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Processing of Holliday junctions by the *Escherichia coli* RuvA, RuvB, RuvC and RecG proteins

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Abstract. Recent work has led to significant advances in our understanding of the late steps of genetic recombination and the post-replicational repair of DNA. The RuvA and RuvB proteins have been shown to interact with recombination intermediates and catalyse the branch migration of Holliday junctions. Although both proteins are required for branch migration, each plays a defined role with RuvA acting as a specificity factor that directs RuvB (an ATPase) to the junction. The RuvB ATPase provides the motor for branch migration. The next step is catalysed by RuvC protein which recognises Holliday junctions and promotes their resolution by endonucleolytic cleavage. New data indicates an alternative pathway for Holliday junction processing. This pathway involves RecG, a branch migration protein which is functionally analogous to RuvAB, and a protein (activated by a *rus* mutation) which works with RecG to process intermediates independently of RuvA, RuvB and RuvC.
Key words. Recombination; DNA repair; ATPase; branch migration; resolution.

Introduction

E. coli RecA protein plays a central role in recombination and is responsible for homologous pairing and strand exchange leading to the formation of recombination intermediates in which interacting DNA helices are linked by a Holliday junction (for reviews see 7, 31, 46; Stasiak and Egelman, Kowalczykowski in this issue). Our understanding of the later steps of recombination advanced recently with the isolation of proteins that could specifically recognise and process recombination intermediates into recombinant DNA products. This review will describe the properties of these novel enzymes and provide an overview of the late steps of recombination in *E. coli*.

Branch migration of Holliday junctions – a catalysed process?

First proposed in 1964, the Holliday junction[11] is a central intermediate in homologous recombination and represents the point of strand exchange between homologous duplex DNA molecules. A model for the structure of the Holliday junction (fig. 1A) proposed that two parallel duplex DNA helices could be linked by crossed strands without disruption of base-pairing at the point of exchange[38]. To generate increasing lengths of heteroduplex DNA, the junction needs to be moved along DNA (fig. 1B), and theoretical studies led to the suggestion that heteroduplex formation occurs by rapid spontaneous branch migration of the Holliday junction, by a reaction which involves the rotation of two duplex DNA helices[23].

Early studies attempted to measure the speed of spontaneous branch migration in vitro. Figure-8 DNA molecules, formed in vivo during the growth of bacteriophage G4 DNA[41,45], were isolated and converted into X-structures by restriction digestion. Branch migration of the Holliday junction led to dissociation of the X-structures into linear monomers, as followed by electron microscopy[41] or agarose gel electrophoresis[45]. The X-structures were found to be unstable at 25 °C and dissociated even more rapidly at higher temperatures[41]. From the experimental data, the step rate of branch migration (the speed of movement of the Holliday junction across 1 base pair) was determined. The step rate was temperature-dependent and increased from 60 bp/sec at 10 °C to 10,000 bp/s at 30 °C[45]. The step rate does not directly translate into the speed of branch migration, since branch migration occurs by a

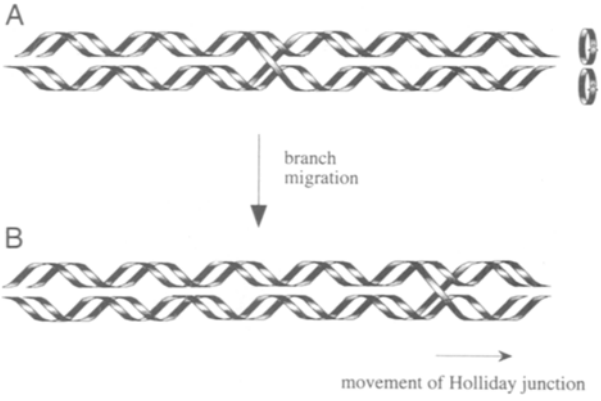

Figure 1. Model of a heteroduplex joint in which 2 duplexes are connected by a Holliday junction. Spontaneous branch migration of the Holliday junction (in the indicated direction) may involve the synchronous rotation of the 2 duplexes. Protein-mediated branch migration may require the separation of both duplexes followed by reannealing into heteroduplex DNA (not shown).

random walk, but allows a prediction of the probability of a junction moving a certain distance within a certain time (at 10,000 bp/s, a junction has a probability of 0.32 to migrate across 2.5 kilobases within 10 min[45]). Although these values were lower than earlier estimates[23], they were high enough to lead the authors to conclude that spontaneous branch migration is fast and accounts for the formation of heteroduplex DNA in vivo[41,45].

