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DNA Triple Helices: biological consequences and therapeutic potential

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Abstract

DNA structure is a critical element in determining its function. The DNA molecule is capable of adopting a variety of non-canonical structures, including three-stranded (*i.e.* triplex) structures, which will be the focus of this review. The ability to selectively modulate the activity of genes is a long-standing goal in molecular medicine. DNA triplex structures, either intermolecular triplexes formed by binding of an exogenously applied oligonucleotide to a target duplex sequence, or naturally occurring intramolecular triplexes (H-DNA) formed at endogenous mirror repeat sequences, present exploitable features that permit site-specific alteration of the genome. These structures can induce transcriptional repression and site-specific mutagenesis or recombination. Triplex-forming oligonucleotides (TFOs) can bind to duplex DNA in a sequence specific fashion with high affinity, and can be used to direct DNA-modifying agents to selected sequences. H-DNA plays important roles *in vivo* and is inherently mutagenic and recombinogenic, such that elements of the H-DNA structure may be pharmacologically exploitable. In this review we discuss the biological consequences and therapeutic potential of triple helical DNA structures. We anticipate that the information provided will stimulate further investigations aimed toward improving DNA triplex-related gene targeting strategies for biotechnological and potential clinical applications.

Keywords

triplex DNA; DNA triple helices; triplex-forming oligonucleotides; H-DNA; unusual DNA structures; genetic instability

1. Introduction

The DNA of a single cell contains all of the genetic information necessary for life's processes. Friedrich Miescher discovered DNA in 1868, yet it took more than 70 years to demonstrate that it is the molecule that carries genetic information [1]. Once this was realized, tremendous effort has been made to better understand both the structure and function of DNA. Not only does the DNA primary nucleic acid sequence define the genetic code, its secondary structure plays important roles in regulating gene expression such that the formation of multi-stranded DNA structures at specific sites in the genome can influence many cellular functions. DNA

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can form multi-stranded helices through either folding of one of the two strands or association of two, three, or four strands of DNA. A well-established multi-stranded DNA structure, triple helical DNA (triplex DNA), both naturally occurring intramolecular H-DNA structures, and triplex-forming oligonucleotide (TFO)-targeted intermolecular triplexes will be the focus of this review.

Triple-helical nucleic acids were first described in 1957 by Felsenfeld and Rich [2], who demonstrated that polyuridylic acid and polyadenylic acids strands in a 2:1 ratio were capable of forming a stable complex. In 1986, it was demonstrated that a short (15-mer) mixed-sequence triplex-forming oligonucleotide (TFO) formed a stable specific triple helical DNA complex [3]. The third strand of DNA in the triplex structure (*i.e.* the TFO) follows a path through the major groove of the duplex DNA. The specificity and stability of the triplex structure is afforded via Hoogsteen hydrogen bonds [4], which are different from those formed in classical Watson-Crick base pairing in duplex DNA. Because purines contain potential hydrogen bonds with incoming third strand bases, the binding of the third strand is to the purine-rich strand of the DNA duplex [5,6].

2. Classification of DNA triple helices

Since the original discovery of triple helical nucleic acids, a number of triplex DNA structures that form under various conditions *in vitro* and/or *in vivo* have been identified (reviewed in [7–9]). These include intermolecular triplexes (with a pyrimidine third strand “Y:RY”, a purine strand or mixed pyrimidine/purine third strand “R:RY”), and intramolecular triplexes (H-DNA).

2.1. Intermolecular triplexes

Intermolecular triplexes are formed when the triplex-forming strand originates from a second DNA molecule (Figure 1). Intermolecular triplexes have attracted much attention because of their potential therapeutic application in inhibiting the expression of genes involved in cancer and other human diseases, for targeting disease genes for inactivation, for stimulating DNA repair and/or homologous recombination pathways, for inducing site-specific mutations, and for interfering with DNA replication. An example of triplex formation with a polypurine TFO sequence specific for the human *c-MYC* P2 promoter is shown in Figure 2.

Triplex formation occurs in two motifs, distinguished by the orientation of the third strand with respect to the purine-rich strand of the target duplex. Typically, polypyrimidine third strands (Y) bind to the polypurine strand of the duplex DNA via Hoogsteen hydrogen bonding in a parallel fashion (*i.e.* in the same 5’ to 3’, orientation as the purine-rich strand of the duplex), whereas the polypurine third strand (R) binds in an antiparallel fashion to the purine strand of the duplex via reverse-Hoogsteen hydrogen bonds [6,10,11]. In the antiparallel, purine motif, the triplets are G:G-C, A:A-T, and T:A-T; whereas in the parallel, pyrimidine motif, the canonical triples are C^+:G-C and T:A-T triplets (where C^+ represents a protonated cytosine on the N3 position) The hydrogen bonding schemes found in purine and pyrimidine motif triplexes are depicted in Figure 3. Antiparallel GA and GT TFOs form stable triplexes at neutral pH, while parallel CT TFOs bind well only at acidic pH so that N3 on cytosine in the TFO is protonated [12], substitution of C with 5-methyl-C permits binding of CT TFOs at physiological pH [13,14] as 5-methyl-C has a higher pK than does cytosine. For both motifs, contiguous homopurine-homopyrimidine runs of at least 10 base pairs are required for TFO binding, since shorter triplexes are not substantially stable under physiological conditions, and interruptions in optimum sequence can greatly destabilize the triplex structure [15–20]. If purine bases are randomly distributed between two duplex strands, the consecutive third-strand bases should switch from one strand of the duplex to the other, resulting in a structural distortion of the sugar-phosphate backbone and lack of stacking interactions. This is energetically

unfavorable, and therefore the most appropriate duplex target for triplex formation contains consecutive purine bases in one strand. Thus, an ideal target for triplex formation is the presence of a homopurine sequence in one strand of duplex and a homopyrimidine sequence in the complementary strand.

Triplex formation is kinetically slow compared to duplex annealing [21–25]. However, once formed, triplexes are very stable, exhibiting half-lives on the order of days [21,23]. Formation of both intramolecular and intermolecular triplexes depends on several factors including length, base composition, divalent cations, and temperature [7]. The affinity and specificity of TFO binding are critical features to their success of a gene targeting molecules. The dissociation constants (Kd_s) of TFOs for their target duplexes typically range from 10^{-7} to 10^{-10} M, making them feasible gene targeting agents.

2.2. Intramolecular triplexes (H-DNA)

In an intramolecular triplex or H-DNA structure, the third strand is provided by one of the strands of the same duplex DNA molecule at a mirror repeat sequence (Figure 4). Four isomers of intramolecular triplexes can exist depending on the strand that serves as the third strand. Intramolecular triplexes are also known as H-DNA or *H-DNA, depending on whether the third strand of the triplex is Py- or Pu-rich, respectively. These structures will be discussed at length in Section 5–Section 9.

