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The search for a human Holliday junction resolvase

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Abstract

Four-way DNA intermediates, known as Holliday junctions, are formed during mitotic and meiotic recombination, and their efficient resolution is essential for proper chromosome segregation. Bacteria, bacteriophages and archaea promote Holliday junction resolution by the introduction of symmetrically related nicks across the junction, in reactions mediated by Holliday junction resolvases. In 2008, after a search that lasted almost 20 years, a Holliday junction resolvase was identified in humans. The protein, GEN1, was identified using MS following the brute-force fractionation of extracts prepared from human cells grown in tissue culture. GEN1 fits the paradigm developed from studies of prokaryotic Holliday junction resolvases, in that it specifically recognizes junctions and resolves them using a mechanism similar to that exhibited by the *Escherichia coli* RuvC protein.

Keywords

GEN1; Holliday junction; resolvase; xeroderma pigmentosum complementation group G (XPG)

Introduction

Early models for meiotic recombination predicted the formation of a crossed-strand exchange, or Holliday structure, as an intermediate that linked two synapsed DNA molecules [1]. Such a structure provided a relatively simple explanation for a variety of gene-conversion events that were observed during meiosis in fungi, and DNA forms resembling Holliday intermediates were subsequently visualized by electron microscopy. The mechanism by which these intermediates are dealt with by the cell to enable the separation of intact chromosomes, a process that is essential for chromosome segregation, has been a topic of interest ever since. The present article summarizes some of the historically important steps in the search for enzymes that resolve Holliday junctions, and, in particular, focuses on the identification of the long sought-after human Holliday junction resolvase GEN1.

A brief history of the Holliday junction

In 1964, Robin Holliday at the John Innes Institute in the U.K., was interested in the mechanisms of recombination and repair that took place in the smut fungus *Ustilago* and the budding yeast *Saccharomyces cerevisiae*. To account for the genetic characteristics of spores formed in lower eukaryotes, he proposed a model for recombination that provided a molecular basis for both gene conversion and crossing over (i.e. genes originally linked on the same chromosome could segregate from each other) [1]. The Holliday model suggested that, after DNA replication, which generates two copies of each of the two heterozygous alleles, recombination is initiated by the introduction of nicks at the same position in two DNA molecules with different alleles. The breaks were thought to allow single strands from one interacting DNA to anneal with complementary sequences in the other duplex. The switching of strands between DNA molecules results in the formation of a cross-stranded structure that physically links the two interacting DNA molecules. The structure at the point of strand exchange later became known as a Holliday junction, and is embedded in history as a central intermediate in the process of homologous recombination.

A critical aspect of the Holliday model invokes cutting (or 'resolution') of the crossover so that the two DNA helices can separate. Owing to the symmetry of the junction, it was proposed that there might be two possible orientations of resolution, each with a different outcome. If the breaks are introduced into the strands that are complementary to the initiating nicks, the arms flanking the junction are crossed over. In contrast, cutting of the other pair of strands results in recombination without crossing over. In both cases, however, the strand exchange produces heteroduplex DNA containing mismatched nucleotides. Holliday postulated that mechanisms must exist to repair these mismatches, a suggestion that was later proved to be true when the enzymes of mismatch repair were discovered [2]. A few years after Holliday's proposal of a model for recombination, electron-microscopy studies provided physical evidence for the existence of crossed-stranded intermediates during the recombination of bacteriophages S13 and ØX174 in *Escherichia coli* [3-5], and in yeast during the recombination of 2µ plasmid DNA [6].

Although Holliday's proposals for the mechanism by which recombination is initiated were not quite correct (we now know that recombination generally initiates at double-strand breaks in DNA), his model was important for driving forward our understanding of how recombinants might arise. In the 1980s, major advances were seen with the identification, purification and characterization of key recombination proteins from *E. coli* [7-9]. Most importantly, studies with purified RecA protein (the *E. coli* recombinase that promotes homologous pairing and strand exchange) showed that this protein alone could generate Holliday junctions *in vitro* [10,11]. The concept of the Holliday junction was, by this time, well accepted by the scientific community, and it became embraced as a key intermediate in recombination.

