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Resolution of synthetic Holliday structures by an extract of human cells

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ABSTRACT

Virtually all models for recombination between homologous DNA sequences invoke a branched intermediate known as a Holliday structure. The terminal steps of recombination are postulated to involve a specific cleavage through the four-way junction of a Holliday structure, in a process known as resolution. We have constructed a synthetic Holliday structure in which the position of the junction of the DNA duplexes can branch migrate through approximately 185 bp. Using this structure, we have found that a component of a cytoplasmic extract of Hela cells is capable of cleaving the central junction of the substrate in a manner consistent with resolution. The activity requires a divalent cation but does not require an exogenous energy source. This is the first reported resolution activity from a mammalian source.

INTRODUCTION

Homologous recombination is a process in which similar DNA sequences interact and undergo a transfer of information, producing new "recombinant" sequences containing information from each of the original molecules. Recombination has been implicated in many cellular processes, including gene expression and the generation of genetic diversity. Much of our knowledge of the biochemistry of homologous recombination comes from studies of the RecA protein of E. coli. RecA protein catalyzes what are believed to be early steps in recombination, namely, the pairing of homologous DNA molecules and exchange of single strands of DNA to form heteroduplex (1,2). Activities that catalyze pairing and strand exchange between homologous DNA molecules have also been identified in eukaryotes (3-7).

Homologous recombination undoubtedly requires additional enzymes that catalyze later steps of the process, such as the processing of recombination intermediates into recombinant products. Virtually all models for homologous recombination invoke

a branched intermediate that has become known as a "Holliday" structure. The structure is named for Robin Holliday, who in 1964 (8) proposed such an intermediate. It is envisioned that during recombination between two duplexes of DNA, the two DNA molecules become covalently bonded to one another by a crossing-over of single strands. It is the joint molecule that is formed by such single strand crossing-over that is known as the Holliday structure, and the crossover point has been referred to as the "Holliday junction." A Holliday structure can be drawn in an open configuration in which it resembles an "X". Depicted in such a way, the structure can be seen as consisting of four duplex arms of DNA radiating from a central four-way junction (8).

"Resolution" of recombination has been defined as cleavage through the Holliday junction to produce two distinct duplex DNA molecules (8). Endonuclease VII from bacteriophage T4 and endonuclease I from T7 both have been shown to be capable of cleaving structures designed to mimic Holliday intermediates (9,10). The lambda Int protein can cleave and rejoin Holliday junctions constructed specifically from att sites (11). Recently, activities that catalyze the in vitro cleavage of Holliday junctions or Holliday junction analogs have been isolated from yeast (12,13). There have been two reports of proteins from mammalian cells that have the ability to specifically bind to DNA molecules resembling Holliday structures in vitro, but neither of these proteins was shown to possess any catalytic activity (14,15).

In previous demonstrations of "resolution," some of the substrates differed from predicted recombination intermediates in a potentially important way--the junctions of the DNA molecules had little or no ability to branch migrate, due to lack of significant homology of the sequences comprising the structures. The possible importance of symmetry elements and branch migration to the resolution process is an issue that has been raised previously (16). In this work, using a novel synthetic Holliday structure that has the capability of branch migration through 183 bp, we present the first evidence for an enzyme from mammalian cells that can cleave a Holliday structure in a manner expected for a resolving enzyme.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes, bacterial alkaline phosphatase, Klenow fragment, and T4 polynucleotide kinase were purchased from New England Biolabs or Bethesda Research Laboratories and were used as described by the supplier.

