Chapter 1

Introduction

Introduction

The information encoding the regulation and expression of the estimated 20,000–25,000 genes in the human genome and their products is contained in the nucleotide sequence of DNA[1,2]. At the simplest level, the information encoded by nucleotide sequences is expressed through a process of transcription of DNA information to an RNA message, followed by translation into the final protein product. States of human disease are frequently the result of gene expression disregulation, and the discovery and development of technologies capable of granting control over the process of gene expression would have wide-ranging applications in human health.

Structure of DNA

DNA is comprised of two complimentary polydeoxyribonucleotide strands paired in an antiparallel fashion through formation of hydrogen-bonding contacts between the nucleotide bases: adenine pairs with thymine, and guanine with cytosine (figure 1.1).[3] Although DNA can adopt a number of conformations, the biologically relevant is B-form DNA, in which the nucleotide pairing produces a double-helical strand with a wide and shallow major groove, and a deep, narrow minor groove between the phosphate backbones. To a rough approximation, the DNA double helix is uniform, but a series of hydrogen-bonding donors and acceptors located on the edges of nucleotide bases are presented in the major and minor grooves that function as "handholds" for DNA-binding proteins and natural products.

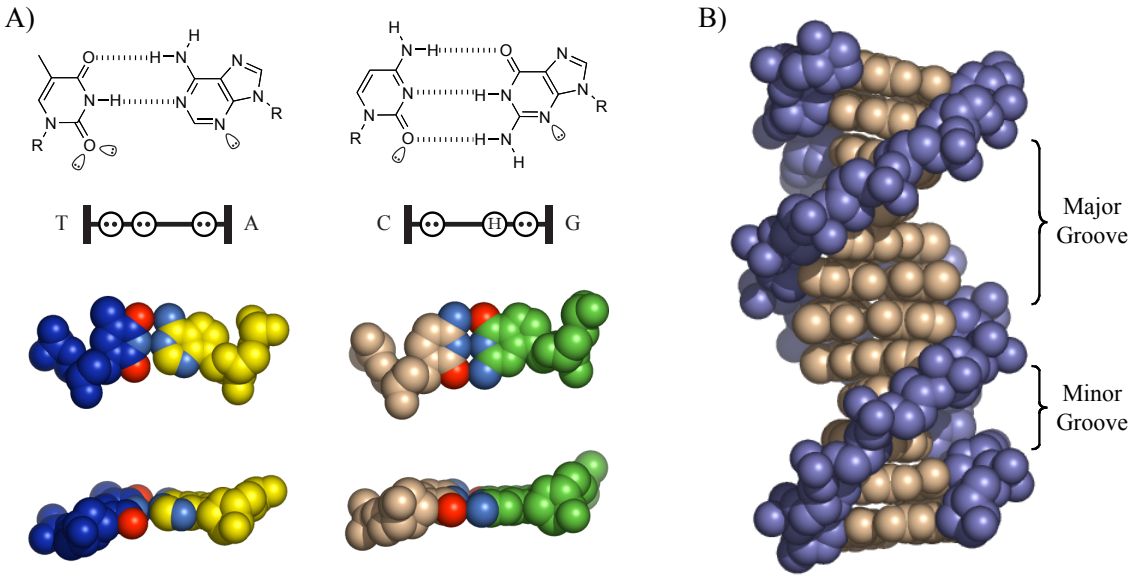

Figure 1.1. Schematic illustration of B-form DNA. A) Schematic of hydrogen-bond formation between a T•A basepair and a C•G basepair. Also shown is a schematic representation of the hydrogen-bonding donor and acceptor pattern presented by the basepair edges into the minor groove. Lone pairs are represented by two dots in an open circle, and hydrogen bonding donors represented by an "H" in an open circle. B) Structure of B-form DNA to illustrate the major and minor grooves of B-form DNA.

DNA-Binding Natural Products

Two natural products that bind DNA with some degree of sequence specificity are Hoechst 33258 and distamycin (figure 1.2).[4-7] Courtesy of their crescent shape, both these relatively simple heterocycle oligomers fit neatly into the minor groove of DNA to form hydrogen bonds with the nucleotide edges. Distamycin can form either a 1:1 complex or a 2:1

complex with DNA: the latter is illustrated in figure 1.2B). Distamycin has a preference for A,T tracts of DNA due to hydrogen-bonding contacts between the amide-bond hydrogen and minor-groove lone pairs; G•C pairs present an energetically unfavorable steric clash between the exocyclic amine of guanine and hydrogens on the pyrrole ring. Replacement of a pyrrole ring with an N-methylimidazole ring, however, enabled G•C recognition via the Nitrogen lone pair, which forms a hydrogen bond with the guanine exocyclic amine.[8]