Holliday junction structure

In 1983, Ned Seeman and his colleagues described the formation of a synthetic Holliday junction simply by annealing four partially complementary oligonucleotides [12]. Although

the importance of these structures was perhaps not fully appreciated at the time, their formation was actually a defining moment that enabled workers in the field to determine the three-dimensional structure of the junction, as well as to develop simple assays for their enzymatic resolution. But initial studies of Holliday junction structure were both surprising and controversial. Gel electrophoresis and FRET (fluorescent resonance energy transfer) analyses (a technique that measures the distance between the excited states of two fluorescent dyes) revealed that the four-way junction could exist in a variety of structures, none of which resembled the expected Holliday junction structure in which the two interlinked DNA helices were aligned parallel to each other. Controversially, in the presence of a bivalent metal ion such as Mg^{2+} , the junction appeared to adopt a 2-fold-symmetric X-shape in which the DNA helices lie antiparallel to each other [13-15] (Figure 1A). In this antiparallel orientation, the exchanging strands did not cross over each other. The torsional angle of the sugar-phosphate backbone of the DNA at the point of exchange adapts a *gauche* conformation, instead of the *trans* conformation which is found in normal duplex DNA, allowing it to bend into a U shape and pair with the complementary strand running in the opposite direction. But this was no artefact, as the antiparallel structure of the Holliday junction was later confirmed and extended by X-ray crystallographic analyses [16-18].

In contrast with the antiparallel X-structure observed in the presence of bivalent cations, an unstacked 4-fold-symmetric square-planar structure was observed in the absence of ions. These differences indicate that the metal ions serve to neutralize electrostatic repulsions caused by the phosphates in the DNA backbone at the point of the crossover [19]. In a biological sense, it is possible that this unstacked structure might be the more physiologically relevant form of the junction, because enzymes that bind Holliday junctions and catalyse the key reactions that are involved in junction processing bind and stabilize the more open planar structure.

Holliday junction resolvases

One logical prediction of the Holliday model, which first invoked the formation of the four-way junction, is that there must be some way for the cell to resolve these crossovers to allow chromosome segregation. But it was not until 1982 that biochemical evidence suggested that there might be specific enzymes that could target and cut such structures. However, the first enzyme was not a cellular enzyme, as it was encoded by a bacteriophage. The phage was T4 and the protein, endonuclease VII, was the product of gene 49. In pioneering work, Kiyoshi Mizuuchi et al. [20] showed that T4 endonuclease VII cut Holliday junctions in a structure-specific manner, and that this reaction was critically important for the formation of unit-sized genomes that could be packed into phage heads. Subsequently, a similar, but somewhat more promiscuous, nuclease was identified from bacteriophage T7 [21].

By this time, the search for cellular Holliday junction resolvases was becoming an intensive area of research and Richard Kolodner's group were among the first to detect resolution activities catalysed by cell-free extracts from *S. cerevisiae* [22,23]. This endonuclease was subsequently identified as Cce1, but it was something of a surprise that this activity turned out to be a mitochondrial, rather than a nuclear, resolvase [24]. Subsequently, a similar

mitochondrial Holliday junction resolvase was identified from *Schizosaccharomyces pombe* and is known as Ydc2 [25,26].

The first cellular Holliday junction resolvase to be described was the RuvC protein from *E. coli*. By fractionating cell-free extracts, Bernadette Connolly in my group detected Holliday junction resolvase activity [27], and, in collaboration with Robert Lloyd's laboratory, we discovered that the activity was the product of the *ruvC* gene [28,29]. Soon, the protein was purified, and it was possible to begin the characterization of the first cellular Holliday junction resolvase [30]. These were exciting times, and our studies were carried out in parallel with those by Hideo Shinagawa and his colleagues in Japan [31,32].

The mechanism of Holliday junction resolution by RuvC can be broken down into a number of experimentally separable steps, including DNA binding, modification of DNA structure and cleavage [33]. RuvC binds specifically to a Holliday junction as a dimer and unfolds the junction into an open-planar configuration (Figure 1B). In the presence of bivalent cations, the resolvase introduces symmetrical nicks in strands of the same polarity. Although the protein binds junctions in a structure-specific manner, the cleavage reaction exhibits an extra level of selectivity at the sequence level, such that the degenerate sequence 5'-A/TTT↓G/C-3' is preferred over other sequences. The mechanism of resolution is similar to that exhibited by T4 endonuclease VII, T7 endonuclease I, CceI and Holliday junction resolvases subsequently identified in archaea [34,35]. Collectively, the resolvases recognize Holliday junctions and cut them by the introduction of symmetrically positioned nicks in strands with the same polarity to form nicked duplex products that can be repaired in a simple nick-re-ligation reaction.

