(2′-F) or 2′-methoxy (2′-OMe) has also been carried out. These electronegative substituents induce a preference for the 3′-endo conformation of ribose (Figure 6.9) which is the structural conformation in A-type duplexes. PNA, LNA, and 2′-substituted nucleic acid analogs have all been pursued as potential drug candidates, as well as diagnostic tools.

A range of other analogs in which the ribose is replaced by six-membered ring structures includes hexitol nucleic acids (HNA) and cyclohexene nucleic acids (CeNA) (Figure 6.9). The hexitol moiety of HNA adopts an anhydrohexitol chair conformation which is a faithful mimic of the ribose C3′-endo conformation in RNA (Figure 6.9). Thus, HNA can form duplexes that are highly similar to the A-type helical structure and the bases of HNAs are oriented in an axial position leading to stronger base pairing. In contrast, CeNAs are more flexible around the C2′–C3′ bond as the cyclohexenyl moiety easily adopts different conformations. Importantly, both HNAs and CeNAs are resistant to nuclease degradation.

Generation of XNA polymers was until recently, generally achieved by chemical synthesis since most unnatural nucleotide analogs are poor polymerase substrates. Pioneering work on re-design of RNA polymerases identified enzyme variants that were capable of synthesizing XNAs nearly fully substituted with nucleotides harboring 2′-OMe modified sugars. More recently, a combination of rational design and directed evolution of DNA polymerases enabled the synthesis of eight different XNAs including HNA, CeNA, LNA, and 2′-F substituted DNA. Notably, the XNAs could be inversely transcribed into DNA and evolved into high-affinity aptamers, demonstrating that the XNAs comprise the capacity for both heredity and evolution. Subsequently, six different XNA enzymes (XNAzymes) were discovered including HNAzymes and CeNAzymes capable of cleaving and ligating RNA.

Chemical modification of nucleobases generally does not modulate stability to nucleases, but allows for functional versatility, and thus for complementary storage of genetic information. With only four different bases or nucleotides, compared to the 20 different amino acids in proteins, the functional diversity of nucleic acids is confined. In an attempt to address this, a substantial number of unnatural base pairs (UBPs) have been developed. This has been successfully achieved, for example, by reshuffling of the hydrogen-bond donor and acceptor patterns (Figure 6.10). Even though some of these lack the complementary hydrogen bonds that underlie Watson–Crick base pairing, it has been demonstrated that they can be replicated in vitro with efficiencies approaching the natural bases. Similarly, other UBPs have been efficiently incorporated and replicated in vitro, but incorporating unnatural bases into DNA in vivo is a substantial bigger challenge. A number of criteria need to be fulfilled: first, the unnatural nucleoside triphosphate building blocks have to be introduced into the cells; second, the endogenous polymerases must incorporate the unnatural building block within a complex intracellular environment; third, the UBP needs to be formed during replication enabling duplex formation and finally, the UBP must be resistant toward the endogenous DNA repair mechanisms. Remarkably, the incorporation of an unnatural base pair in *Escherichia coli* was achieved by importing the unnatural building blocks via an exogenously expressed nucleotide triphosphate transporter into cells transformed with an artificial plasmid coding for the UBP. In this way, a semi-synthetic organism with an expanded genetic alphabet was generated. This could enable the site-specific replacement of a wide range of chemical functionalities in standard DNA, thereby enhancing the functional diversity of, for example, DNA aptamers and DNAzymes.

6.4.2 RNA Interference

The first evidence for small regulatory RNAs was demonstrated with the discovery of Lin-4 which is a miRNA that controls the temporal development of the nematode *Caenorhabditis elegans*. It was later demonstrated that Lin-4-mediated gene silencing was triggered by double-stranded RNA (dsRNA) precursors that inhibited protein translation by base-pairing to complementary mRNA in a process termed RNA interference (RNAi) (Figure 6.11). Subsequently, small synthetic dsRNAs were used to demonstrate that RNAi pathways are also present in mammalian cells.