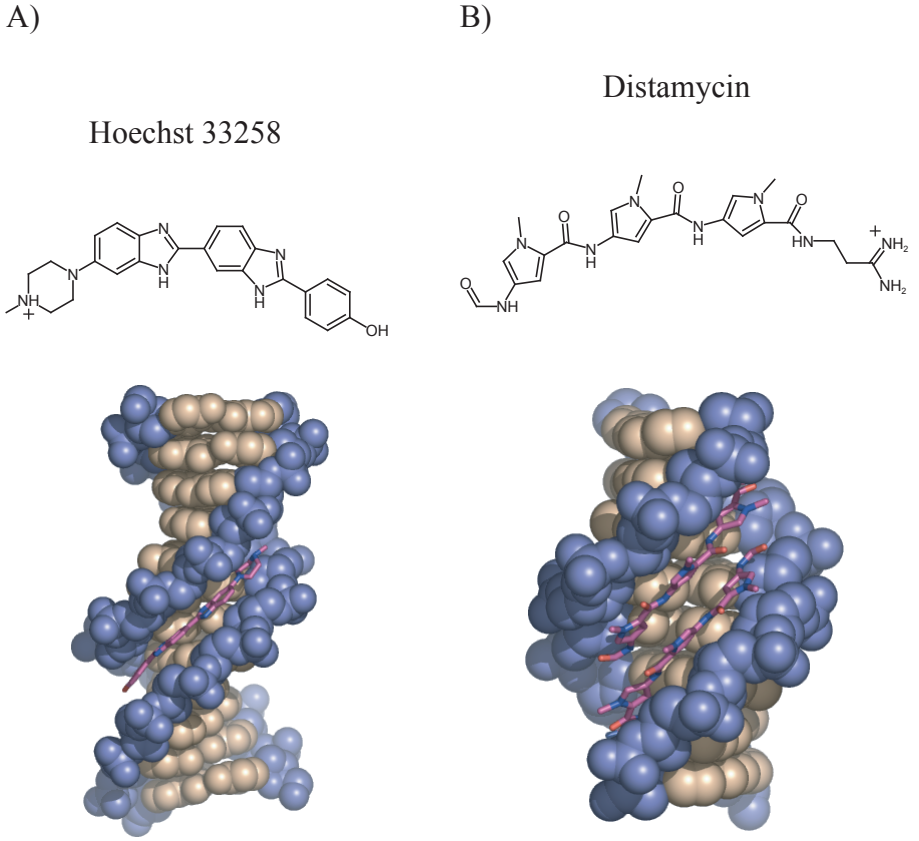

Figure 1.2. Schematic illustration of the chemical structure of Hoechst 33258 and Distamycin, and structures of DNA-Hoechst 33258 and DNA-distamycin complexes.

Polyamide Recognition of DNA

Polyamides are a class of synthetic small molecules that bind DNA in a sequence-specific manner modeled on Distamycin-DNA recognition through hydrogen-bonding interactions with hydrogen-bonding donors and acceptors in the floor of the DNA-minor groove. Polyamides are oligomers of N-methylpyrrole (Py), N-methylimidazole (Im), N-methyl-3-hydroxypyrrole (Hp), and 3-chlorothiophene (Ct) carboxamides linked through amide

bonds.[9-16] DNA binding can occur in a 1:1 or a 2:1 fashion, with polyamide strands generally aligning N → C with the 5' → 3' DNA strand direction. The sickle shape of polyamides closely matches the radius of the DNA minor groove, and allows polyamides to approach the DNA minor groove floor closely enough to engage in these hydrogen-bonding interactions.