3. Potential applications of DNA triplex formation in therapeutics

3.1. Targeting genes as an approach to molecular-targeted therapeutics

The ability to target specific genes to modulate their structure and/or function in the genome has far-reaching implications in biology, biotechnology, and medicine. TFOs represent near-ideal molecules for this purpose because of their ability to bind duplex DNA with high affinity and specificity. Facile chemistries for TFO modification are also available, allowing the covalent attachment of DNA damaging agents, for example, to target damage to specific sites in a genome. Oligonucleotides have been designed to target nucleic acids as well as proteins in a variety of applications. A potential advantage of targeting DNA rather than RNA or protein is the limited number of copies to be targeted. In addition, TFOs unlike antisense oligonucleotides or siRNAs can be used to target damage specifically to mutate or inactivate a gene. Thus, in a biological context, antigene approaches may provide some advantages over antisense strategies. Other general positive features of oligonucleotide-based approaches are: facile synthesis of the reagents; the availability of a variety of chemical modifications (to the bases, sugar-phosphate backbone, and the 5’ and 3’ ends to improve cellular uptake, target binding, specificity, and stability; and availability of efficient delivery systems.

3.2. Abundance of TFO binding sites in mammalian genomes

The gene products involved in many important biological processes such as cell signaling, proliferation, and carcinogenesis have now been identified, providing plausible targets for gene modulation. Triplex technology represents an approach to regulate these processes by manipulating the structure and/or function of these critical genes. However, in order to target any gene of interest, there must be unique TFO binding sites in those genes. To determine the number of potential TFO binding sites available in mammalian genomes, we designed an algorithm to search the entire human and mouse genomes for such sites, and were surprised to find ~2 million in each of these mammalian genomes [26]. We found that most annotated genes in both the mouse and human genomes contain at least one unique TFO binding site, and these sites are enriched in the promoter and /or transcribed gene regions.

3.3. Modulating gene expression via triplex formation

The first demonstration of TFO-directed transcription inhibition was reported by the Hogan group two decades ago [5]. Since that time, many groups have published reports demonstrating the ability of TFOs to inhibit the expression of a number of genes in a variety of systems. Mammalian genes that have been targeted by TFOs are summarized by Wu *et al*. (2007) [27]. However, obstacles to this approach have limited the potential of triplex technology as a consistent and reproducible method of transcriptional regulation. Several limitations to this technology include TFO delivery and uptake into cells, TFO stability once in the cells, lack of optimal target site binding affinity and specificity due to intracellular salt concentrations and pH, displacement by DNA metabolic activities (*e.g.* transcription, replication, and repair), and chromatin structure which may present a barrier to target site accessibility. As examples, physiologic concentrations of potassium can facilitate the formation of G-quadruplex structures on G-rich purine TFOs, thereby preventing triplex formation [15,21,28], and in the case of pyrimidine TFOs, binding can be inhibited by physiologic pH due to the requirement for cytosine protonation at N3 [14]. It is also possible that alternative activities of the TFO in the cell preclude its intended purpose in binding its duplex target to inhibit gene expression. TFOs have been demonstrated to act as “decoy” oligonucleotides, which bind transcription factors, such that they are not available to bind their duplex consensus sequences for transcription activation [29]. Aptamer effects of TFOs have also been observed in which the oligonucleotides can bind proteins and inhibit their activity. Efforts have been made to overcome these limitations, largely through chemical modification of the TFO, which we discuss in detail in Section 4.

3.4. Directing site-specific DNA damage

Another potential strategy for the development of TFOs as “therapeutic agents” is their utility as targeted DNA damaging agents. This approach differs from TFO-directed transcription inhibition in that TFO-directed DNA damage has been shown to stimulate mutation, recombination, and DNA repair at the targeted sites. Thus, this application has the potential to directly inactivate genes, rather than transiently regulating gene expression. As an example, triplex formation can be used to direct site-specific DNA damage and thereby induce DNA repair synthesis locally, independent of replication synthesis [30–32]. We have used TFOs targeted to the *c-MYC* gene to stimulate repair synthesis in the presence of an antitumor antimetabolite, gemcitabine, to increase its incorporation into DNA. We found that when used in combination, *c-MYC*-specific TFOs significantly increased the effectiveness of gemcitabine in inhibiting the growth of human breast tumor cells [33].

Physical studies of the triplex structure reveal that binding of the TFO induces structural distortions in the underlying duplex even though the Watson-Crick hydrogen bonding is preserved [34,35]. These features, along with facile chemistries to covalently couple DNA damaging agents to the TFOs, make triplexes attractive probes for directing site-specific DNA damage [36–38]. Targeting DNA damage to a specific site via triplex formation can be used to induce mutations [32,39,40] and/or recombination *in vitro* and *in vivo* [41–43], presumably through recognition of the triplex structures as damage by the repair machinery of the cell [31,32,44–46]. Thus, triplex technology provides a mechanism to modify gene structure and function in living organisms [39,47]. Conjugation of TFOs with photoactivatable chemical groups such as psoralen (which requires UVA irradiation for activation) allows one to control the timing of damage (*i.e.* after the TFO has bound its target) to reduce non-specific, collateral damage to the rest of the genome [37,38,48,49]. These features make TFOs powerful reagents for controlled gene manipulation in mammalian systems.

4. Approaches to Improve the Efficacy of TFOs in Biological Systems

As discussed above (Section 3.3), there are many factors that can limit the efficacy of triplex technology in cellular systems. Improvements in TFO chemistries are under active investigation, as these modifications could considerably increase the efficacy of antigene oligonucleotide therapeutics.

4.1 Chemical modifications of TFOs

To improve the binding affinity, selectivity and stability of oligonucleotides inside the cell, a number of modifications have been made to the bases, the backbone, the 5' or/or 3' ends, and/or the sugar moiety of oligonucleotides. Some examples of base modifications to TFOs designed to bind in the antiparallel purine triplex motif include the substitution of guanine by 6-thioguanine [28,50] or the substitution of adenine by 7-dezaxanthine [51]. These modifications have assisted in preventing the formation of unwanted intramolecular secondary structures within the TFOs. In the parallel pyrimidine triplex motif, the substitution of cytosine by 5-methylcytosine, N^6-methyl-8-oxo-2-deoxyadenosine [52,53] or pseudoisocytodines [54] has been used to reduce the pH dependence of triplex formation. At neutral pH, the substitution of thymine by 5-propynyluracil stabilizes triplex formation [55].

