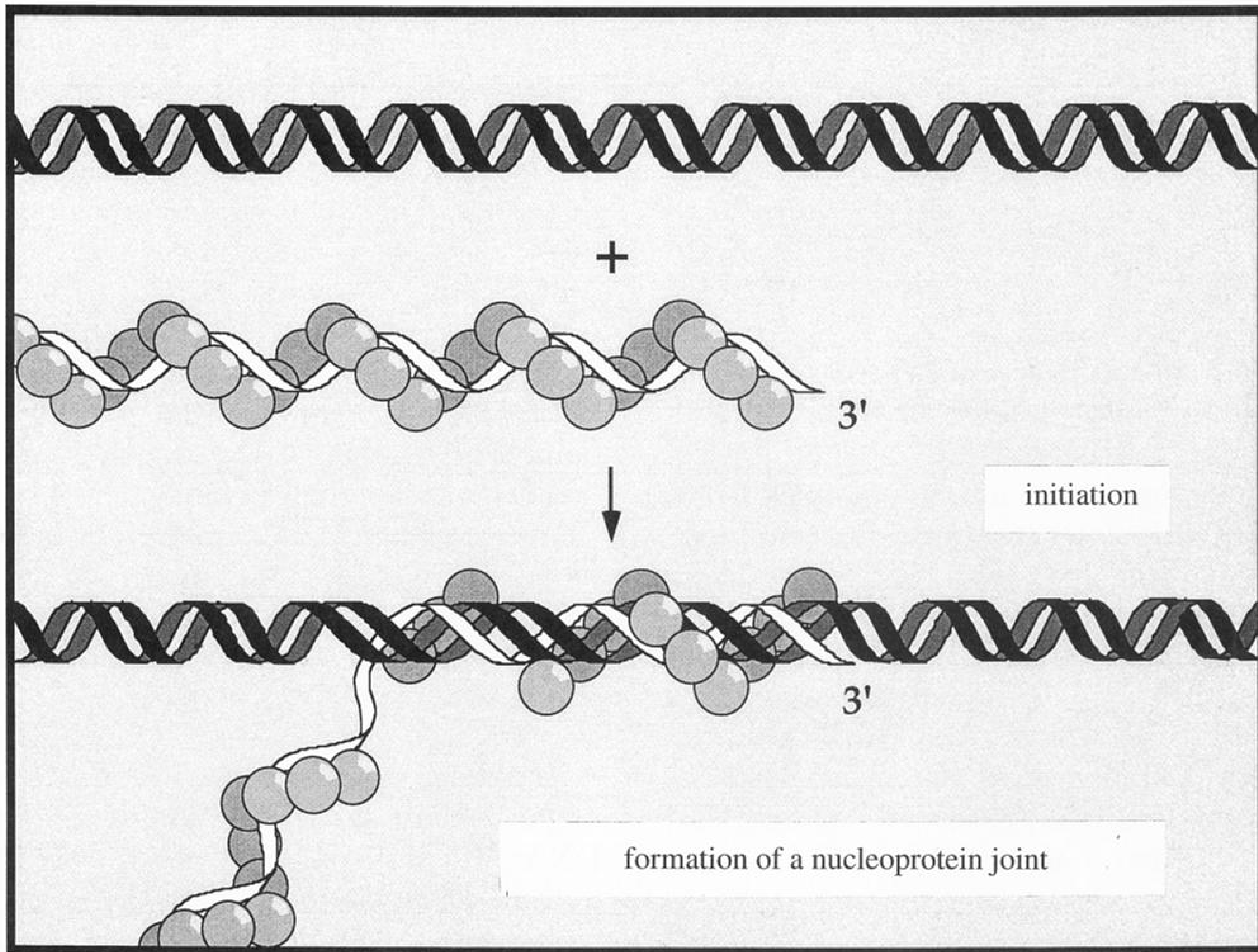

igure 2. Recognition of homology in duplex DNA by the RecA nucleoprotein filament.