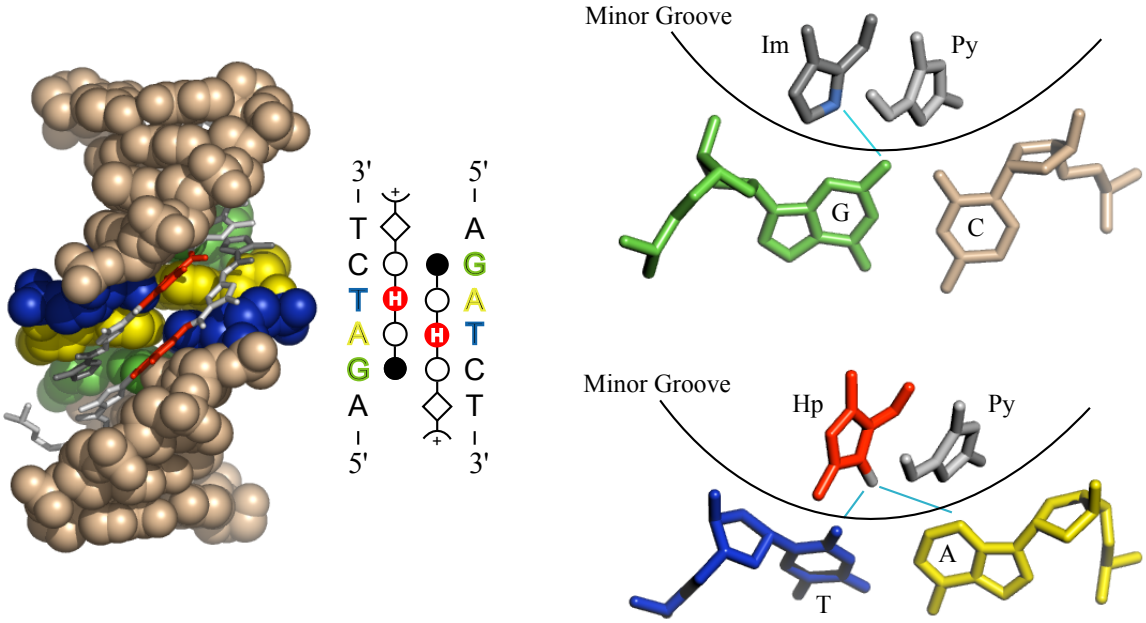

Figure 1.3. Structure of a 2:1 polyamide-DNA complex, and illustration of hydrogen-bonding interactions between the polyamide heterocycle core and the minor groove floor.

Polyamide sequence specificity is programmed through side-by-side pairings of heterocyclic amino acids in the DNA minor groove: Im/Py recognizes G•C over C•G; Py/Py is degenerate for A•T and T•A; Hp/Py and Hz/Py distinguish T•A for A•T; and Ct/Py prefers T•A over A•T in the cap position. These pairing guidelines were revealed through quantitative DNase I footprinting titrations, as well as NMR and crystallographic structural data. These data have also elucidated the peculiarities of polyamide-DNA interactions that give rise to the sequence specificity. A network of hydrogen bonding interactions is created between the polyamide amide bonds and base-pair edges, while hydrogen bonding between the nitrogen lone pair of Im and the exocyclic amine of guanine forms the basis for G•C recognition (figure 1.4).[17-19] Hp/Py recognizes T•A through hydrogen bond formation between the Hp hydroxyl and the thymine-O2 lone pair along with shape-

selective recognition of an asymmetric cleft between thymine-O2 and adenine-C2.[17, 19] The proposed recognition of T•A at the amino terminus by Ct/Py projection of the 3-chloro substituent awaits structural verification.[20]

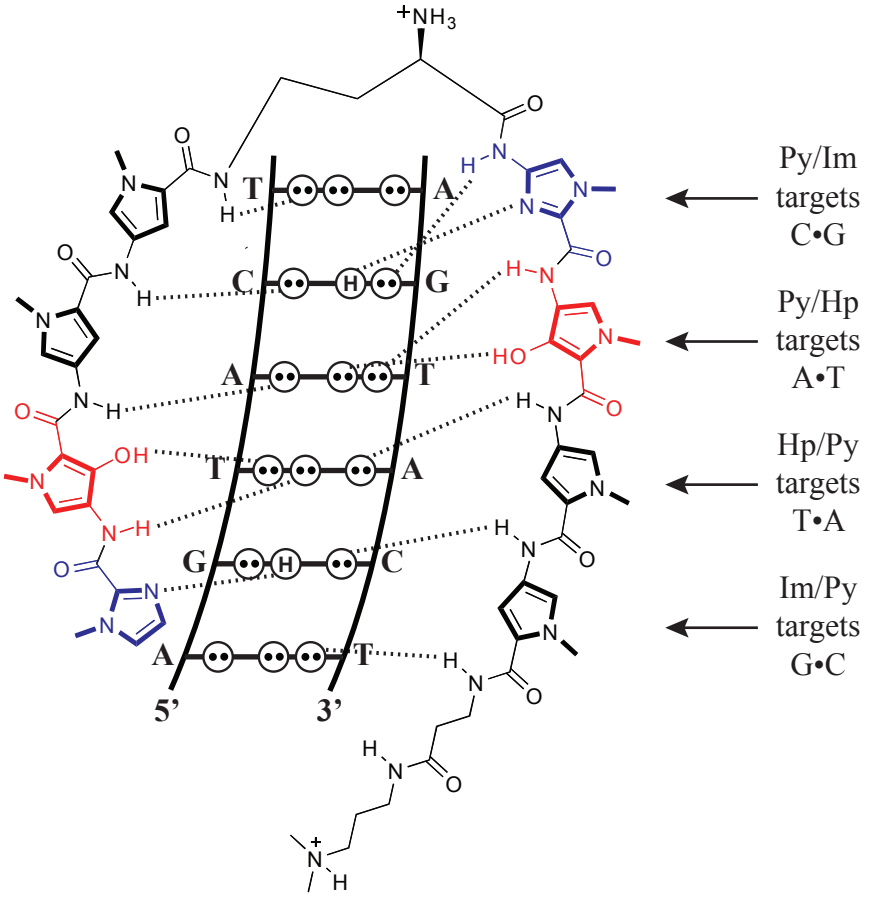

Figure 1.4. Schematic illustration of the system of hydrogen bonding pattern between the DNA nucleotide edges and an eight-ring hairpin polyamide that gives rise to DNA recognition by polyamides.