Modifications of the natural phosphodiester backbone have been designed to improve TFO binding affinity and stability. Among these are chemical modifications that result in neutral or cationic backbones to reduce the electrostatic repulsion between the negatively charged phosphodiester backbone of the TFO with that of the target DNA duplex. Examples of such modifications include thioate linkages [56,57], $N^{3'} - P^{5'}$ phosphoramidates [58,59], and morpholino phosphoramidate linkages [60]. The cationic phosphoramidate linkages, N,N-diethylethylenediamine and N,N-dimethylaminopropylamine, confer increases in TFO binding affinity and intracellular activity [61]. Other promising oligonucleotide backbone modifications that allow for improvements in triplex formation include peptide nucleic acids (PNAs) and locked nucleic acids (LNAs). PNAs are non-ionic nucleic acid analogs in which the sugar-phosphate backbone is replaced by an N-aminoethyl-glycine-based polyamide structure. PNAs bind to single–stranded DNA via Watson-Crick base pairing and can also form triple helices through Hoogsteen base pairing with the DNA/PNA duplex [62,63]. The neutral polyamide backbone was designed to minimize non-specific electrostatic effects that often are observed with DNA oligonucleotides. PNAs, under certain conditions, can bind to any DNA duplex sequence by a strand-invasion mechanism. Based on this property, PNAs have great potential as DNA targeting "drugs" [64]. Moreover, PNAs are resistant to both nucleases and proteases, and their neutral backbone increases their hybridization affinities to complementary RNA and DNA strands [65]. PNAs have been used successfully to target chromosomal DNA at transcription start sites to inhibit gene expression in cells [66–68]. In LNA molecules, the deoxyribose moiety is modified by introducing a methylene bridge between the 2'-O, 4'-O, and 4'-C. This bridge results in a locked 3'-endo conformation, which reduces the flexibility of the ribose, and allows for stable triplex formation [69–72]. LNA modifications within a TFO can increase the binding affinity to the target duplex DNA, and can increase resistance to digestion by nucleases [73]. Beane *et al*., have demonstrated that LNAs can recognize chromosomal target sequences and efficiently block endogenous expression of the progesterone and androgen receptors [74]. An LNA analogue ENA, containing a 2'-O, 4'-Cethylene bridge has also been reported to form stable triplex at physiological pH [75].

Because RNA:DNA duplexes are more stable than DNA:DNA duplexes, many researchers are interested in modifying the ribose moiety in oligonucleotides to test the effect of binding and stability in triplex formation. Inoue *et al*., have reported that 2'-O-methyloligoribonucleotide derivatives are not subject to cleavage by RNase H, and that a 2'-O-methylribonucleotide:RNA duplex is thermally more stable than an RNA:RNA duplex [76]. 2'-O-methylribose (2'-OMe)

and 2'-O-aminoethylribose (2'-AE) modified oligonucleotides have been synthesized and successfully used to induce mutations in cells via the formation of DNA:DNA:RNA triple helices [77,78]. Interestingly, extensive modification of TFOs with 2'-OMe and 2'-AE residues leads to increased binding affinity *in vitro*, but reduced activity *in vivo* [79]. Bridged nucleic acids (BNAs) have been used to overcome the requirement for long purine runs for efficient triplex formation [80]. For example, a 2'-O,4'-C-methyleneribonucleic acid containing 2-pyridone-modified bases can recognize target duplexes containing a CG inversion with high affinity, without compromising selectivity, thereby increasing the TFO binding code TFO binding code [81].

The vast majority of 5' or 3' end modifications include covalent attachment of DNA damaging agents to direct site-specific DNA damage via triplex formation. Examples of reactive molecules that have been conjugated to TFOs include 2-amino-6-vinylpurine, haloacetyl amide, aryl nitrogen mustard, N_4,N_4-etheno-5-methyldeoxycytidine, and various psoralen derivatives. All of these induce site-specific cross-linking and/or alkylation in the target duplex DNA molecule [37,38,82–86]. Moreover, end modifications have also been used to prevent cellular exonucleases from degrading the TFO once inside cells. A successful example of such a 3'-end modification includes a 3'-phosphopropyl amine, which prevents TFO degradation following intravenous or intraperitoneal injection into mice [87].

4.2. Delivery of TFOs to nuclear target sites

A variety of delivery reagents are available to facilitate the cellular uptake of oligonucleotides in tissue culture and *in vivo* studies. Cationic lipids, cationic polymers, and cell penetrating peptides represent a few examples. While these strategies have been widely and successfully applied to deliver oligonucleotides into cells, uptake still remains a limitation and improvements to these techniques are being developed. A number of chemical modifications have been incorporated into TFOs to facilitate their uptake. These include, but are not limited to: polyamine analogs [88] cholesterol derivatives [89] nuclear targeting peptide conjugates [90]; 6-phosphate-bovine serum albumin [91]; and polypropylenimine dendrimers [92].

In eukaryotic cells DNA is packaged into chromatin, such that this packaging of the DNA into nucleosomes may present a limitation to TFO accessibility to their duplex targets *in vivo*. Chemical modifications to TFOs (discussed above) may allow TFOs to compete with the chromatin structure, thereby enhancing the efficacy of triplex technology in biological systems. Interestingly, a tetrapeptide within the DNA binding domain of human high mobility group B1 (HMGB1) protein has been shown to stabilize triplex formation in the purine motif [93]. HMGB1 has also been demonstrated to specifically bind to triplex structures with high affinity [94]. HMGB1 is a very abundant non-histone chromatin-associated protein, thus it is possible that this protein may play a role in triplex formation and stabilization in chromatin.

A better understanding of the molecular mechanisms of action and the requirements for optimization of cellular stability, nuclear delivery, and DNA binding of these oligonucleotide therapeutics will certainly enhance their clinical potential.

5. H-DNA conformation and its occurrence in genomic DNA

Eukaryotic genomes contain many S1 nuclease sensitive sites with a common feature being runs of polypurine-polypyrimidine sequences. These types of sequences are capable of adopting non-canonical DNA structures. For example, H-DNA, or intramolecular triplex DNA is a structure in which half of the pyrimidine tract swivels its backbone parallel to the purine strand in the underlying duplex, or the purine strand (in *H-DNA) binds to the purine strand of the underlying duplex in an antiparallel orientation, to form a triple helical DNA structure [95]. The complimentary strand remains single stranded [96], and is therefore sensitive to S1

nuclease activity. In an H-DNA structure, the conformation is maintained by T-A*T or C-G*C + Hoogsteen hydrogen bonding in the major groove of the DNA. Similar to intermolecular triplexes, formation of a stable C-G*C+ hydrogen bond requires protonation of cytosine at N3, which explains the pH dependency of this type of H-DNA structure. In fact, this structure was called H-DNA based on the requirement of H^+ to protonate the cytosines in the swiveled third strand. The other form of intramolecular triplex, *H-DNA, which is maintained by T-A*A or C-G*G base triplets, can be formed at neutral pH and requires bivalent cations such as Mg^{2+} for stability. Hence, a polypurine-polypyrimidine stretch with mirror repeat symmetry can form H-DNA readily. Interestingly, protonated base triads such as C-G* A+ can be incorporated into an intermolecular pyrimidine-purine*purine triplex conformation. For example, this triad can be formed between plasmids containing a d(C)n(G)n duplex sequence and a d(AG)n oligonucleotide as the third strand, or intramolecularly on a $G_{10}TTAA(AG)_5$ sequence in a supercoiled plasmid, forming a triplex structure containing C-G*G and C-G*A+ base triads under acid conditions, but without a requirement for bivalent cations [97]. Therefore, the formation of an *H-DNA structure is more versatile and does not require sequences containing perfect mirror symmetry. For both types of H-DNA (H-DNA and *H-DNA, both will be referred to as H-DNA in the following text), two isoforms are possible depending on whether the 3’ half or the 5’ half of the third strand is involved in the triplex structure formation. However, the isoform in which the 3’ half of polypyrimidine strand is included in the triplex structure, or when the 5’ half of the polypurine strand is part of the triplex is favored [98,99].