Eukaryotic resolvases and mechanisms of resolution

The quest for nuclear resolvases in both yeast and mammalian cells has been an arduous task (Figure 2). However, a resolvase activity that fits the RuvC paradigm (symmetrical cleavage that leaves ligatable nicks) was detected in 1990 during the fractionation of cell-free extracts prepared from homogenized calf-thymus tissues [36]. The same activity was subsequently seen in extracts prepared from cells grown in tissue culture and shown to be nuclear rather than mitochondrial [37,38]. However, because of its low abundance, the gene encoding this resolvase remained unknown for many years.

The difficulty in identifying this gene led some to wonder whether eukaryotic cells really had a true RuvC-like resolvase, and, indeed, the generality of this mechanism of resolution was thrown into question by the realization that eukaryotes had other ways in which to resolve Holliday junctions (Figure 3). In particular, it was shown that *S. pombe* mutants defective in Mus81–Eme1, a heterodimeric 5'-flap endonuclease, exhibit very pronounced meiotic defects consistent with problems in the processing of recombination intermediates. Because the meiotic phenotype could be partially rescued by ectopic expression of the bacterial Holliday junction resolvase, these observations led to view that Mus81–Eme1 is the *S. pombe* Holliday junction resolvase [39]. However, *in vitro* analysis of the fission yeast protein indicated that recombinant purified Mus81–Eme1 was quite poor at cutting Holliday junctions, and instead preferred D-loops and nicked junctions, so it remained a possibility

that these structures more truly reflect the DNA species that are cleaved during meiotic recombination [40,41]. Similar substrate specificities have been observed with purified *S. cerevisiae* Mus81-Mms4 (Mms4 is the orthologue of Eme1) and with human Mus81–Eme1 [42,43]. Surprisingly, the meiosis-defective phenotype seen in *S. pombe* differs from that which is seen in other organisms [44], as severe defects in crossover formation are not observed in *S. cerevisiae* *mus81* mutants, indicating that Mus81-Mms4 do not provide the only mechanism for Holliday junction resolution and crossover generation [45,46]. *Mus81*-knockout mice exhibit minor meiotic defects, so it is unlikely that MUS81-EME1 makes a primary contribution to meiotic recombination in vertebrates [47,48]. Whether or not they play a role in mitotic recombination remains to be elucidated, as one report has shown that RNA interference-mediated depletion of MUS81 in human somatic cells caused a 2-fold reduction in the frequency of mitotic recombination [49], whereas another indicates that mouse *Eme1*^{-/-} embryonic stem cells do not display any recombination defects [50].

In recent years, it has also become apparent that Holliday junctions can be dissociated by yet another pathway that is present in eukaryotic cells, in this case mediated by BLM (Bloom's syndrome protein) in combination with topoisomerase III α and RMI1 (formerly known as BLAP75) [51-53]. In this case, the demise of the crossover is catalysed by BLM-driven junction migration followed by topoisomerase-mediated decatenation in a reaction that is defined as Holliday junction dissolution rather than resolution (Figure 3). As Holliday junction dissolution by BLM and topoisomerase III α gives rise to non-crossover products, the description of this activity provides a satisfying explanation for the elevated levels of crossovers that are observed in cells carrying *BLM* mutations. The *S. cerevisiae* homologues Sgs1 and Top3 promote similar reactions in yeast [54], so in some situations (probably when recombination occurs at blocked replication forks), this system appears to provide an alternative pathway to process Holliday junctions.

The search for RuvC-like resolvases in eukaryotic cells

Even though the Holliday junction resolvases from different species are functionally conserved, they bear little similarity to each other at the level of amino acid sequence. This makes it particularly difficult to use sequence homology and database analyses to identify related nucleases from higher organisms. The lack of primary sequence conservation indicates that they are unlikely to have evolved from the same ancestral gene, which makes us wonder why the Holliday junction resolvases, which act in highly conserved recombination/repair processes, are so evolutionarily diverse?