A number of polyamide architectures have been explored, and the most commonly deployed is the eight-ring hairpin polyamide with a binding site of six basepairs (figures 1.4, 1.5). Linking two antiparallel heterocycle strands through an aliphatic linker group to create a "hairpin" polyamide results in substantial gains in DNA-binding affinity ($K_a(M^{-1})$).[21] This gain in binding affinity is thought to occur as a result of reduction in the entropic cost of polyamide-DNA binding. Linking the two strands additionally enforces side-by-side heterocycle pairing rather than the slipped binding modes available to unlinked heterocycle strands.[21, 22] The most commonly employed turn group has been the γ-amino butyric acid, optionally with a chiral amine moiety at the α carbon.[23] The presence of this amine moiety

imbues the turn residue with a strong preference for T•A and A•T pairs over G•C and C•G pairs.[21]

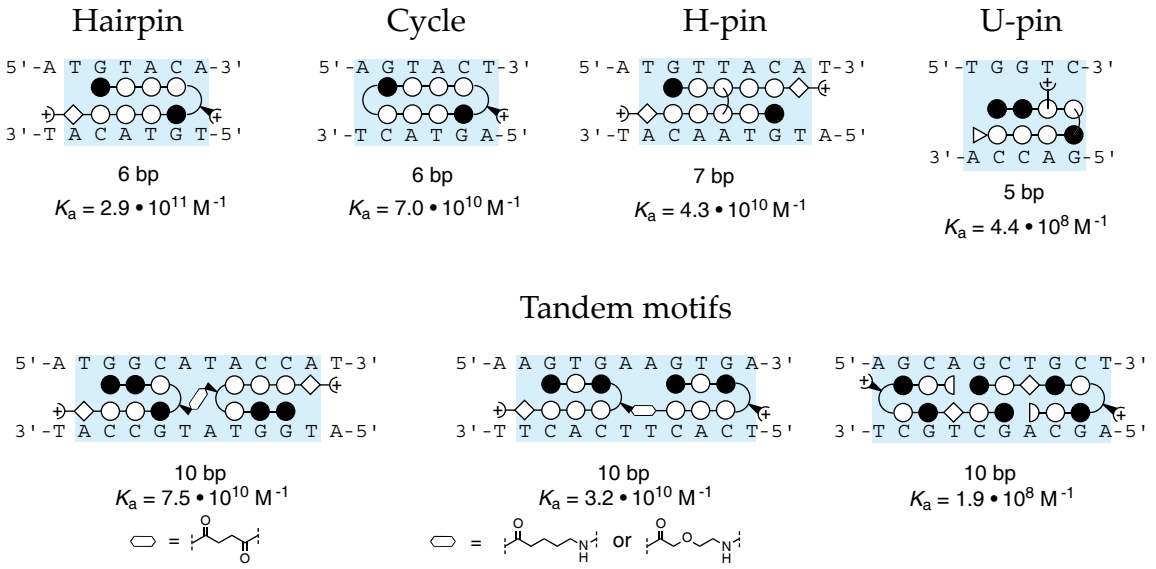

Figure 1.5. Schematic ball-and-stick representations of various polyamide structural motifs. Below each motif is given the binding site size and binding affinities (K_a (M^{-1})).

The modular nature of polyamides makes their synthesis amenable to solid-phase synthesis methods on resins including β-Ala-PAM and Kaiser oxime resin; use of β-Ala-PAM resin results in a polyamide product with a C-terminal β-alanine moiety that is specific for A•T basepairs (figure 1.6).[24, 25] Following resin loading with the initial C-terminal, standard Boc coupling chemistry is used to build the polyamide in the C→N direction. Aminolysis provides the cleaved polyamide, and judicious selection of the cleaving group allows further elaboration of the polyamide with all manner of C-terminal tail groups. Solid-phase synthesis has been successfully exploited to access rapidly a wide range of polyamide heterocycle cores in the relatively small quantities required for exploratory research, but can be adapted in a relatively straightforward fashion to produce larger quantities of polyamide product. Production of large quantities of particular cores is facilitated through synthesis of heterocycle trimers or tetramers, which can be readily introduced into the polyamide core on resin. Recently, the first solution-phase synthesis of a polyamide in our group yielded gram-scale polyamide product.