Construction of a Synthetic Holliday Structure

DNA fragments containing attachment site sequences from bacteriophage HP1c1 and its host Haemophilus influenzae Rd., as well as the left and right junctions between phage and host sequences from a lysogen, were isolated from plasmids that have been described previously (17). These DNA fragments as well as the construction of the Holliday structure are depicted schematically in Fig. 2A. (The 800 bp phage fragment shown is a Bgl I - Bgl II fragment of pWPF18; the 750 bp right junction fragment of phage and Haemophilus sequences is a Bgl II - Hind III fragment of pHPC101; the 850 bp left junction fragment is a Pst I - Bgl I fragment of pHPC103; the 800 bp fragment of Haemophilus sequences is a Pst I- Hind III fragment of pHPC102 (17)). HP1c1 and Haemophilus attachment sites contain a core region of 183 bp of homology. The core region consists of 93 consecutive base pairs of identity between phage and host, a 27 bp region containing six mismatches, and 63 consecutive identical residues (17). As previously described by Bell and Byers (18), an equimolar mixture of the four fragments was heat denatured and slowly reannealed to generate the two forms of Holliday structures depicted in Fig. 2A. The slowly-migrating Holliday structures were cut from a low melting point 1% agarose (Bio-Rad) gel in a small piece of agarose in order to isolate the substrate from the starting fragments. The excised agarose slice was melted at 65° C and loaded onto a standard 1% agarose (Sigma) gel from which, following a second round of electrophoresis, the Holliday structure was electroeluted. After electroelution, the Holliday structure DNA was ethanol precipitated, resuspended in TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA), and stored frozen at -20° C.

Preparing Radiolabelled Holliday Structures

Labelled Holliday structures were prepared in two different ways. In some experiments, the 750 bp junction fragment was end-labelled with ^{32}P by treatment with bacterial alkaline

phosphatase followed by incubation with gamma-[^{32}P]ATP and T4 polynucleotide kinase. This labelled DNA fragment was then mixed with the three additional, unlabelled fragments and Holliday structures were produced as above. In other experiments, pre-formed, unlabelled Holliday structures were end-labeled by treatment with Klenow fragment, alpha-[^{32}P]dCTP, and three unlabelled deoxynucleoside triphosphates. This procedure labelled the arms of the structure that had recessed 3'-OH groups at their termini.

Preparation of Nuclear and Cytoplasmic Extracts

Hela cells (line S3) were used as the source of the extracts. Cells were grown either as a monolayer in DMEM supplemented with 10% newborn calf serum (Gibco), or were purchased frozen from the MIT Cell Culture Center. Purchased cells had been grown at MIT in suspension in DMEM supplemented with 5% horse serum. Approximately 3-5 x 10^8 cells (1 gram) were collected and resuspended in 10 ml of (20 mM Tris-Cl pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 200 mM sucrose) and incubated on ice for 10 min. (The remainder of the preparation was conducted at 4° C.) The cells were sedimented, resuspended in 10 ml of buffer B (the above buffer minus the sucrose and containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) and disrupted by 20-30 strokes in a Dounce homogenizer, using an A pestle. Disrupted cells were centrifuged at 8000 x g for 10 min to sediment nuclei. The supernatant was saved as the cytoplasmic fraction. The nuclear pellet was resuspended in 5 ml buffer B containing 500 mM NaCl, 10 mM EDTA, sonicated for 30 seconds, and the fraction was clarified by centrifugation for 30 min. at 12,000 x g. Nuclear and cytoplasmic fractions were dialyzed for 16 hours against 200 volumes of buffer C (20 mM Tris-Cl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1mM PMSF, 10% glycerol).

Resolution Assay

To assay for resolution, reaction mixtures (100 ul, in buffer C) containing 0.5 ng Holliday structure DNA and the indicated amount of extract were incubated at 37° C for 1 hour. Reactions were terminated by the addition of SDS to 0.5% and proteinase K to 100 ug/ ml and incubation at 37° C for one hour. Nucleic acid was retrieved by ethanol precipitation, rinsed with 70% ethanol, and resuspended in an appropriate volume of TE. Samples

were subjected to electrophoresis through 1% agarose gels, transferred to nitrocellulose filters (19), and hybridized with a probe specific for the Holliday structure sequences. When assays were performed using labelled substrates, agarose gels were dried down and subjected to autoradiography directly.

S1 Nuclease Digestion of Substrate

0.5 ng of Holliday structure substrate was incubated for 15 min. at 37°C in 150 ug of Hela cytoplasmic protein (that had previously been boiled for 15 min. to destroy endogenous enzymatic activities) containing 1 mM $ZnCl_2$ or 1mM $ZnCl_2$ plus 400 units of S1 nuclease (Sigma). Reactions were terminated and processed as described above. In these experiments, controls using double- and single-stranded lambda phage DNA were performed to ascertain that the S1 nuclease was active and specific for single-stranded DNA. The reaction conditions were far from the optimal conditions for S1, and this necessitated the use of a large excess of enzyme. Enough enzyme was used to completely digest 1 ug of single-stranded lambda DNA under the reaction conditions employed.