More recently these rates of spontaneous branch migration have been questioned. Using recombination intermediates made in vitro by *E. coli* RecA-mediated strand exchange (fig. 2A), we found that spontaneous branch migration was slow with little dissociation at physiological temperatures. Indeed, at 37 °C, >90% of the intermediates were still present after 6 hours of incubation, and at 72 °C, ≈30% of the intermediates were still present after 1 hour[24]. It is possible that Holliday junctions made in vitro by RecA are different from those made in vivo, though we believe this to be unlikely. Indeed, confirmation of the slow rate of branch migration has been provided in several recent studies using annealed structures containing Holliday junctions[14,28]. In the presence of Mg^{2+}, a joint molecule of 300 bp was found to be stable for >6 hours at 37 °C (I. G. Panyutin and P. Hsieh, pers. comm.). The rate of spontaneous branch migration is affected by buffer conditions with faster rates observed in the absence of divalent cations[14] (I. G. Panyutin and P. Hsieh, pers. comm.).

The influence of mismatches on branch migration has also been investigated. Using Holliday junctions formed by annealing short DNA fragments (<50 nucleotides), a single mismatch, insertion or deletion was found to be sufficient to impede spontaneous branch migration[28]. These results indicate that spontaneous branch migration occurs only over short stretches of DNA and is severely inhibited by non-homologous sequences. The reason for the discrepancy between the more recent observations and the earlier experiments is unknown, but may be due to the presence of contaminating protein activities in the early figure-8 preparations.

An important consequence of the low rate of spontaneous branch migration is that the process requires catalysis. Recently, two enzymes have been found that promote ATP-dependent branch migration: the *E. coli* RuvA and RuvB proteins, and the *E. coli* RecG protein.

RuvA and RuvB proteins

To investigate the roles of RuvA and RuvB during recombination, the two proteins were overexpressed in *E. coli* and purified to homogeneity[12,36,42]. In solution, the purified 22 kDa RuvA protein forms a tetramer[35,42] and the 37 kDa RuvB protein forms a dimer[35,42]. In the presence of Mg^{2+}, RuvA and RuvB interact to form a complex whose sedimentation coefficient is compatible with a composition of 1 RuvA tetramer and 1 RuvB dimer[35]. However, as described below, functional RuvB is likely to be a much larger complex. Since the two proteins exhibit independent properties yet interact to promote branch migration, we describe the properties of the proteins separately, followed by the reactions that they catalyse.

RuvA-DNA interactions

RuvA protein binds to single-stranded DNA, duplex DNA and, with higher affinity, to Holliday junctions. Initially, the interaction of RuvA with supercoiled plasmid DNA was analysed by gel retardation and nitrocellulose filter-binding assays[36]. However, RuvA binds Holliday junctions with a much greater affinity, as shown using synthetic Holliday junctions produced by annealing 4 oligonucleotides[13,29,30]. On the basis of the observation that RuvA forms a tetramer in solution[35,42], we assume that the RuvA-junction complexes consist of DNA bound by 1 or 2 tetrameric proteins (K. Hiom and S. C. West, unpubl. observ.). RuvA binds in a structure-specific manner since DNA-binding is sequence-independent. The protein can also bind Y-structures[20]. The interaction of RuvA with DNA does not require ATP and is most stable in the absence of divalent cations or at low Mg^{2+} concentrations[26,29].

RuvB-DNA interactions

Although under many conditions the interaction between RuvB and DNA is mediated by RuvA, RuvB alone will bind DNA in an ATP- and Mg^{2+}-dependent manner[26]. DNA binding occurs in the presence of ATP and is strongest in the presence of ATPγS, a non-hydrolysable ATP analogue. ADP cannot substitute for ATP or ATPγS. In contrast to RuvA, RuvB binding requires Mg^{2+} and is detected at $MgCl_2$ concentrations ≥10 mM[26]. In the presence of ATPγS, RuvB interacts weakly with single-stranded DNA. However, RuvB shows a greater affinity for duplex DNA and, in the presence of ATPγS, stoichiometric amounts of RuvB (1 RuvB monomer/6 nucleotides) protect the DNA from DNAse I digestion[26]. This result indicates that RuvB forms complexes with duplex DNA that shield the phosphodiester backbone of the DNA.