When the crystal structures of various Holliday junction resolvases were solved, it became clear that most belong to two families: the integrase superfamily (*E. coli* RuvC and *S. pombe* Ydc2) or the nuclease superfamily (T7 endonuclease I and archaeal Hjc) [55-60]. So, even though the resolvases are not conserved at the amino acid level, there are some similarities at the structural level. By contrast, T4 endonuclease VII and *E. coli* RusA (a cryptic resolvase that is phage-derived) seem to have evolved independently. So exactly what the Holliday junction resolvases have in common remains a difficult question to answer, as the only obvious feature is a catalytic domain containing of clusters of basic aspartate and glutamate residues that are required for metal ion binding.

Without clues from structural studies or genetics, we have, for several years, used a brute-force biochemical approach to try to identify the human Holliday junction resolvase known as ResA. Our studies were more recently complemented by carrying out a screen for a resolvase activity in *S. cerevisiae*, using a library of strains in which each gene is TAP (tandem affinity purification)-tagged and expressed from its endogenous locus. Remarkably, both approaches led us to the same protein, and the identification of this eukaryotic resolvase is a great credit to the perseverance and skills of three young postdocs: Stephen Ip, Uli Rass and Miguel Gonzalez Blanco [61].

To identify ResA, nuclear extracts prepared from large volumes of HeLa cells were assayed for Holliday junction resolvase activity using small radiolabelled synthetic junctions as the substrate (Figure 4A). Dependent upon the use of particular column matrices, it was possible to develop purification schemes that selectively purify two distinct activities capable of Holliday junction resolution (Figure 4B). One resolvase activity was shown to be associated with the MUS81-EME1 complex, as determined by MS and Western blotting of peak fractions from the final column step. Exactly how MUS81-EME1 from human cells can resolve Holliday junctions much more efficiently than the equivalent enzyme purified in recombinant form remains a puzzle, although similar observations have been made with Mus81-Eme1 purified from *S. pombe* [62]. The second resolvase activity, corresponding to ResA, was more difficult to identify from the final chromatographic fractions and necessitated the use of renaturation following SDS/PAGE separation. Fortunately, renaturation was successful and the resolvase activity was found to be present in a single gel slice in which proteins had an expected mass of approx. 60 kDa. MS analysis of this slice revealed the identity of an N-terminal fragment of an uncharacterized protein (FLJ40869) that contains an obvious nuclease domain [61]. This protein was identified as GEN1, which belongs to the Rad2/XPG (xeroderma pigmentosum complementation group G) nuclease family.

In parallel with the above approach, the *S. cerevisiae* TAP-fusion library was screened for proteins capable of Holliday junction resolution [63,64]. Strains harbouring TAP-fusion proteins were grown and individual lysates were prepared from each culture. TAP-fusion proteins were immunoprecipitated from the lysates using IgG-Sepharose, and tested for resolution activity using synthetic Holliday junctions. Rather than screen all TAP-fusions, our screen was biased towards the low expression categories, on the basis of our prediction that Holliday junction formation is likely to be a relatively rare event in vegetative cells and that the Holliday junction resolvase would therefore not be an abundant protein. We also excluded proteins that had well established and unrelated functions. In total, we screened 1100 strains, and found one novel resolvase activity, which is the product of the *YEN1* gene [61]. Remarkably, the Yen1 protein is the *S. cerevisiae* homologue of human GEN1, the Holliday junction resolvase activity identified from fractionated HeLa cells. Subsequent studies showed that both Yen1 and GEN1 are Holliday junction resolvases that cut junctions symmetrically to give rise to nicked duplex products that can be re-ligated.

The identification of eukaryotic Holliday junction resolvases represents a major breakthrough that should advance our understanding of the biochemical mechanisms of recombination and recombinational repair. Surprisingly, although GEN1/Yen1 promote

Holliday junction resolution by a symmetrical cleavage mechanism analogous to that exhibited by RuvC, Cce1, Hjc and the bacteriophage resolvases, they do not show any sequence or structure conservation with these proteins. Instead GEN1/Yen1 belong to the Rad2/XPG family of nucleases [65], and contain the characteristic XPG N-terminal (N) and internal (I) nuclease domains and a helix-hairpin-helix domain (Figure 5A).