5.1. Abundance of genomic H-DNA-forming sequences

Computer generated sequence analysis of genomic DNA from various species resulted in several interesting observations related to DNA structural elements. Regions of 15–30 contiguous purine or pyrimidine tracts are greatly overrepresented in all eukaryotic species examined, ranging from yeast to human [100,101]. In the *N. tobacum* chloroplast genome, the most abundant regions of contiguous purine or pyrimidine tracts are found in the following order: intergenic regions; 3’-downstream and 5’-upstream (promoter) regions; 5’ and 3’ untranslated regions; introns; and coding regions [101]. However, only very few of these tracts were found in prokaryotic genomes (and were most often located in intergenic regions [101]). Naturally occurring sequences capable of adopting H-DNA structures are very abundant in mammalian cells (~1 in every 50,000 bp in humans [102]). Genome-wide scanning of the human genome for long (>100 bp) polypurine-polypyrimidine sequences suggested that most polypurine.polypyrimidine sequences were located in introns, followed by promoters and 5’ or 3’ untranslated regions, and were enriched in genes involved in cell signaling and cell communication [103]. In addition, the genes containing long polypurine-polypyrimidine sequences tended to undergo alternative splicing, were more susceptible to chromosomal translocations, and were expressed at lower levels compared to other genes [103]. Interestingly, there seems to be a different distribution of potential H-DNA-forming sequences in *S. cerevisiae* [104]; the occurrence of polypurine-polypyrimidine sequences with the length of mirror repeat >10 bp was highest in the promoter region, followed by exons, while very few were found in intron and intergenic regions, which is very different from the other reports of such sequences in eukaryotic genomes. It is not yet clear if this discrepancy reflects differences between the yeast and human genomes, or if this difference is due to a bias in the searching programs used.

5.2. Detecting H-DNA in vitro and in vivo

There are a variety of methods to test the presence of an H-DNA conformation *in vitro* or on constructs containing the sequences of interest in *E. coli* using chemicals such as diethyl pyrocarbonate, chloroacetaldehyde, osmium tetroxide (OsO_4), dimethyl sulfate, or psoralen to modify nucleotides specifically in single-stranded DNA or double-stranded DNA [105,106]. Raghavan *et al*. (2006) recently summarized chemical probing procedures to detect non-B

DNA structures in mammalian genomic DNA using either bisulfite or $KMnO_4/OsO_4$ [107]. Triplex-specific monoclonal antibodies have been developed and used to probe the conformation of H-DNA *in vivo* [108,109]. The triplex-specific antibodies showed significant binding to the nuclei and this binding could be inhibited by competing triplex DNA [110]. Another approach to identify H-DNA conformations *in vivo* takes advantage of the single-stranded region exposed in an H-DNA structure, which is capable of hybridization with complementary fluorescence modified single-stranded DNA by “in situ non-denaturing" hybridization. Results from both hybridization probing signals and H-DNA antibody immunostaining were found to be consistent [111]. These studies provide evidence for the existence of H-DNA structures *in vivo*. However, these experiments were not performed under physiological conditions. It is very difficult to detect H-DNA *in vivo* in living cells under native conditions due to the transient and dynamic nature of the H-DNA conformation, the complexity of genomic DNA, the presence of DNA binding proteins, and the lack of specific probes that are highly selective to H-DNA.

6. H-DNA induces genetic instability

Bacolla *et al.* (2006) found that genes carrying long polypurine-polypyrimidine sequences are more susceptible to chromosomal translocations [103]. Certain “fragile site” or “hotspot” regions of the genome are mapped in or near sequences that have the potential to adopt non-B DNA conformations. For example, a segment in the promoter of the human c-*MYC* gene capable of adopting H-DNA [112], overlaps with the one of major breakage hotspots found in c-*MYC*-induced lymphomas and leukemias [113–117]. An H-DNA structure is also found in the *BCL-2* gene major breakpoint region in follicular lymphomas, and disruption of the H-DNA conformation markedly reduces the frequency of translocation events, supporting a role for the H-DNA structure in the formation of the oncogenic translocations [118]. Autosomal dominant polycystic kidney disease (ADPKD) is one of the most frequent inherited single gene disorders, and mutations in the *PKD1* gene account for up to 85% of ADPKD cases [119]. The 21st intron of the human *PKD1* gene was found to contribute to the high mutation rate of the gene in both the germ line and somatic cells [120]. This unstable region contains a 2.5 kb polypyrimidine tract composed of 97% C+T sequences, and 23 H-DNA-forming mirror repeats [121] which forms H-DNA when tested *in vitro* [122]. In addition to the truncation and missense mutations, further analysis revealed complex germ line rearrangements in a 5.8 kb fragment harboring the polypyrimidine tracts of introns 21 and 22 in patients [123]. We have demonstrated that H-DNA structures are intrinsically mutagenic in mammalian cells [124]. Either the endogenous H-DNA-forming sequence from the human *c-MYC* promoter where a breakpoint hotspot is found in diseases such as Burkitt’s lymphoma [125–127], or model H-DNA-forming sequences, induced mutation frequencies ~20-fold above background levels in a *supF* reporter gene in COS-7 cells. Approximately 80% of the mutations were large-scale deletions and/or rearrangements. The structures of the junctions at deletion breakpoints suggested that the H-DNA-forming plasmids had undergone DNA double-strand breaks (DSBs) that were subsequently processed *via* a non-homologous end-joining pathway. In Figure 5 we outline potential H-DNA-induced deletion or translocation pathways. Further, DSBs were found near the H-DNA locus on the plasmids recovered from mammalian cells [124]. Similarly, Bacolla *et al* (2004) found that the 2.5-kbp polypyrimidine sequence in the human PKD1 gene (as discussed above), induced DSBs at the regions that form H-DNA, and resulted in large-scale deletions in *E. coli* [128]. The mechanisms by which H-DNA induces DSBs and genetic instability are still under investigation, though there is evidence that the DNA replication, transcription, and repair proteins may be involved.