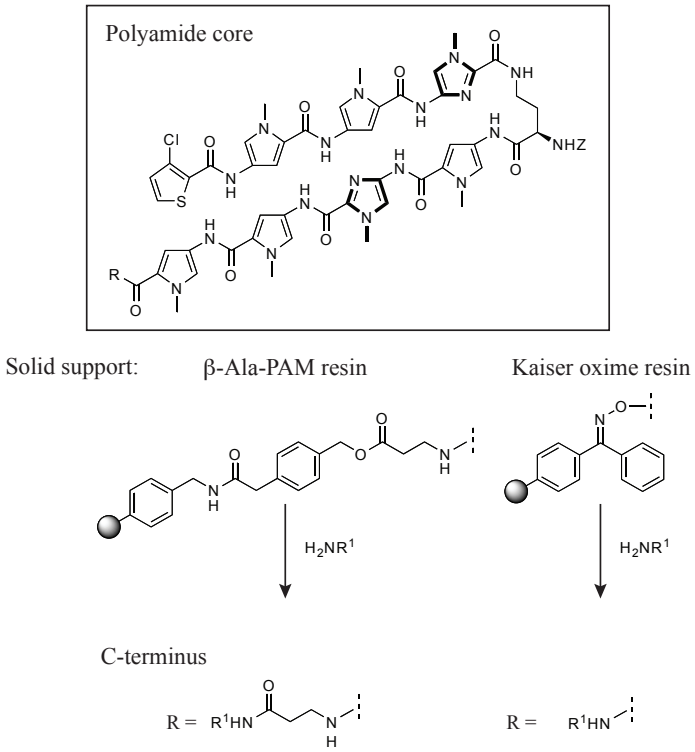

Figure 1.6. Schematic illustration of solid-phase synthesis of polyamides.

Regulation of Gene Expression with Polyamides:

The sequence-specificity of polyamides presents a potentially powerful tool for modulating gene expression in pursuit of disease treatment. Two possible mechansims by which gene regulation could be achieved are illustrated schematically below (figure 1.7). The first involves direct steric blockade of transcription factor binding in the promoter region of a gene, and the second mechanism is based on recruiting transcriptional machinery to the promoter through use of activator domains.

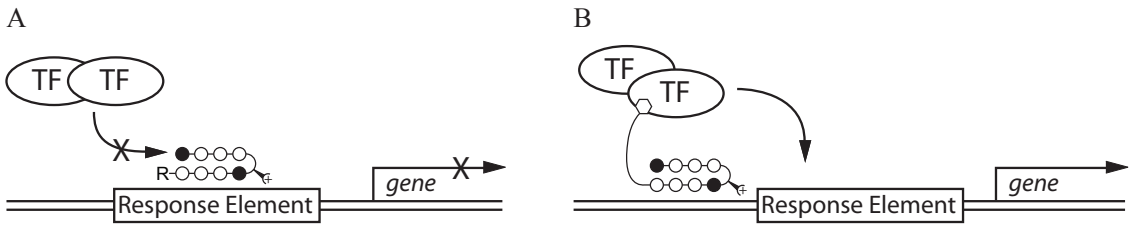

Figure 1.7. Schematic of proposed mechanisms of polyamide gene expression regulation through modulating the DNA-protein interface. A) Inhibition of transcription factor (TF) protein-DNA binding through direct steric blockade at a TF recognition element (RE). B) Polyamide elaboration with protein recruitment domains to increase local concentration of targeted DNA-binding proteins.

The binding affinity of most eight-ring hairpin polyamides is on the order of 10^9-10^{10} M^{-1}, which makes them competitive with DNA-binding proteins such as transcription factors and transcriptional activators (figure 1.8).[26] By targeting polyamides to protein binding sites or adjacent sequences, *in vitro* studies have demonstrated the ability of polyamides to inhibit DNA binding of proteins through direct steric or allosteric effects.[27] Major-groove binding proteins have been shown to co-occupy DNA sequences with minor-groove binding polyamides, but they can be displaced by intercalator-polyamide conjugates.[28-30]