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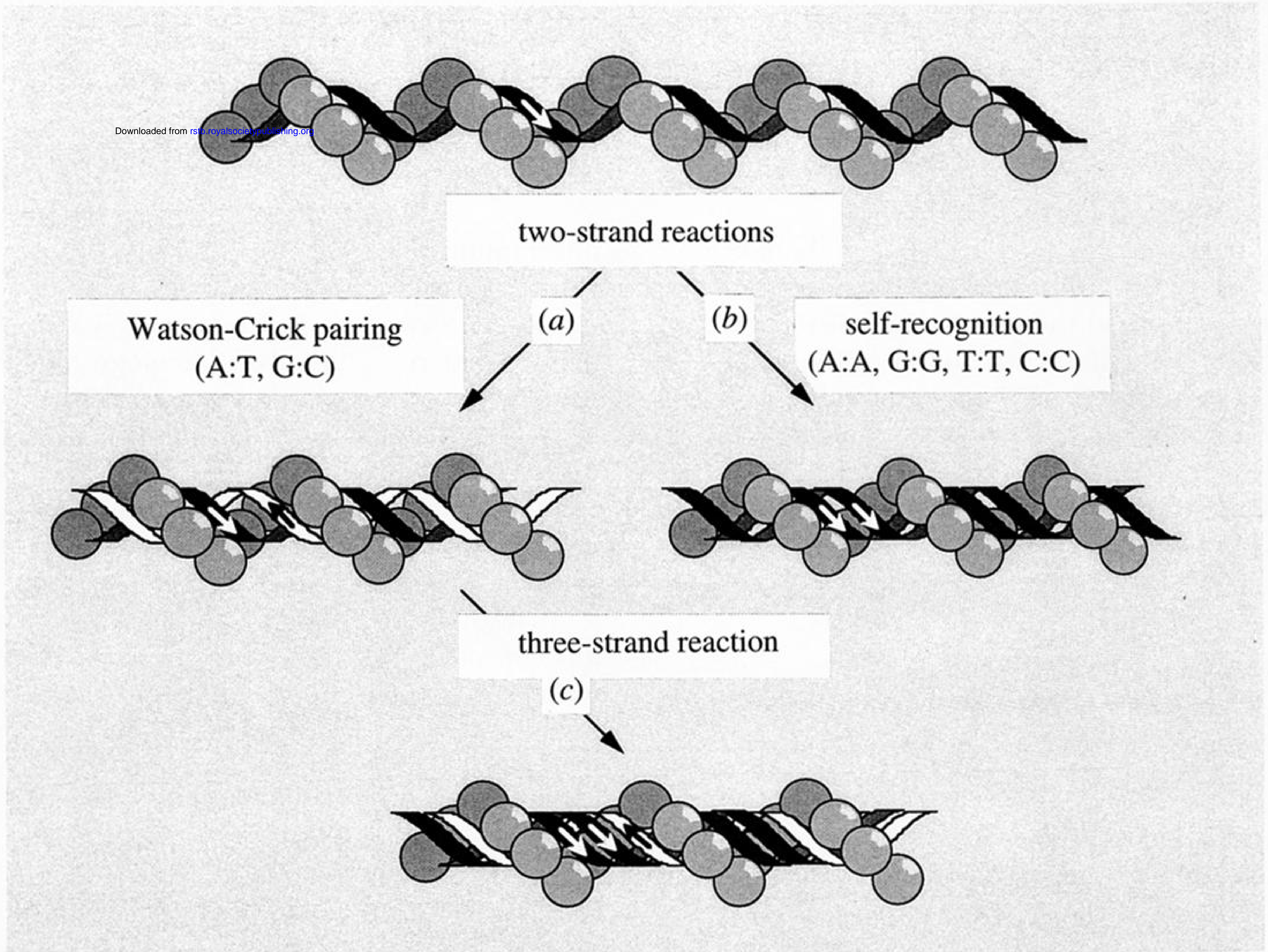

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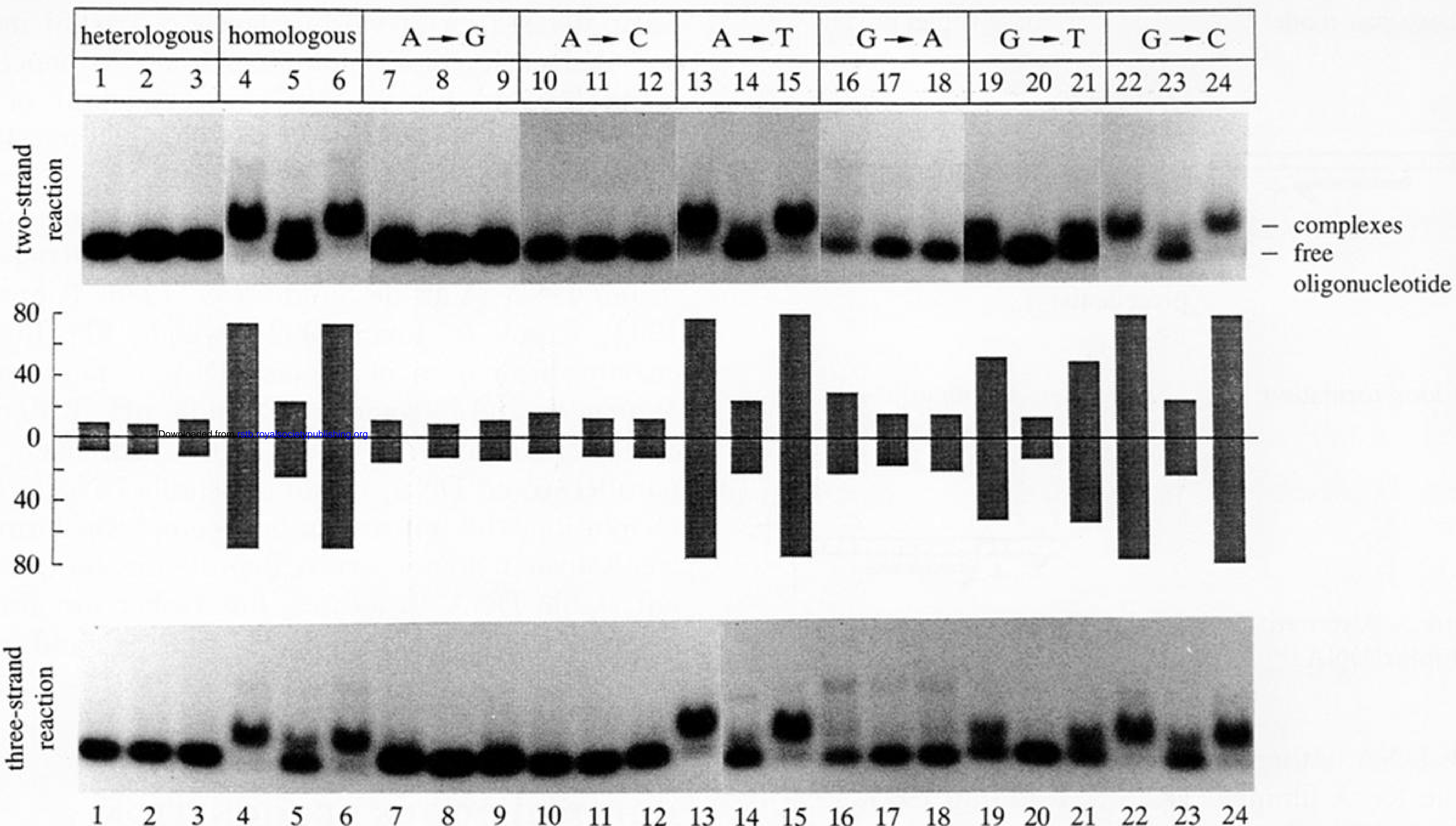
igure 4. Experimental models of homologous recognition in the RecA filament. (*a*) Watson–Crick pairing of a omplementary oligonucleotide with a single strand in the RecA filament; (*b*) Self-recognition of an oligonucleotide identical sequence; (*c*) Recognition of a third strand by a RecA filament that contains a pair of complementary rands.



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'igure 5. Comparison of the effects of base substitutions in an oligonucleotide on self-recognition (figure 4*b*) versus he recognition of that same oligonucleotide by duplex DNA in the RecA filament (figure 4*c*) (Rao & Radding 1994). 'he top of each panel shows the effects of base substitutions on the two-strand reaction, the bottom on the three-trand reaction. For the two-strand reaction, a RecA filament was formed on an 83 mer oligonucleotide in the resence of ATPγS; for the three-strand reaction, the filament was formed on the same 83 mer to which a omplementary 43 mer had been annealed. Pairing was initiated by adding 5′ labelled 33 mer homologous ligonucleotide, a homologous oligonucleotide carrying base substitutions, or a heterologous 33 mer oligonucleotide, n the presence of a 24-fold excess of heterologous unlabelled carrier oligonucleotide. Each pairing reaction was done n three ways: without any additional competitor (first lane in each set of three, e.g. lane 4), with a tenfold excess f unlabelled competitor of the same sequence as the labelled 33 mer (second lane in each set of three, e.g. lane 5), r with another heterologous competitor (third lane in each set of three, e.g. lane 6). Pairing was done for 6 min at 7 °C, the samples were analysed by a gel retardation assay and quantitated by scanning the gel (PhosphorImager, Aolecular Dynamics) (Rao & Radding 1993). The quantitative data from the three-strand reactions are plotted as nverted bars for ease of comparison with the two-strand reactions. Additional controls included: omission of RecA rotein, omission of ATPγS, and deproteinization of wild-type reactions with SDS and proteinase K as described Rao & Radding 1993), all of which resulted in no detectable pairing. (Copyright permission from *Proc. natn. Acad. Sci. U.S.A.*, 1994.)