6.1. Influences of H-DNA on DNA replication and repair

The existence of H-DNA structures may effectively impede the activity and accuracy of DNA polymerases in replication. *In vitro*, H-DNA structures impose a very strong barrier for Taq DNA polymerase [129]. In a primer extension assay, purified DNA polymerase β showed a 4-fold decrease of polymerase accuracy at a template AG_{11} versus its complementary CT_{11} sequence, and was suggested to be initiated by polymerase dissociation and re-association events due to the H-DNA structure formed at the AG_{11} repeats during synthesis [130]. Purified calf thymus polymerase á was arrested at microsatellite sequences capable of forming H-DNA structures, and also exhibited higher error frequencies [131]. Long GAA repeats (> 40 repeats), which form H-DNA, stalled replication fork progression on plasmids in *S. cerevisiae* [132]. An H-DNA-forming sequence GA(20) in the simian virus 40 (SV40) genome slowed growth in monkey CV1 cells, resulting in lower titers and smaller plaques [133]. GA repeats reduced the rate of nucleotide incorporation into the SV40 genome, resulting in stalled replication forks [133,134]. *E. coli* strains transformed with plasmids containing the 2.5-kbp polypyrimidine sequence from the human PKD1 gene grew slower than those had shorter inserts, and this effect correlated with the level of negative supercoiling of the plasmid DNA *in vivo*, suggesting that the H-DNA structures formed were responsible for the cell growth retardation [135]. Although direct evidence is still not available, it is possible that H-DNA structures formed in mammalian genomes could modulate DNA replication and result in DNA breakage and genetic instability.

Interestingly, our unpublished data suggest that H-DNA is able to induce DNA breakage and mutagenesis in HeLa cell extracts in the absence of replication (Wang and Vasquez, unpublished results), indicating that other factors, such as the DNA transcription, or repair machinery might be involved in the mutagenesis. In *E. coli*, the H-DNA-forming sequence from the human PKD1 gene activated an SOS response and induced significant NER-dependent cell lysis [135]. The same H-DNA-forming sequence also induced deletions in the adjacent GFP reporter gene in *E. coli*, but the mutation frequency was greatly reduced in mismatch repair (MMR) MutS and MutL deficient strains, implicating the MMR pathway in H-DNA structure formation, recognition, and/or processing in bacteria [128].

H-DNA structures have been implicated in stimulating homologous recombination. The single-stranded region in the H-DNA structure could potentially invade a duplex containing a homologous sequence, thereby forming a D-loop structure, which could induce homologous recombination. Alternatively, the single-stranded regions from two H-DNA structures could form Watson-Crick base pairs, and subsequently convert into a Holliday junction, an intermediate that leads to homologous recombination (Figure 6) [105,136]. Furthermore, H-DNA-induced DSBs in mammalian cells are also sources of recombination. In fact, H-DNA-forming sequences are often hotspots of recombination, for example, H-DNA sequences were found at sites of unequal sister chromatid exchange in the Cγ2a and Cγ2b heavy chain genes in the MPC-11 mouse myeloma cell line [137]. H-DNA-forming sequences cloned in plasmids near a neomycin resistance gene stimulated homologous recombination between plasmids in the EJ human bladder cancer cell line, and the level of recombination stimulation was proportional to the ability of the sequences to adopt an H-DNA conformation [138].

7. H-DNA is implicated in transcription regulation

Sequences that are capable of forming H-DNA are found in promoter regions of genes more frequently than expected by random distribution of bases in eukaryotic genomes, suggesting that they may be involved in the regulation of gene expression [139]. There are many published reports that H-DNA can either up-regulate or down-regulate gene expression, depending on a number of factors, including the location of H-DNA in a gene, and the adjacent sequences and elements. In bacteria, when an H-DNA-forming sequence was inserted in a β-*lactamase* promoter, *lacZ* gene expression was increased significantly [140]; when an H-DNA structure

was located at the coding region or between the promoter and coding sequence, a strong down-regulation of gene transcription was observed [141–143].

While a considerable body of evidence exists to support the notion that H-DNA within or near genes can affect gene expression, the mechanisms that are involved are complex and largely undefined. Brahmachari *et al*. (1997) reported that insertion of H-DNA-forming sequences within a *lacZ* reporter gene did not significantly inhibit gene expression in mammalian COS cells [142]. However, the presence of similar sequences upstream of a *lacZ* reporter gene led to a several-fold reduction of gene expression in mammalian cells, which was in contrast to the results from similar studies in *E. coli* [130]. Several studies performed either *in vitro* or *in vivo* in eukaryotic systems resulted in different conclusions, demonstrating either up-regulation [144,145], down-regulation [142,146–149] or no effect of H-DNA on gene transcription [150,151]. One obvious explanation for these differences is that there may be very different mechanisms involved under each particular condition and thus, should be studied case by case.

The transcriptional activity of minimal mouse albumin promoters in HeLa cells containing various mutant H-DNA-forming sequences derived from the human *c-MYC* promoter can be predicted by the ability of the particular sequences to form H-DNA and not by repeat number, position, or the number of mutant base pairs [144]. The H-DNA-forming sequence from the *c-MYC* promoter serves as a cis-acting element interacting with ribonucleoprotein and other transcription factors in mammalian cells and cell nuclear extracts [152]. When an H-DNA-forming sequence is located in close proximity to a TATA box, it results in an unwound region adjacent to the TATA box, destabilizing the T-A hydrogen bonds. This structural alteration in the TATA region may inhibit the initial recognition process by transcription factors at the transcription initiation site which may require double-stranded DNA [98]. H-DNA is a relatively rigid structure; once formed a 130° bend is induced in the DNA strand, which may bring distant cis-elements in close proximity to promoters to regulate transcription initiation [149,153]. Alternatively, an H-DNA structure formed between two adjacent cis-elements may disrupt the cooperation between proximal elements [149]. For example, an H-DNA-forming sequence located ~1.8 kbp upstream of the transcription start of the rat hsp70.1 stress gene, increased transcription of a reporter gene by abolishing the effect of an adjacent putative silencing element in rat hepatoma cells [154].

Although transcribed regions are not the most H-DNA-enriched regions in mammalian genomes, the ability of H-DNA-forming sequences to adopt H-DNA conformations may be enhanced in these regions. H-DNA formation can be induced by the negative supercoiling generated by the transcription machinery, and in some cases, further stabilized by the binding of nascent purine-rich RNA to the single-stranded DNA in H-DNA structure. Once formed, these structures may inhibit or block the transcription machinery [147]. We have recently reported that an S1-sensitive element from the *c-MYC* promoter, which has the potential to form either a H-DNA or a quadruplex structure, blocks T7 RNA polymerase in an *in vitro* assay, resulting in partial transcription product arrest. Further, various nucleotide substitutions were introduced to specifically destabilize either the triplex structure or the quadruplex structure, and the result suggested that the triplex structure, but not quadruplex, was responsible for the transcription arrest [155].

8. Modulating H-DNA structure as a potential gene targeting strategy

Anticancer agents that target DNA are among the most effective agents in cancer therapeutics, but are often extremely toxic due to lack of specificity for the tumor cells. Although the mechanisms by which H-DNA influences DNA metabolism are not well understood, it is clear that it plays important roles in a variety of DNA processes, and the unique structure of H-DNA

provides a potential target for a the development of a new class of more selective DNA-based therapeutics.