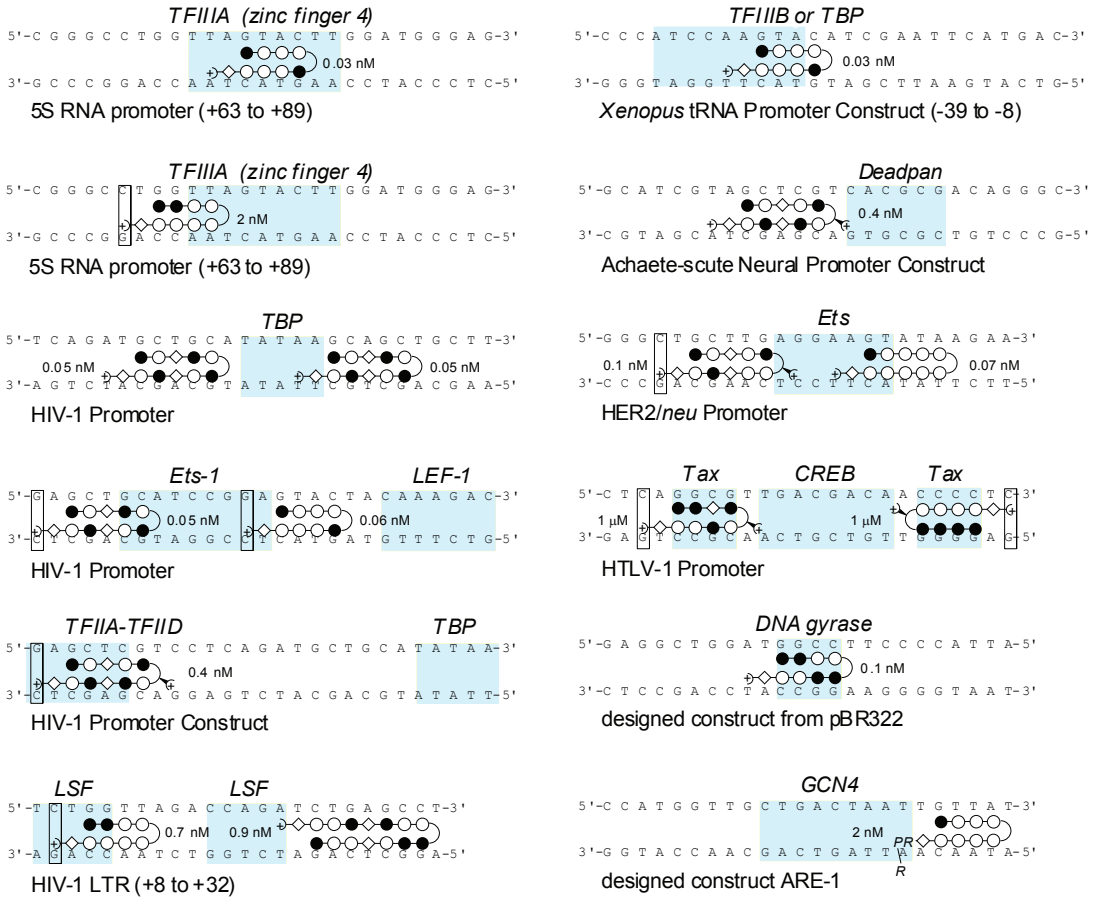

Figure 1.8: Schematic illustration in the ball-and-stick fashion of inhibition of protein binding by polyamides. Indicated by light-blue boxes are the protein binding sites to show overlap between polyamide and protein binding sites. Also given are polyamide dissociation constants (K_a (M^{-1})).

A further point of comparison between polyamides and DNA-binding proteins is their relatively limited recognition sequences. Given the size of the genome, it is clear that a much longer sequence than the six bases recognized by an eight-ring hairpin polyamide would be required to target a unique DNA binding site. The limited size of polyamide

binding sites may result in promiscuous polyamide binding thruought the genome and numerous "off-target" effects. This concern is mitigated by consideration of known DNA-protein interactions. Protein-DNA recognition, e.g. transcription factor-DNA recognition, is the result of a combination of electrostatic interactions with the polyanionic-DNA backbone and Van der Waals forces acting between the protein and the major or minor groove floors.[31-36] In the cellular context, the DNA double helix is not suspended in the nucleus in isolation, but is constantly interacting with proteins and other molecules in the nuclear millieu. These protein binding sites are generally no more than four basepairs, the same size as eight-ring hairpin polyamide binding sites.[37]