Assays for Nicks in Resolution Products

In order to determine if the fragments produced by resolution of the Holliday structure contained DNA strand discontinuities, two approaches were taken. Using unlabelled substrates, resolution reactions were carried out as described above except that the samples were electrophoresed through alkaline agarose gels prepared as described previously (20). Following electrophoresis, the gel was neutralized, blotted to nitrocellulose, and hybridized as above. The resulting autoradiograph was analyzed for the presence of fragments in the range of 300 bp - 500 bp, as discussed in the text. Alternatively, as a more quantitative assay, the Holliday structures were end-labelled by a fill-in reaction using alpha-[^{32}P]dCTP and Klenow fragment. Resolution reactions were carried out using 0.5 ng of the labeled substrate (2 x 10^4 cpm). Reactions were terminated as described above and samples were electrophoresed through a neutral agarose gel to separate substrate from product. The gel was soaked in alkaline electrophoresis buffer (20) for one half hour; the gel was rotated by 90 degrees, and the samples were electrophoresed under alkaline conditions in the second dimen-

sion, for a distance of 8 cm. The substrate and product lanes were cut into 0.5 cm slices and counted in scintillation fluid (Optifluor, United Technologies).

Protein Determinations

Protein was determined by the Bio-Rad Protein Assay using ovalbumin (Sigma) as a standard.

Electron Microscopy

Electron microscopy was performed by T. Kelly (Johns Hopkins). DNA spreads were prepared by the formamide technique of Davis et al. (21). Spreads were shadowed with platinum and paladium prior to microscopy.

RESULTS AND DISCUSSION

Construction of a synthetic Holliday structure

In choosing a synthetic Holliday structure to be used in the identification and isolation of a Holliday structure-resolving activity from mammalian cells, several parameters were considered. It seemed reasonable to use a substrate that would appear to closely mimic an authentic recombination intermediate. We reasoned that an authentic intermediate would likely contain a fair amount of homology within the arm sequences since recombination normally occurs between homologous sequences. Homology in the arms of a Holliday stucture may influence the resolution process by allowing branch migration, providing symmetry elements, and/or allowing variable geometry at the four-way junction. In addition, we reasoned that there might be some minimal length requirements for the arms of the substrate. In vivo, the effective arm length is probably quite large. Of relevance to these conjectures is the observation that in vitro resolution of Holliday structures by an enzyme from yeast was shown to be apparently influenced by homology in the arm sequences (22). In addition, others have shown that a yeast enzyme that can cleave Holliday structures having arm lengths of several hundred bp and the capability of limited branch migration (23) could not cleave Holliday structures with arms only 12 bp in length and with no capability of branch migration (16). Also of interest are the recent reports (14,15) of proteins from mammalian cells that can bind to, but not cleave, small Holliday structure analogs constructed from oligonucleotides that contain no homology ele-

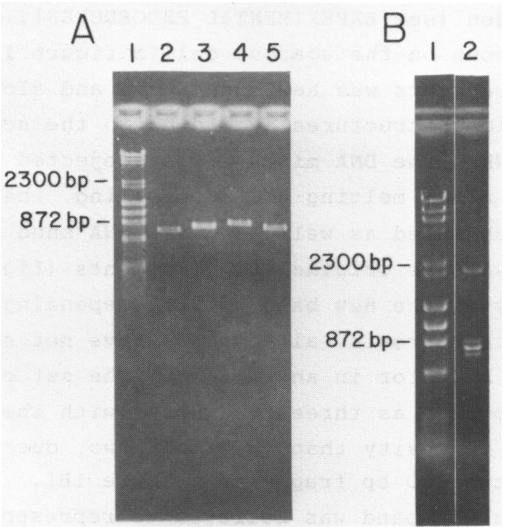

Figure 1. Production of synthetic Holliday structures. Panel A) Agarose gel electrophoresis of four restriction fragments containing attachment site sequences from bacteriophage HP1c1 and host Haemophilus influenzae Rd. from which Holliday structures were made. Approximately 20-40 nanograms of each fragment were displayed on a 1% agarose gel. Lane 1, molecular weight standards; lane 2, 750 bp junction fragment; lane 3, 800 bp fragment containing host attachment site; lane 4) 850 bp junction fragment; lane 5, 800 bp fragment containing phage attachment site. Panel B) Lane 1, molecular weight standards; lane 2, mixture of the four fragments displayed in panel A after melting and slow reannealing. See EXPERIMENTAL PROCEDURES and figure 2 for more details.

ments. Finally, in order to minimize interference of our studies by topoisomerases and nicking activities, it seemed wise to choose a substrate whose structure was not dependent upon the maintenance of torsional stress, such as would be the case with cruciform substrates produced by extrusion of palindromes from supercoiled molecules.