8.1. Targeting the H-DNA structure

A potential promising therapeutic target is the single-stranded DNA region exposed in an H-DNA structure. When a plasmid containing H-DNA and a polypurine oligonucleotide complementary to the single-stranded polypyrimidine region of the H-DNA structure were transfected into HeLa cells, up to 90% of the oligonucleotides could be recovered after 24 hours, while the same oligonucleotides were predominantly eliminated when co-transfected with a control plasmid unable to form H-DNA [156]. Ohno *et al.* (2002) used complementary fluorescence-modified single-stranded DNA to detect H-DNA conformation *in vivo* [111]. Although the biological function of this oligonucleotide binding was not explored in these particular studies (*e.g.* whether or not complementary oligonucleotide binding can stabilize the H-DNA structure, the effect on gene expression, DNA replication and genetic instability), the high specificity and affinity of binding and the stability of the oligonucleotide once bound to H-DNA makes this a reasonable approach to develop potential gene targeting therapies. Expanded GAA.TTC trinucleotide repeats in intron 1 of the frataxin gene are involved in Friedreich's ataxia (FRDA) disease etiology, and it is known that these repeats can reduce gene expression by forming H-DNA structures [157]. The use of an oligonucleotide that binds the single-stranded GAA sequence during transcription resulted in inhibition of H-DNA formation in the frataxin gene, and to a subsequent frataxin gene specific increase in full-length transcript, providing a therapeutic strategy to prevent or treat FRDA [158]. In addition to the effect of destabilizing H-DNA formation to regulate gene expression, H-DNA complementary oligonucleotides may also be covalently attached to DNA damaging agents to direct DNA damage to the corresponding H-DNA structure, similar to the sequence-specific delivery of DNA-damaging agents by TFOs (see Section 3.4 above).

8.2. Stabilizing H-DNA

Triplex-specific monoclonal antibodies have been developed to detect H-DNA structure *in vivo*, and to regulate H-DNA-related effects on DNA metabolic processes, *via* either structure stabilization, destabilization, and/or interference with the related trans-acting proteins. Introduction of triplex-specific monoclonal antibodies into permeabilized mouse myeloma cells inhibited DNA replication and total transcription in the nuclei by ~20%, resulting in a significant decrease in cell growth without an increase in cell death [110].

In addition to interacting with the H-DNA structure directly, molecules that interfere with H-DNA-binding proteins may also be useful in modulating H-DNA-related metabolism. Cellular nucleic acid binding protein (CNBP) binds to the polypurine single-stranded region of H-DNA and increases H-DNA-induced transcription *in vivo*. Purine-rich oligonucleotides that effectively competed with the binding of CNBP to the H-DNA-forming sequence in the human *c-MYC* promoter eliminated ~90% of H-DNA-mediated transcription in an *in vitro* transcription reaction [159].

Positively charged Lys- and Arg-rich oligopeptides, and spermine have been shown to stabilize the H-DNA conformation by interacting with the unpaired single stranded region and by neutralization of electrostatic repulsion of negatively charged phosphates in triplex molecule [160]. Small chemical compounds such as these have the advantage of efficient cellular uptake. A promising class of H-DNA-modulating agents includes DNA minor groove binders or intercalators which can bind to triplex structures. While minor groove binders typically destabilize triplexes, intercalators often stabilize this conformation by providing an aromatic surface area for stacking with triplex bases in an intercalation complex [161]. Most of these compounds were evaluated on intermolecular triplex structures, however, such molecules have

the potential to modulate H-DNA as well, and it will be of interest to evaluate them on H-DNA structures. The antitumor agent, benzo[e]pyridoindole (BePI), has a higher affinity for triplex than for duplex DNA, and stabilizes H-DNA structures formed on plasmids inserted between the promoter and the coding sequence of the β-*lactamase* gene. The presence of BePI enhanced the H-DNA-induced transcription inhibition through the β-*lactamase* gene in *E. coli* cells, demonstrating the potential of small molecules as H-DNA modulators in cells [143].

9. Concluding Remarks

The formation of triplex DNA, either in an intramolecular fashion from the same DNA molecule, or in an intermolecular fashion by delivery of a TFO into cells, has very attractive application potential. Naturally occurring intramolecular triplexes play important roles in regulating DNA metabolism and gene function, and are inherently mutagenic and recombinogenic. Regulating H-DNA conformation or specifically interfering with H-DNA-related interactions using small molecules or oligonucleotides presents a promising gene targeting strategy. In the past decades, numerous published findings support the utility of TFOs as sequence-specific gene targeting agents for modulating gene function and/or modifying DNA sequence, and great efforts have been made to increase the efficiency and specificity of this gene targeting approach. However, the mechanisms by which (inter- and intramolecular) triplex structures regulate DNA metabolism, such as replication, transcription, recombination, and mutation, are still under investigation. An important topic of future efforts toward this goal includes identifying the proteins that recognize and bind to triplex DNA, stabilize or unwind triplex DNA, and that cleave or repair the triplex structure. Indeed, rather remarkable first steps have already been made toward characterizing the structure and function of triplex DNA. These efforts have been valuable in designing more specific and efficient triplex-related gene targeting agents.

Abbreviations

2’-OMe, 2’-O-methylribose
2’-AE, 2’-O-aminoethylribose
ADPKD, autosomal dominant polycystic kidney disease
BePI, benzo[e]pyridoindole
BNAs, bridged nucleic acids
CNBP, cellular nucleic acid binding protein
DSBs, double-strand breaks
FRDA, Friedreich’s ataxia
HMGB1, high mobility group B1
LNAs, locked nucleic acids
MMR, mismatch repair
NER, nucleotide excision repair
OsO_4, osmium tetroxide
PNAs, peptide nucleic acids
TFOs, triplex-forming oligonucleotides