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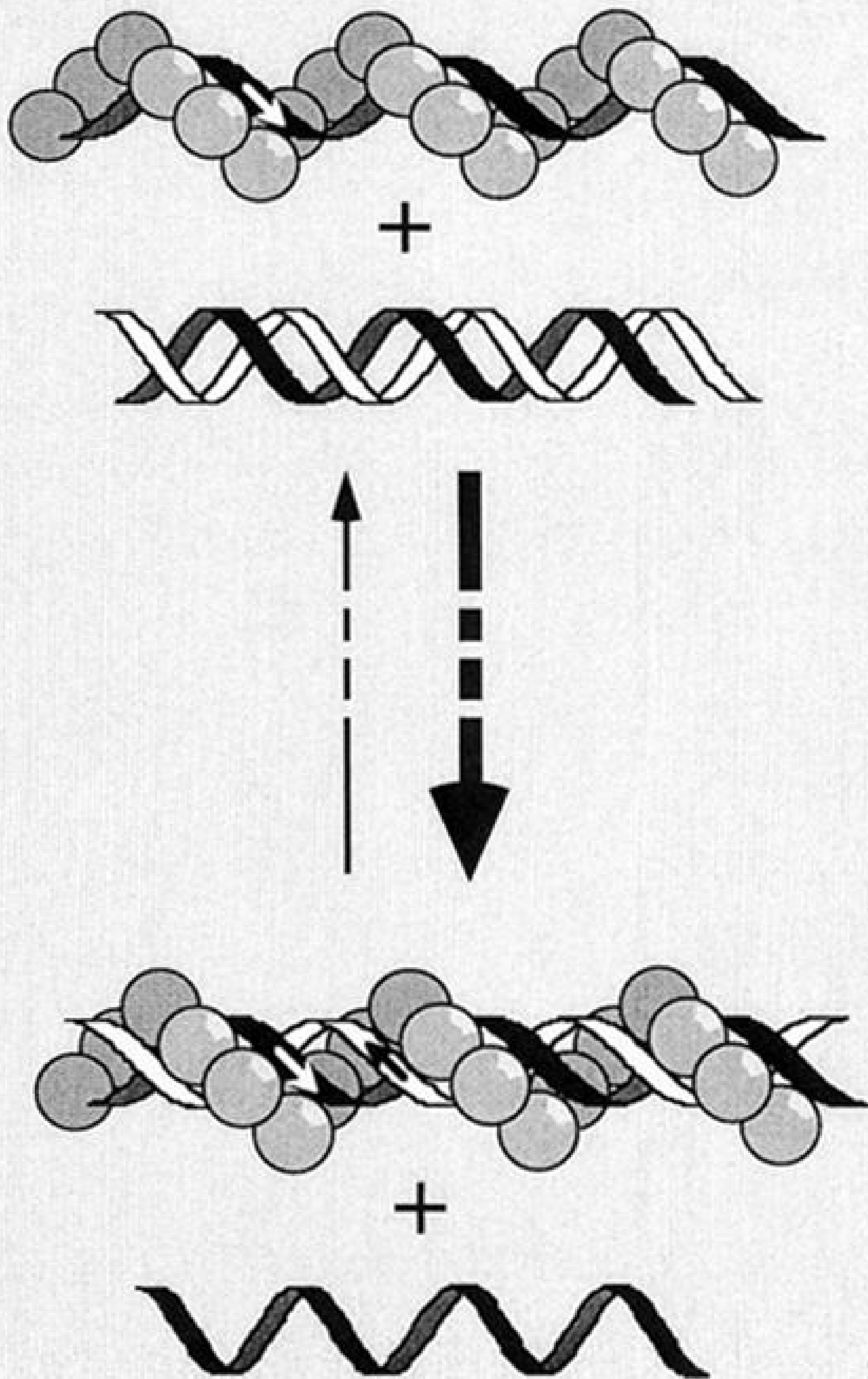

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gure 6. The reversibility of strand exchange when a duplex igonucleotide is one of the substrates.