The attachment sequences from lysogenic bacteriophage HP1c1 and its host, Haemophilus influenzae Rd., provided a unique opportunity to construct a suitable synthetic Holliday structure having arms of both substantial length and homology. To construct such a substrate, four DNA fragments were used that contained the phage attachment site, host attachment site, and the left and right junctions between phage and host sequences isola-

ted from a lysogen (see EXPERIMENTAL PROCEDURES). These four fragments are shown on the agarose gel in figure 1A. A mixture of these four fragments was heat denatured and slowly reannealed to produce Holliday structures according to the scheme depicted in figure 2A. When the DNA mixture was subjected to agarose gel electrophoresis after melting and reannealing, the four original fragments were observed as well as a new DNA band migrating more slowly than any of the original DNA fragments (figure 1B). The precise mobility of the new band varied, depending upon the conditions of electrophoresis, although we have not studied the electrophoretic behavior in any detail. The set of four original fragments appeared as three fragments, with the middle fragment of greater intensity than the other two, due to the comigration of the two 800 bp fragments (figure 1B). Since the slowly-migrating DNA band was believed to represent the two forms of the Holliday structure shown in figure 2A, this DNA band was purified from agarose gels.

When the isolated putative Holliday structure DNA was examined by gel electrophoresis following an additional round of melting and reannealing, the slow migrating band as well as the set of four original fragments were produced (data not shown). This confirmed that the putative Holliday structures did indeed contain all of the DNA strands comprising the four original fragments. When the isolated structures were examined by electron microscopy, molecules resembling "X" forms were observed (data not shown). With these observations, we were satisfied that we had in fact isolated synthetic Holliday structures.

The junction of the synthetic Holliday structure had the potential to branch migrate through approximately 183 bp due to the homology of the phage and host sequences, as schematically illustrated in figure 2A. Migration to the ends of the arms was blocked by ~300 bp of heterology on each arm. The isolated structures were stored at -20°C in TE. Under these conditions the structures were stable; the structures could be frozen, thawed, and incubated at 37°C for 1 hour with no detectable melting of the molecules, as assayed by agarose gel electrophoresis, (data not shown). The structures were also resistant to digestion by S1 nuclease at 37°C (see below).