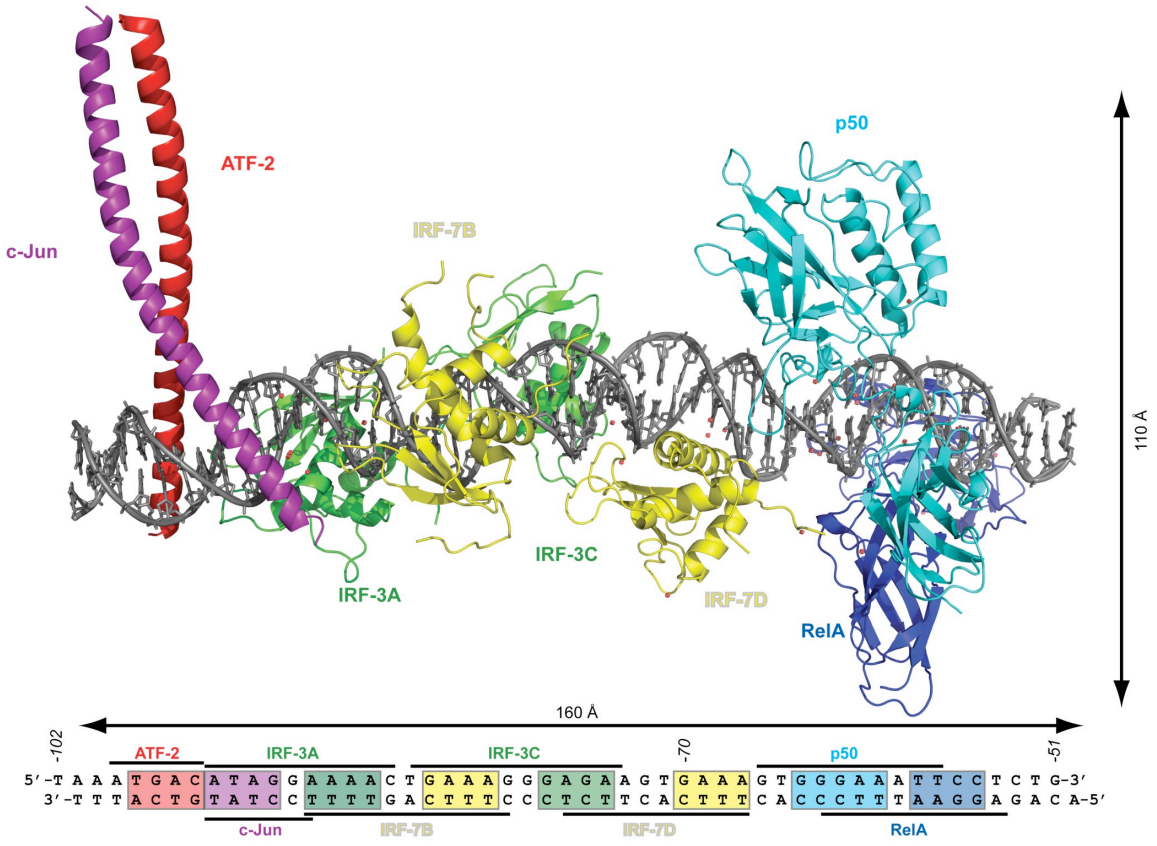

Figure 1.9. Structure of the interferon-b enhanceosome complex to demonstrate the relatively limited size of protein DNA binding sites, and protein-induced perturbations in the DNA double helix. Figure based on Figure 4 in Reference 37, and generated in PyMOL using a composite binding model obtained from the pdb file in the Supplemental Information.

Nuclear Localization of Polyamides:

A central aim is the use of polyamides in biological systems and whole organisms to regulate endogenous genes that are critical mediators of health and disease. To realize this

goal, polyamides must be able to enter the cell and accumulate in the nucleus in sufficient quantities to bind to the appropriate DNA sequence and disrupt the protein-DNA interface. The mechanism by which polyamides enter the cell and translocate to the nucleus is not well understood. Previous work in the Dervan group has shown that polyamide cell uptake is an energy-dependent process, as it is impeded by lowered temperatures and in glucose-lacking cell culture media[38]. Further, internal data have demonstrated that the biological activity of polyamides is enhanced in some cases by co-administration of verapamil, which inhibits function of drug-efflux pathways.[39]