Of particular interest is the way in which RuvA targets RuvB to a Holliday junction, as seen by gel retardation or by junction-specific ATPase activity[30]. The role of RuvA may thus be that of a 'molecular matchmaker' whose role it is to deliver an ATPase to a site of action[32]. RuvAB-Holliday junction complexes were detected by gel retardation assays in the presence of non-hydrolysable nucleoside cofactors[30]. In these experiments, glutaraldehyde fixation was required to stabilise the complexes. However, more recent studies indicate that RuvAB-Holliday junction complexes are stable during glycerol gradient centrifugation without fixation

(K. Hiom and S. C. West, unpubl. observ.). Complex formation requires the presence of $MgCl_2$. These results indicate that the first step of branch migration involves RuvA directing RuvB to the Holliday junction, leading to the formation of a RuvAB-Holliday junction complex.

ATPase activity of RuvB

RuvB protein hydrolyses ATP, dATP, dCTP and TTP[12,30]. Although it can bind dGTP, it does not promote its hydrolysis. The ATPase activity of RuvB protein is stimulated by DNA[26,36]. At low Mg^{2+} concentrations (<10 mM $MgCl_2$), ATP hydrolysis is RuvA-dependent and occurs in the presence of single-stranded DNA, linear duplex DNA or circular duplex DNA[36]. These results are consistent with the proposal that RuvA-mediates the binding of RuvB to DNA, thereby leading to increased ATP hydrolysis by RuvB.

At Mg^{2+} concentrations that permit direct RuvB-DNA interactions (≥ 10 mM $MgCl_2$), the DNA-dependent ATPase of RuvB occurs in the absence of RuvA[26]. The ATPase activity is greatest with circular duplex DNA. The difference between linear and circular duplex DNA is particularly interesting since, at least in the presence of ATPγS, RuvB shows the same binding affinity for circular or linear DNA[26]. In view of the structure of RuvB, as described in a later section, it is possible that DNA binding by RuvB results in the formation of a functional RuvB complex which moves along the DNA at the expense of ATP hydrolysis. We assume that the low amount of ATP hydrolysis observed with linear DNA results from the dissociation of RuvB from DNA ends. The low ATPase observed with single-stranded circles most likely reflects the low affinity of RuvB for single-stranded DNA.

Branch migration mediated by RuvA and RuvB

In vitro, RuvA and RuvB promote ATP- and Mg^{2+}-dependent branch migration of Holliday junctions. The first indication of such a RuvAB-mediated reaction was the removal of a cruciform structure from supercoiled plasmid DNA[36]. Subsequently, branch migration was demonstrated directly using 2 types of DNA substrates: 1) recombination intermediates made by RecA-mediated strand exchange (fig. 2A)[25,43] or 2) model Holliday junctions formed by annealing 4 partially complementary oligonucleotides (fig. 2B)[13,29,30]. The use of these two alternative systems allowed us to determine some of the roles played by the two proteins during branch migration. As described above, RuvA and RuvAB are able to form specific complexes with Holliday junctions in vitro[29,30]. In the presence of Mg^{2+} and ATP, the combined action of RuvA and RuvB results in dissociation of the DNA by branch migration of the Holliday junction (fig. 2A and B). Branch migration is dependent upon the hydrolysis of a nucleoside triphosphate (ATP = dATP > dCTP > TTP)[30].