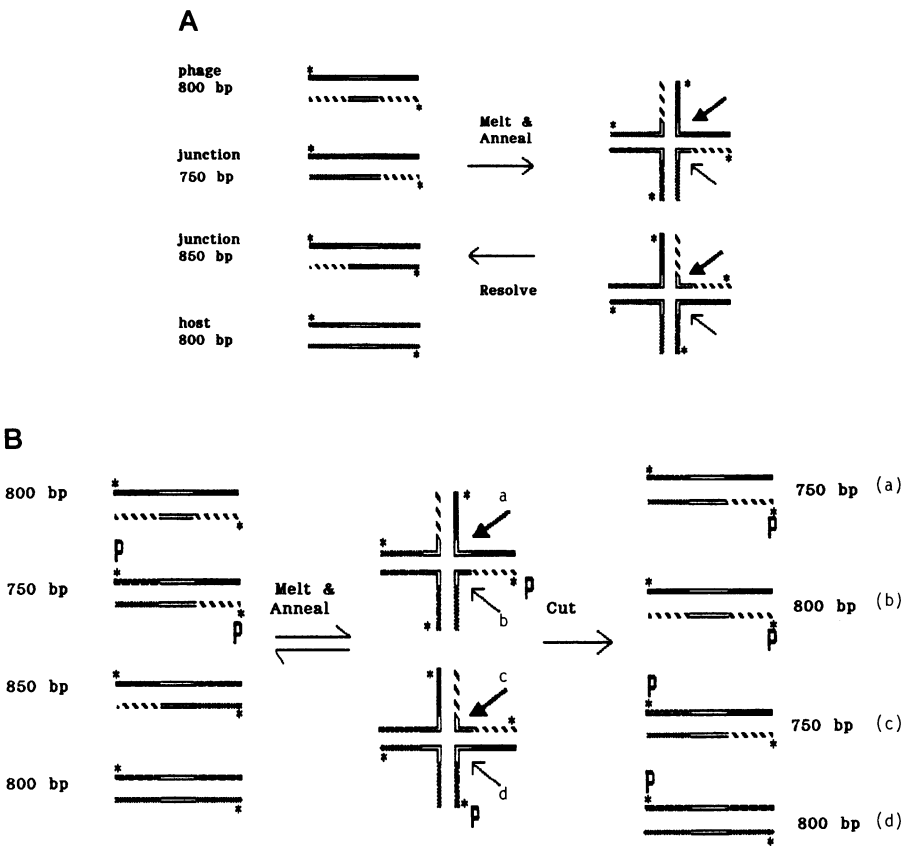

Figure 2. Construction of synthetic Holliday structures and assay for resolution. A) Shown is a schematic illustration of the four fragments displayed in figure 1A and the manner in which they can form Holliday structures by melting and reannealing. The stars (*) indicate the 5' ends of strands. The 183 bp core region of homology between HP1c1 and Haemophilus attachment sites is represented by the white segment at the center of each fragment. The various other patterns represent unique sequences of individual DNA strands. Resolution, or cutting through the four-way junction in one of two fashions (indicated by bold and thin diagonal arrows), results in the production of the starting fragments. B) Holliday structures were constructed starting with the 750 bp fragment 5'-end-labelled with ^{32}P, indicated by P. As indicated, the four different cleavage reactions, a,b,c,and d, would result in the formation of 750 bp labelled fragments (reactions a and c) and 800 bp labelled fragments (reactions b and d). Additional unlabelled fragments that are not shown in the figure would be produced by such cleavage reactions.

Specific processing of Holliday structures by Hela cell extracts

Nuclear and cytoplasmic fractions were prepared from Hela cells that were grown either as monolayer or suspension cultures. The cellular fractions were assayed for the ability to resolve the synthetic Holliday structure substrate into the set of four fragments from which the substrate was originally constructed, as illustrated in figure 2A. As shown, (figure 3, lanes 1 and 2), cytoplasmic fractions of Hela cells were capable of converting the substrate into DNA fragments consistent with resolution. All four predicted fragments appeared to be produced in the correct stoichiometry, as judged by the tell-tale pattern of three bands migrating at 850 bp, 800 bp, and 750 bp, with the 800 bp band more intense than the other two (compare with figure 1B, lane 2). We estimated that approximately 5% of the substrate was resolved under the reaction conditions employed in the experiments displayed in figure 3, lanes 1 and 2. We did not observe the production of any smaller discrete products, such as arm-length fragments (which would migrate at approximately 400 bp). We did observe smearing of the band corresponding to the Holliday structure. This might have been due to exonucleolytic digestion of the substrate or heterogeneity in the mobility of intact structures.

Curiously, nuclear fractions reproducibly contained little or no apparent resolving activity (data not shown). Nuclear fractions did not appear to contain inhibitors of the resolving activity since addition of nuclear extract to cytoplasmic fractions did not reduce the amount of detectable cytoplasmic activity (not shown). Finding an apparent resolving activity in the cytoplasm rather than the nucleus was somewhat unexpected; however, there is precedence for proteins with known nuclear functions, such as DNA polymerase-alpha, to be found in the cytoplasm due to leakage of nuclei during cell fractionation (24). Alternatively, it was possible that the detected activity was mitochondrial or that the largest pool of resolving activity in fact resides in the cytoplasm and is recruited to the nucleus.

To rule out the possibility that the production of discrete products by the cytoplasmic fraction was due to a sequence-specific reaction analogous to a restriction digestion, the four

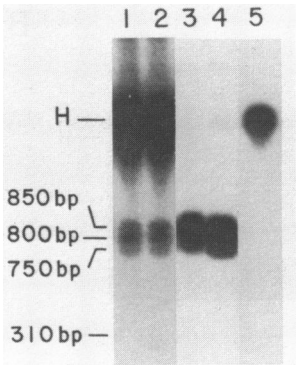

Figure 3. Specific processing of Holliday structures by cytoplasmic extract. Synthetic Holliday structures were incubated in cytoplasmic extract (200 ug protein), and reaction products were analyzed by Southern blotting. Lanes 1 and 2, 0.5 ng of Holliday structure DNA after incubation in cytoplasmic extract of Hela cells (note production of fragments of molecular weights 750 bp - 850 bp.); lane 3, four original DNA fragments (0.5 ng total) after incubation in cytoplasmic extract (200 ug protein); lane 4, four DNA fragments untreated; lane 5, 0.5 ng of Holliday structure substrate untreated. "H" denotes the mobility of the Holliday structure.