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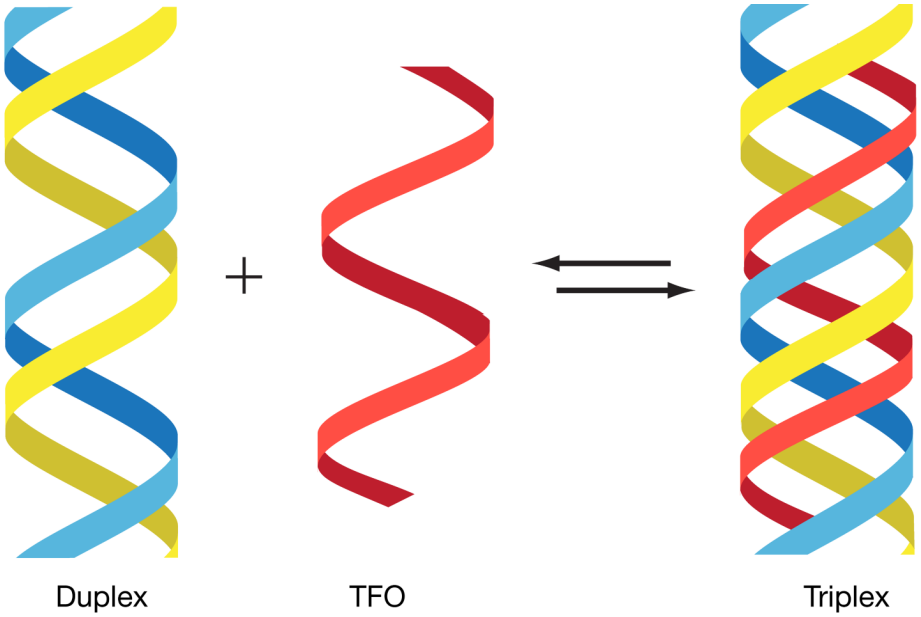

Figure 1. Schematic representation of intermolecular DNA triplex formation

In the target duplex, the purine and pyrimidine strands are shown in blue and yellow, respectively. The TFO, which binds to the purine-rich strand of the target duplex through the major groove, is indicated in red.

Figure 2. Triplex-forming sequences in the human *c-MYC* gene

The TFO is placed in an antiparallel orientation relative to the target duplex from the human *c-MYC* P2 promoter. Vertical lines indicate Watson-Crick hydrogen bonds and stars indicate reverse Hoogsteen hydrogen bonding.

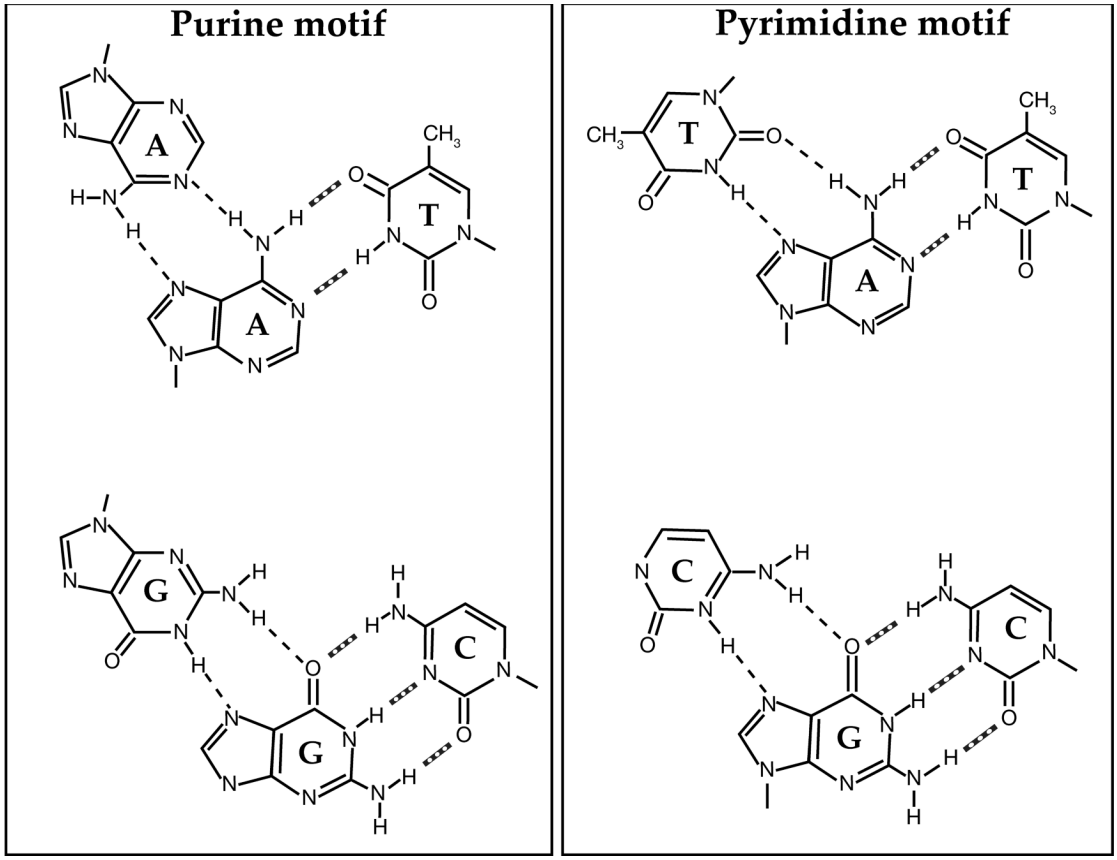

Figure 3. Schematic representation of canonical base triplets formed in purine and pyrimidine triplex motifs

Watson-Crick base pairing is illustrated by dotted lines, and Hoogsteen base pairing by broken lines.

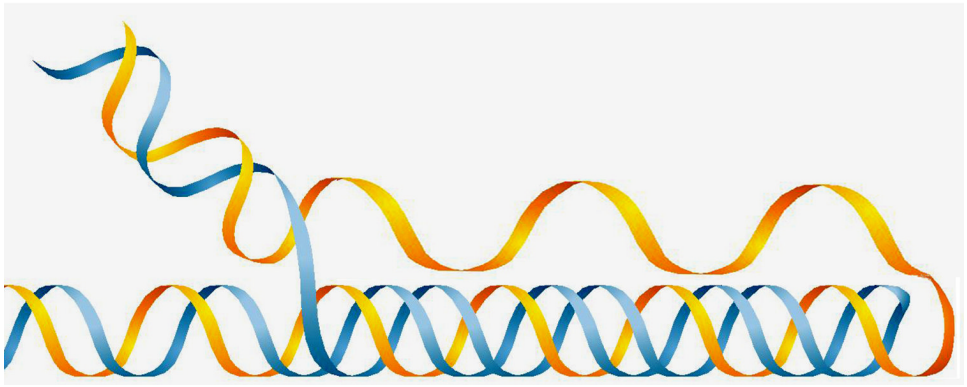

Figure 4. H-DNA (intramolecular triplex DNA)

In the polypurine-polypyrimidine tract with mirror repeat symmetry, one of the single strands (shown in blue) folds back and forms triplex structure and the other strand (shown in yellow) is left unpaired.