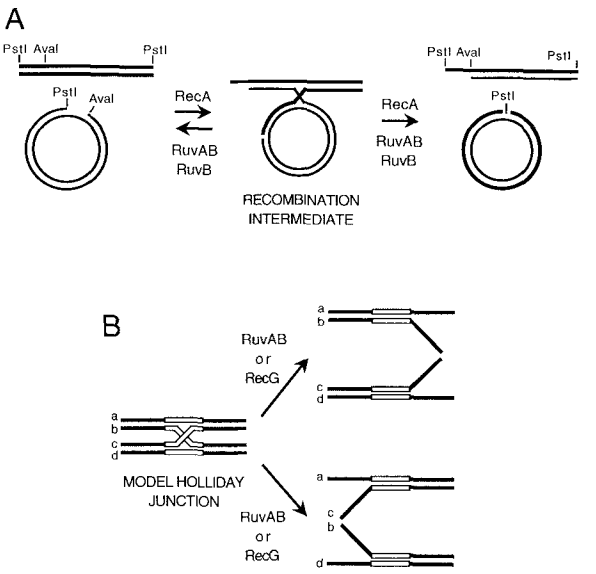

Figure 2. Model systems for studying branch migration. *A* Substrates, intermediates and products of RecA-mediated in vitro recombination between homologous duplex DNA molecules. The substrates, gapped and linear duplex DNA (left), are paired by RecA protein and strand exchange leads to the formation of recombination intermediates containing a Holliday junction (centre). The products of RecA-mediated strand exchange are gapped linear and nicked circular duplex DNA (right). Products can also be formed by the action of RuvA and RuvB on deproteinised recombination intermediates. In this case, however, branch migration goes forwards (to products) or backwards (to starting substrates). *B* Branch migration of synthetic Holliday junctions. Holliday junctions (left) are formed by annealing 4 partially complementary oligonucleotides (≈ 60 nucleotides in length). The junction shown here has a homologous core (white) and 4 heterologous arms. Branch migration by RuvAB or RecG occurs without polarity and leads to dissociation of the junction into the indicated pairs of DNA products (right).

Surprisingly large amounts of Ruv proteins are required for branch migration and the stoichiometry corresponds to about 20 RuvB monomers per synthetic Holliday junction (60 bp in length)[30]. The significance of this stoichiometry becomes clear in view of the structure of RuvB (as described in a later section). Branch migration occurs in both possible orientations and without any apparent bias or polarity (fig. 2B)[13,29]. In contrast, a Y-junction is dissociated in one direction only such that a unique single-strand of DNA is displaced[19] (I. R. Tsaneva and S. C. West, unpubl. observ.). The lack of polarity observed with a Holliday junction is presumably due to the two-fold symmetry of the junction.

In reactions with deproteinised recombination intermediates (fig. 2A), RuvB alone was found to promote branch migration (RuvB-mediated reaction)[25,43]. RuvB-mediated branch migration required saturating amounts of protein (≈ 1 RuvB monomer/6 nucleotides) and Mg^{2+} concentrations that permit direct DNA binding (≥ 10 mM $MgCl_2$)[25,44]. This result shows that RuvB (the ATPase) is the motor for branch migration. When

the RuvB concentration was lowered (or at <10 mM Mg^{2+}) the reaction showed an absolute requirement for RuvA (RuvAB-mediated reaction)[25]. These results confirm our proposal that RuvA acts as a molecular matchmaker since it reduces the requirement for RuvB by specifically targetting the protein to the Holliday junction. The minimal protein requirement for RuvA and RuvB in reactions with recombination intermediates (approx. 10,000 bp in length) was about 10 RuvA tetramers and 20 RuvB monomers per molecule of DNA[25]. Branch migration occurred in both possible directions (fig. 2A) with an estimated speed of 10–20 bp/sec[43], faster than the speed of RecA-mediated strand exchange. RuvAB- and RuvB-mediated branch migration reactions were kinetically similar[25]. In contrast to reactions with model Holliday junctions, no junction-specific stimulation of the RuvB ATPase was observed either in RuvB- or RuvAB-mediated reactions[25], probably due to the excess of duplex DNA over Holliday junctions in these reactions.

Sequence comparisons show that RuvB protein contains conserved RNA/DNA helicase motifs[19,44] and led us to test for DNA helicase activity in vitro. Using short fragments (52–558 nucleotides) annealed to circular single-stranded DNA, we observed an intrinsic DNA helicase activity in a reaction that required both RuvA and RuvB proteins[44]. The dissociation reaction occurs with a unique polarity ($5' \rightarrow 3'$ relative to the single-stranded DNA) and requires ATP and Mg^{2+} (ref. 44). In view of the DNA helicase activity, an attractive possibility is that RuvAB-mediated branch migration involves the simultaneous melting of 2 duplex DNA molecules at the Holliday junction, coupled with DNA reannealing into heteroduplex DNA.