original DNA fragments were incubated in the cytoplasmic extract. We did not observe the production of any discrete products generated from these four fragments even though these fragments contained precisely the same sequences as did the Holliday structures (figure 3, lanes 3 and 4). We concluded that the processing of Holliday structures was due to recognition of structure rather than sequence. Additional experiments indicated that synthetic Holliday structures composed of lambda and *E. coli* *att* sequences are also processed by the extract in a manner consistent with resolution (figure 4), suggesting that the apparent resolution reaction was not specific to the original HP1*c*1 substrate and was not dependent upon mismatches that might be present in the original substrate.

As shown in figure 5, the extent of product formation was a function of the amount of cytoplasmic protein used in the reaction, and was greatly reduced when the extract was boiled prior to incubation with substrate. This suggested that the apparent

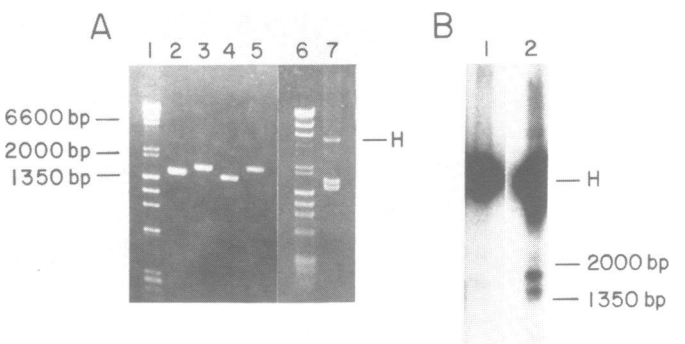

Figure 4. Hela extract catalyzes apparent resolution of a second Holliday structure. In a manner similar to the construction of the original substrate, a second Holliday structure substrate was constructed from DNA fragments containing the attachment site sequences from phage lambda and its host Escherichia coli. The sources of these fragments were plasmids that have been previously described (11). Specifically, a 1500 bp Pst I-Sal I fragment from pWR1 (containing attP), a 1700 bp Eco RI-Bam HI fragment from pWR101 (containing attB), a 1500 bp Sal I-Eco RI fragment from pPH201 (containing attL), and a 1700 bp Pst I-Bam HI fragment from pPH202 (containing attR) were used. A mixture of these fragments was melted and reannealed to produce Holliday structures, which were subsequently gel-purified and then incubated in the Hela cytoplasmic extract. Panel A) Lane 1, molecular weight standards; lane 2, approximately 100 ng of 1500 bp attP fragment; lane 3, 100 ng of 1700 bp attB fragment; lane 4, 100 ng of 1500 bp attL fragment; lane 5, 100 ng of 1700 bp attR fragment; lane 6) molecular weight standards; lane 7, fragments displayed in lanes 2-5 following melting and reannealing. "H" denotes the mobility of the Holliday structures produced. The starting fragments appear as two bands of DNA, one band representing the two comigrating 1700 bp fragments and the other band representing the two comigrating 1500 bp fragments. Panel B) Southern analysis of gel-purified Holliday structures before and after treatment with 200 ug of Hela cytoplasmic extract. Each lane displays 0.5 ng of DNA hybridized with a probe specific for the sequences of the lambda/ E. coli substrate. Lane 1, substrate untreated; lane 2, substrate after incubation in extract. Note production of DNA fragments having the same mobility as the starting fragments (compare with panel A, lane 7). The production of these fragments is consistent with catalysis of resolution.

resolution was due to a protein constituent of the extract rather than simply reaction conditions such as pH, temperature, or ionic strength.