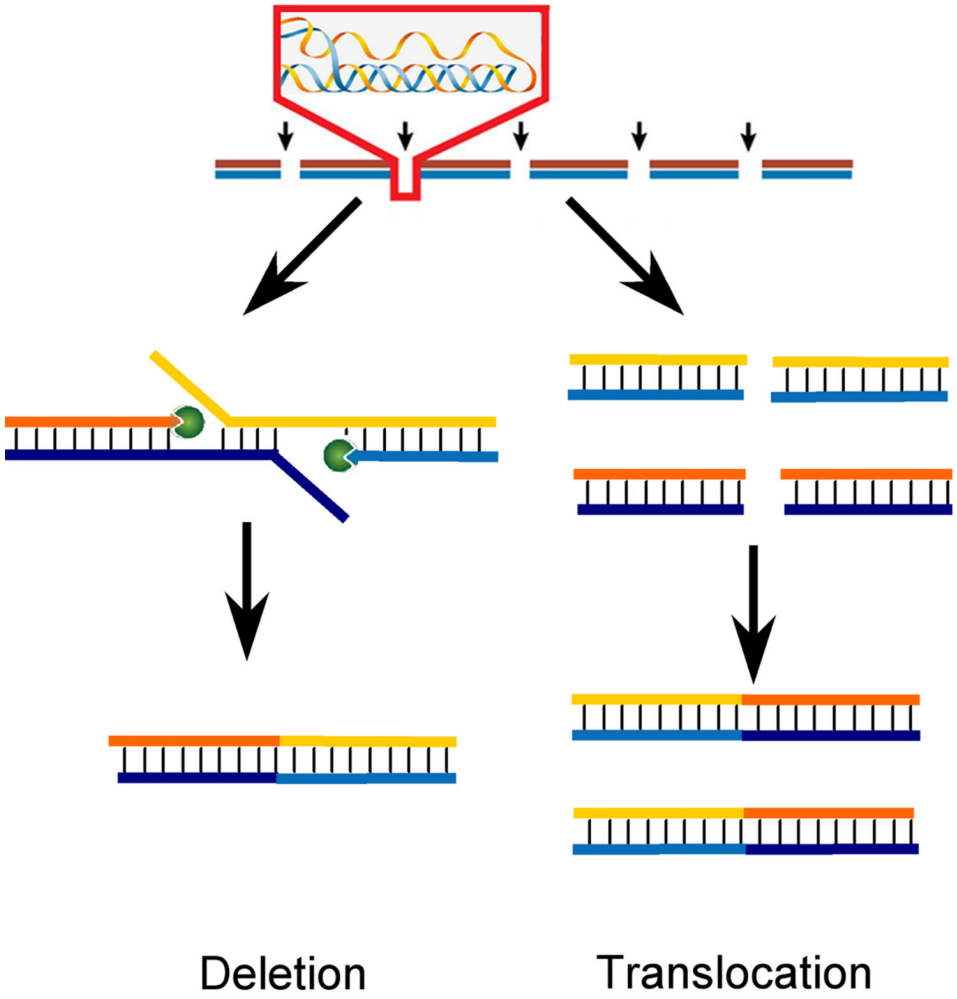

Figure 5. H-DNA structure-induced genetic instability in mammalian cells

DSBs (chromosomal breakage) surrounding the H-DNA are generated by as yet undefined enzymes. Non-homologous end-joining repair at DSBs in mammalian cells can result in large-scale deletions, translocations and rearrangements. Adapted from [162].

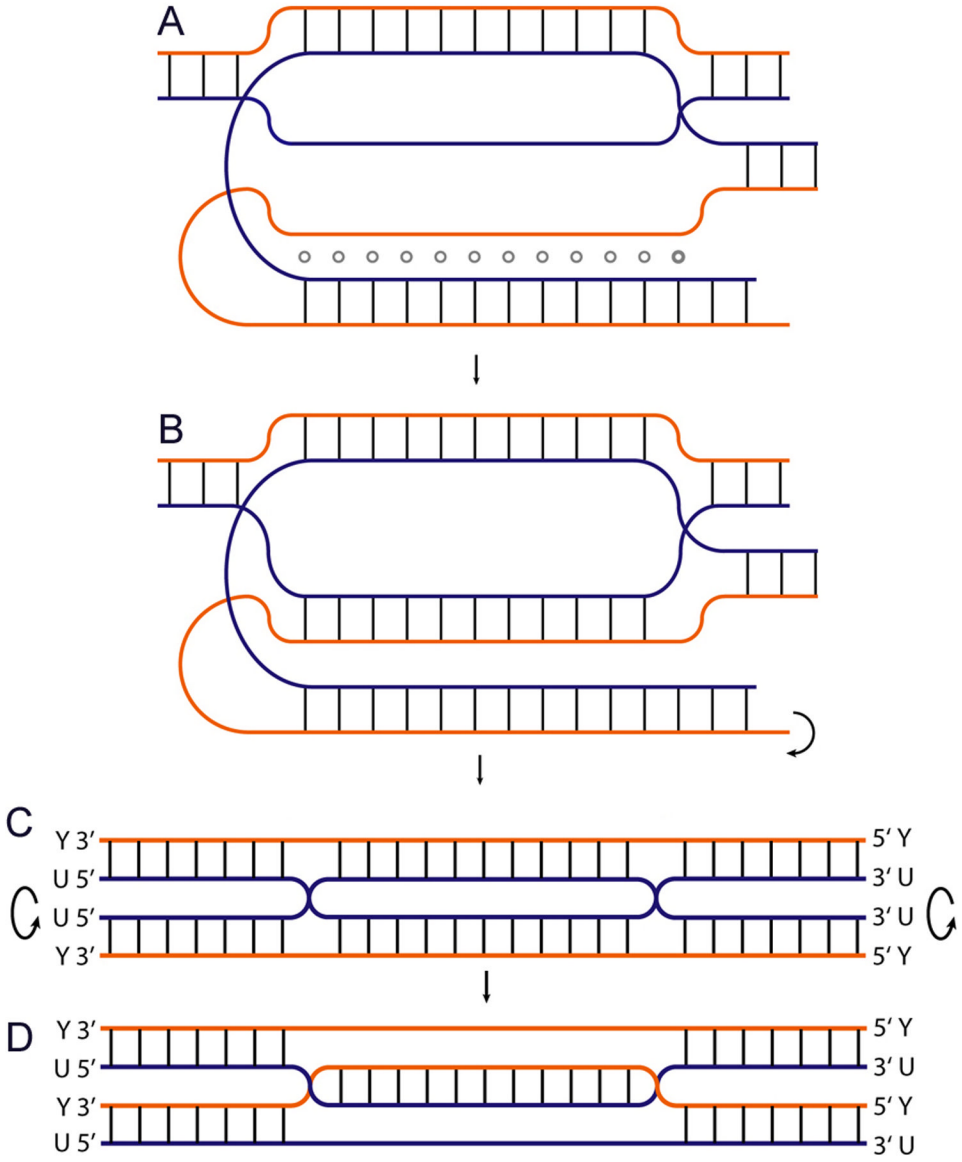

Figure 6. A model for H-DNA induced recombination

(A) The single strand region of the H-DNA structure may invade and pair with a complementary strand of an homologous duplex. **(B)** The third strand in the H-DNA structure could form Watson-Crick base pairs with the released single strand from the homologous duplex to form a double four-way Holliday junction. **(C)** and **(D)** The junction can be rotated and resolved to non-crossover and crossover products. Adapted from [136].