Visualisation of RuvB protein

The RuvB protein has been visualised by electron microscopy following negative staining with uranyl acetate. In the presence of ATP and 15 mM Mg^{2+}, the protein was found to bind relaxed circular duplex DNA to form doublet ring-like structures (A. Stasiak, E. H. Egelman and S. C. West, unpubl. observ.). Preliminary structural analysis indicates multiple subunits per ring. The doublet rings have been cylindrically averaged and three-dimensional image reconstructions indicate that they are bipolar with a deep hollow core through which the DNA may lie.

RecG protein

The *E. coli* RecG protein appears to be functionally similar to RuvAB[20]. The 76 kDa protein has been overproduced and purified[19]. It has a DNA-dependent ATPase activity that is active on single-stranded, linear or supercoiled duplex DNA[19]. Like RuvA, RecG shows a high affinity for synthetic Holliday junctions and forms specific complexes that have been detected by gel retardation[19]. Moreover, in the presence of Mg^{2+} and ATP, Holliday junctions are dissociated by RecG-mediated branch migration to form products identical to those formed by RuvAB[19,20]. The amino acid sequence of RecG shows the presence of RNA/DNA helicase motifs, and it is likely that RecG-mediated branch migration also occurs by a helicase-like mechanism[20]. However, at the present time, classical DNA helicase activity has not been demonstrated with this protein. Thus, RecG protein, similar to RuvAB, binds to Holliday junctions and then dissociates the Holliday junctions by branch migration. The genetics and biochemistry of RuvAB and RecG suggest that they may provide analogous functions within the cell[16,20].

Resolution of Holliday junctions

Following branch migration, genetic recombination requires the resolution of Holliday junctions. To detect resolvase activity, *E. coli* extracts were fractionated to reveal an activity that resolved recombination intermediates made by RecA[6]. This resolvase activity was shown to be the product of the *ruvC* gene[5].

RuvC protein

The purified 19 kDa RuvC protein resolves Holliday junctions in vitro[9]. An improved purification scheme for RuvC has recently been developed[10] and the specificity[3] and mechanics[1] of the Holliday junction resolution reaction has been investigated. DNA binding studies indicate that recognition of a Holliday junction by RuvC protein is structure-specific and occurs in a manner that is independent of divalent cations[1]. This result is interesting since divalent cations play an important role in the folding of a synthetic Holliday junction leading to the formation of a stacked X-structure[8]. In the absence of metal ions, phosphate-phosphate repulsion leads to the disruption of helical stacking interactions such that the junction adopts a more extended (square planar) structure. Our results indicate that RuvC-Holliday junction interactions occur with similar efficiencies at 0 or 500 μM Mg^{2+} (ref. 1), whereas concentrations in excess of 80 μM are required for junction folding[8]. Binding by the resolvase therefore appears to be independent of the state of folding of the junction, or alternatively, the protein may determine the conformation of the junction upon binding.

Whereas junction-binding is independent of cofactors, resolution is strictly dependent upon the presence of Mg^{2+} (ref. 1) or Mn^{2+} (R. Shah, R. J. Bennett and S. C. West, unpubl. observ.). The cation is required for nucleolytic activity rather than DNA folding, since it cannot be replaced by other divalent cations such as Ca^{2+} or Zn^{2+}. Resolution occurs via the intro-

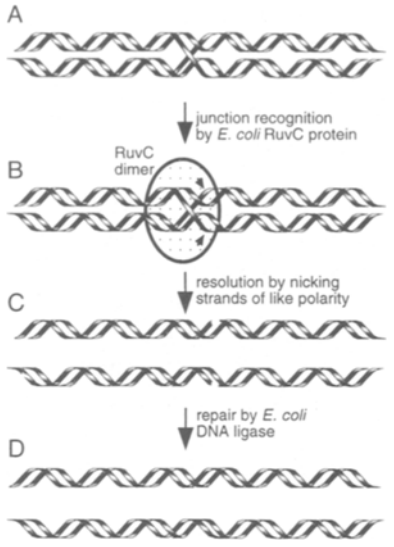

Figure 3. Resolution of a Holliday junction by RuvC. *A* Holliday junction is specifically recognized by RuvC protein. *B* Recognition occurs in a structure-specific manner to form a RuvC-Holliday junction complex. *C* Resolution occurs at specific DNA sequences (3′-side of T) by a dual incision mechanism in which nicks are introduced into two strands of like polarity. *D* The resulting nicked duplex products separate and the nicks are sealed by DNA ligase.

duction of symmetrically opposed nicks into two strands of like polarity (fig. 3), and the products of resolution, 2 nicked duplex DNA molecules, can be repaired in vitro by *E. coli* DNA ligase[1,10]. It is likely that the combined action of RuvC and DNA ligase promote Holliday junction resolution and repair within the cell.