The presence of eukaryotic Holliday junction resolvases in the Rad2/XPG family of nucleases emphasizes the functional diversification of this class of proteins. The phylogenetic tree (Figure 5B) shows the family subdivided into four distinct subgroups: class I, which are characterized by XPG (human) and Rad2 (*S. cerevisiae*), promote endonucleolytic incision at the 3'-side of bubble structures formed during nucleotide excision repair [66]; class II, which are characterized by FEN1 (flap endonuclease 1) (human) and Rad27 (*S. cerevisiae*), utilize their 5'-flap endonuclease activity for Okazaki fragment processing during DNA replication and also act in a variety of specialized DNA-repair reactions that require the trimming of 5'-flaps [67]; class III, which comprise the EXO1-related proteins, which exhibit 5' → 3' exonuclease and weak 5'-flap endonuclease activities, and are required for DNA replication, DNA repair and meiotic recombination [68]; and class IV, which we now know comprise the eukaryotic Holliday junction resolvases [61].

Although genetic studies of the eukaryotic resolvases remain in their infancy, it now appears likely that human cells may have the facility to process recombination intermediates in several different ways involving BLM-topoisomerase III α , GEN1 or MUS81-EME1 (Figure 3). It is possible that different types of recombination intermediate may be channelled into one pathway or another, and that different organisms may show a bias towards one pathway of resolution over another. That *S. pombe* exhibits a heightened dependence on Mus81-Eme1 may be due to the fact that *YEN1* is absent from the *S. pombe* genome, whereas it is present in *S. cerevisiae* and other eukaryotes. Certainly, the identification of GEN1/Yen1 now opens the door to further our understanding of these mechanisms of resolution.

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Biography

Novartis Medal Lecture

Delivered at Robinson College, Cambridge, on 17 December 2008

Stephen West

Abbreviations used

BLM	Bloom's syndrome protein
TAP	tandem affinity purification
XPG	xeroderma pigmentosum complementation group G.

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Figure 1. Holliday junction configurations and mechanism of resolution

The Holliday junction adopts a 2-fold-symmetric antiparallel stacked-X configuration (A). Binding by the RuvC dimer induces structural changes to the junction, which unfolds (B). Resolution occurs by the induction of symmetrically related nicks in strands of like polarity to give rise to nicked duplex products that can be ligated (C).

Figure 2. Timeline of the identification of Holliday junction resolvases

Following Robin Holliday's proposal describing the formation of a four-way DNA intermediate in homologous recombination, resolvases were identified from a variety of organisms. See the text for details.

Figure 3. Mechanisms for the resolution of Holliday junctions

Three possible pathways for the resolution of Holliday junctions in eukaryotic cells. Double-Holliday junctions produced by strand exchanges between homologous molecules (blue and red) can be resolved by ‘dissolution’ mediated by the BLM complex, symmetrical cleavage by GEN1/Yen1 or by asymmetric cleavage mediated by MUS81-EME1 complex. Green arrows indicate junction migration. Red arrows indicate cleavage sites. Broken lines indicate newly synthesized DNA. Yen1 resolvase is absent from *S. pombe*.

Figure 4. Identification of GEN1 as a human Holliday junction resolvase

The assay for Holliday junction resolution involves incubation of partially purified fractions with radiolabelled synthetic Holliday junctions (**A**). The asterisk indicates the radioactive label. Cleavage, by the introduction of symmetrically related nicks (green arrows), produces two nicked duplex molecules. The purification schemes for the identification of MUS81-EME1 and GEN1 (ResA) are shown in (**B**). Figure adapted from [61] with permission. © 2008 Nature Publishing Group. FT, flowthrough; HJ, Holliday junction; ssDNA, single-stranded DNA.

Figure 5. The Rad2/XPG family of proteins in *S. cerevisiae* and *Homo sapiens*

The three characteristic sequence motifs, the N-terminal (green) and internal (red) nuclease domains, and a helix-hairpin-helix domain (blue), are indicated in the proteins shown in (A). The total number of amino acids in each protein is indicated. (B) Phylogenetic relationships of representative Rad2/XPG nucleases from *S. cerevisiae* (*Sc*), *Caenorhabditis elegans* (*Ce*), *Drosophila melanogaster* (*Dm*), *H. sapiens* (*Hs*), and *Arabidopsis thaliana* (*At*). The scale bar indicates distance.