Cytoplasmic extracts promote cleavage rather than melting of Holliday structures

It was possible that the generation of the predicted frag-

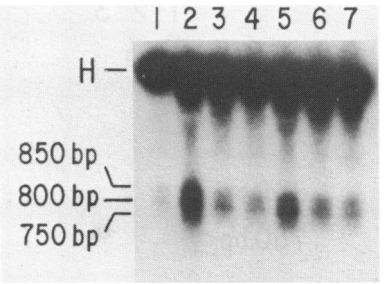

Figure 5. Apparent resolution of synthetic Holliday structures is a function of cytoplasmic protein. Holliday structures were incubated in various amounts of cytoplasmic protein and analyzed by Southern blotting. lane 1) control--300 ug of cytoplasmic protein from Hela cells was boiled for 15 min. prior to addition of ATP and Holliday structure substrate; lanes 2-4) substrate was incubated with 300 ug, 60 ug, or 12 ug, respectively, of cytoplasmic protein from Hela cells; lanes 5-7) substrate was incubated with 125 ug, 25 ug, or 5 ug, respectively, of cytoplasmic protein derived from a different culture of Hela cells. Holliday structure substrate is indicated by "H".

ments from the Holliday structures was due to denaturing of the substrate and reannealing of the strands to produce the fragments. Such melting and reannealing could be catalyzed by a helix-destabilizing or unwinding protein. In order to ascertain that the fragments were produced via cleavage of the Holliday junction and not by strand separation and reannealing, Holliday structures were constructed using a 5' end-labelled 750 bp fragment, as illustrated in figure 2B. We reasoned that cleavage of such a labelled structure through or near the four-way Holliday junction (i.e., resolution) would result in the formation of 750 bp and 800 bp fragments end-labelled with ^{32}P, whereas melting and reannealing of the structure would generate only the original 750 bp labelled fragment.

The result of incubation of the labelled substrate in a cytoplasmic extract is shown in figure 6. In this figure it can be seen that 750 bp as well as 800 bp labelled fragments were produced by action of the extract, indicating that the Holliday structure was in fact cleaved through, or near, the four-way junction. The apparent production of all four fragments (one

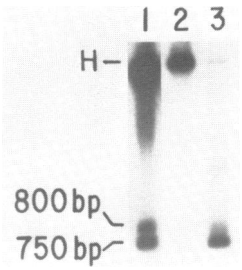

Figure 6. Cytoplasmic extracts promote cleavage through Holliday junctions rather than strand separation and reannealing. Labeled Holliday structures were prepared starting with a labelled 750 bp fragment as illustrated in figure 1B. The labelled substrate was incubated with cytoplasmic extract and the production of a labeled 800 bp fragment, indicative of cleavage, was monitored by electrophoresis. Lane 1) Labelled Holliday structure substrate after incubation with 150 ug of cytoplasmic extract; lane 2) labelled Holliday structure substrate untreated; lane 3) 750 bp labelled fragment produced by melting and slow reannealing of the labelled Holliday structure. Lane 1 was exposed longer than lanes 2 and 3 in order to increase the visibility of the relevant bands.

750 bp, two 800 bp, and one 850 bp; figure 3, lanes 1 and 2) indicated that the resolving activity could carry out either of the cleavages indicated in figure 2A on a given molecule and that both cleavages occurred at approximately equal frequency. The lack of detection of arm length products (figure 3, lanes 1 and 2) suggested that cleavage at the four-way junction predominantly occurred diagonally through the junction and that only one diagonal cleavage was executed per Holliday structure.

The Holliday junction is not S1-Sensitive

We were somewhat concerned that the strands of the Holliday structure might "breathe" during the incubation in the extract, and that such breathing, perhaps in conjunction with extensive branch migration, might make the Holliday junction susceptible to attack by single strand-specific nuclease activities. Cleavage by such a nuclease might fortuitously result in the production of the fragments considered diagnostic for resolution. To explore this possibility, S1 nuclease was added to heat-inactivated extract and the synthetic Holliday structure was incubated in this mixture under typical resolution assay conditions. As shown in figure 7, the four-way Holliday junction was not sensi-

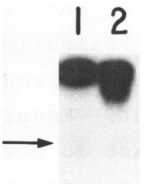

Figure 7. The 4-way Holliday junction is not S1-sensitive. Holliday structures (0.5 ng) were incubated in heat-killed extracts in the presence of S1 nuclease and analyzed by Southern blotting to determine if the junction is cleavable by the single strand-specific nuclease, S1. Lane 1) Substrate incubated with 150 ug of heat-killed extract; lane 2) substrate was incubated with 150 ug of heat-killed extract containing 400 units of S1. Under the conditions employed, control experiments indicated that 400 units of S1 was sufficient to completely digest 1 ug of single-stranded lambda DNA. (The arrow indicates the region of the gel at which the fragments diagnostic for resolution would migrate).