Although binding of a Holliday junction by RuvC is structure-specific, the incision reaction exhibits sequence specificity with the cuts introduced at the 3′-side of thymine residues[1]. The sequence specificity may in part account for the inability of RuvC to cleave junctions without homology[1,9]. For example, the presence of homologous sequences provides perfect symmetry (i.e. opposing thymines) and also allows for branch migration, which may be necessary for the correct positioning of thymines relative to the junction and within the active site of the protein.

Hydroxyl radical footprinting studies show that DNA within the RuvC-Holliday junction complex is distorted such that 2 of the 4 DNA strands become hypersensitive to attack[1]. This may be the result of a local distortion, or bend, of the DNA backbone, since altered sensitivity to hydroxyl radicals has been used as a probe to detect DNA bending[4]. The observed hypersensitivity does not result from RuvC binding per se, but requires the presence of a chelatable metal ion, as demonstrated by inhibition of radical sensitivity (but not RuvC binding) by EDTA[1]. Indeed, the ionic requirements of binding, hydroxyl radical sensitivity and strand cleavage indicate 3 distinct steps in the mechanism of RuvC-mediated Holliday junction resolution – structure-specific recognition, DNA distortion and sequence-dependent cleavage.

The specificity of RuvC for DNA junctions has been compared with the bacteriophage resolvase, T4 endonuclease VII[3]. We found that RuvC protein cleaves 3- or 4-stranded junctions that model recombination intermediates, but is inactive on Y-junctions, DNA containing mismatches or heteroduplex loops, or linear single- or double-stranded DNA. This result contrasts with T4 endonuclease VII which shows broad-range specificity and reflects the specialized role that RuvC plays in recombination and recombinational repair, as opposed to the general repair role that the bacteriophage enzyme performs during DNA packaging[15].

Genetic evidence for Holliday junction processing by RuvA, RuvB, RuvC and RecG

A role for the Ruv proteins in the resolution of Holliday junctions is consistent with the genetic phenotype of *ruv* mutants, which show defects in recombination and DNA repair[17,18,21,27,39]. The *ruv* genes map at minute 41 on the *E. coli* chromosome and are present in 2 operons[33]. The *ruvA* and *ruvB* genes form an operon that is regulated by LexA as part of the SOS-inducible DNA repair response[2,37]. The *ruvC* gene forms a separate operon with a gene (*orf-26*) that encodes a 26 kDa protein of unknown function[33,34,40]. The *ruvC* gene is transcribed independently of *ruvAB* and is not under direct SOS control[34,40].

Although *ruv* and *recG* single mutants are only slightly defective in recombination (30–50% of the normal yield of recombinants are obtained in genetic crosses), *ruv recG* double mutants show a substantial recombination-defective phenotype[16]. In the case of *ruvAB* and *recG* this result is expected due to the functional overlap between RuvAB and RecG in their role as branch migration proteins. However, the genetic overlap also extends to *ruvC* despite the fact that there is no evidence to suggest that RecG protein can cleave Holliday junctions in the absence of RuvC[19]. These results provide new insight into the mechanics of Holliday junction processing. Firstly, they suggest a direct interaction between the RuvA, RuvB and RuvC proteins, and secondly, they indicate that *E. coli* may contain a second Holliday junction resolvase in addition to RuvC. Recently, a supressor of *ruv* was obtained and found to be dependent upon *recG*[22]. This mutation has been provisionally designated *rus* (ruv supressor) and appears to activate a protein that works with RecG[22]. Further studies of this suppressor will help elucidate whether the pronounced recombination deficiency observed in *ruv recG* strains is the direct consequence of eliminating both systems for Holliday junction resolution in *E. coli*.

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