tive to S1. Internal controls using denatured lambda DNA indicated that the S1 nuclease present in the reaction depicted in figure 7, lane 2, was sufficiently active under the conditions employed to completely digest an amount of single-stranded DNA that was 2000-fold greater than the amount of Holliday structure present (data not shown). S1 nuclease apparently attacked only the ends of the arms of the Holliday structure, resulting in a slight smearing of the substrate. Importantly, there was no evidence that the four-way junction was susceptible to attack by a single strand-specific nuclease. This suggested that the Holliday junction did not contain any appreciable, readily accessible single-stranded DNA, reducing the likelihood that the apparent resolution observed was due merely to digestion by a single strand-specific nuclease. In addition, if the four-way Holliday junction were indeed attacked by a single strand-specific nuclease in the extract, then cleavage would probably not have always occurred diagonally through the junction and additional products, such as arm-length fragments, should have been observed.

Resolution products contain no detectable single strand discontinuities

An issue of interest is the question as to whether the nicks introduced during cleavage of the Holliday structure were re-

sealed during the incubation in the cytoplasmic extract, since both cleavage and subsequent religation of a recombination intermediate into genetically sensible products would be required to complete a recombination event. Resolving enzymes from phages T4 and T7 as well as from yeast do not exhibit ligase activities and therefore leave nicks in one strand of the resolution products (9,10,12,13). The lambda Int protein can apparently cleave and seal Holliday junctions constructed specifically from att sites (11).

If each resolution product in our experiments contained a discontinuity in one strand, then that strand should have denatured into two shorter strands upon alkaline agarose gel electrophoresis. Due to branch migration of our substrate, the interruption could, theoretically, have been located anywhere in the core homology region of 183 bp, resulting in strands ranging from ~300 bp - ~500 bp in length. Alkaline electrophoresis of resolution products generated in the absence of ATP, followed by Southern analysis, revealed no evidence for such fragments (data not shown). As a more stringent test for nicks, Holliday structures end-labelled with ^{32}P were incubated in a cytoplasmic extract. The resolution products were separated from the starting material by agarose gel electrophoresis. The gel was rotated and the products were then electrophoresed in the second dimension under alkaline conditions. The gel lane was then cut-up and counted (see EXPERIMENTAL PROCEDURES). In this experiment, starting with 2 x 10^4 cpm of substrate, products containing a total of 1000 cpm of ^{32}P were generated. Greater than 900 cpm out of 1000 cpm, or more than 90% of the radioactivity associated with the product, migrated as full length fragments upon alkaline electrophoresis. This suggested that cleavage of the substrate by the resolving activity resulted in ligatable nicks that were in fact sealed during the course of incubation. Whether cutting and sealing are catalyzed by one or several mammalian enzymes can be answered only by further fractionation of the extract. However, it is interesting to note that when the extract was assayed for the ability to convert nicked circular DNA molecules into covalently closed circles under resolution reaction conditions, no such ligase activity was observed (not shown).

Concluding remarks

This is the first reported demonstration that mammalian cells contain an activity that can process Holliday structures in a manner predicted by virtually all current models for recombination. Preliminary characterization of the Holliday structure resolving activity indicates that pretreatment of protein extracts with RNase T_1 does not reduce the activity. The activity does not require ATP (or any exogenous energy source) and is actually inhibited by the addition of ATP to a concentration of 5 mM in the reaction cocktail. The resolution reaction is apparently dependent upon the presence of Mg^{+2} or some other cation since addition of 10 mM EDTA completely inhibits the reaction. Finally, addition of VM26, a potent inhibitor of eukaryotic topoisomerase II (25) to a level of 200 uM does not inhibit resolution. Further purification and characterization of this Holliday structure-resolving activity from human cells should contribute to our understanding of homologous recombination in mammalian cells. The use of the novel substrate described in this work, which can undergo extensive branch migration, in conjunction with additional substrates that undergo less extensive (or no) branch migration, should allow an assessment of the role(s) that homology and branch migration play in the resolution process.

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