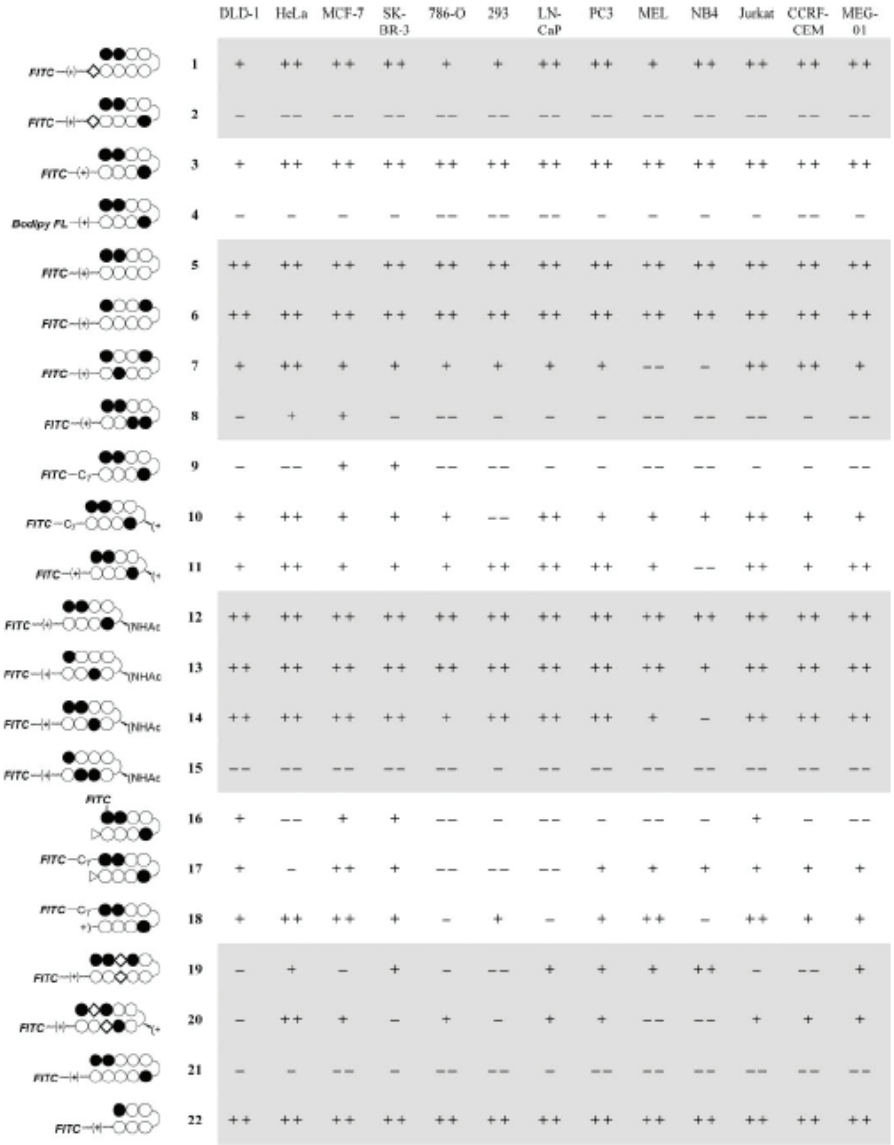

Figure 1.10. Cellular localization of 22 polyamide-fluorophore conjugates in a panel of 13 cultured cell lines. The extent of polyamide nuclear localization is indicated by a +/- nomenclature: ++, nuclear staining exceeds that of the medium: +, nuclear staining comparable to the medium, but still clear: -, very little nuclear staining predominantly cytoplasmic and medium staining; --, no nuclear staining, little cytoplasmic staining.

Extensive attention has been devoted to determining the structural features of polyamides that play a role in nuclear accumulation. Initial investigations were inspired by the work of Bashkin *et al.* demonstrating that a C-terminal fluorescein (FITC) dye enabled demonstrable nuclear localization visualized by confocal microscopy of live, unfixed cells.[39] Following this seminal piece, confocal microscopy techniques were used to examine the nuclear and subcellular localization of polyamides as a function of fluorophore identity, polyamide heterocycle core sequence and composition, and polyamide structure.[38, 40, 41] These investigations revealed that a number of factors influence polyamide cell entry, but did not lead to a set of predictive structural requirements for polyamide nuclear accumulation.

Polyamides in Biological Systems:

Although polyamides had successfully inhibited protein-DNA binding and polyamide–small molecule activator domain conjugates had recruited proteins to DNA *in vitro*, gene regulation by polyamides *in vivo* was less established.[42-48] The most notable example of polyamide gene regulation in cell culture was disruption of HIV-1 replication in human T cells subsequent to dosing with two polyamides targeted to sites adjacent to three transcription-factor binding sites.[49] A critical outgrowth of the extensive confocal microscopy studies of polyamide cell entry and subcellular localization patterns was the revelation that a wide range of heterocycle core-dye combinations are able to cross into the cell.

In collaboration with the group of Dr. Kaelin, a polyamide was designed to bind to the hypoxia-response element in the promoter region of the vascular endothelial growth factor (VEGF) gene.[50] VEGF is among a host of genes regulated by the hypoxia-inducible factor/Aryl-hydrocarbon-receptor nuclear translocator (HIF-1α/ARNT (or HIF-1β)) transcription factor heterodimer.[51] Under normal oxygen tension, the α-subunit of HIF-1α is hydroxylated at two proline residues for subsequent ubiquitination and degradation; under hypoxic conditions, the hydroxylation event does not occur, and instead the HIF-1α/ARNT heterodimer forms and binds to the hypoxia response element (HRE).[52] HIF-1a/ARNT is a master regulator of genes involved in physiological processes including

angiogenesis, glycolysis, cell survival, drug resistance and matrix remodeling.[53-57] As hypoxic conditions are frequently experienced by rapidly growing tissues, such as cancers, HIF-regulated genes and VEGF in particular are of great interest to the medical community for their involvement in cancer progression and survival.[55, 58, 59]

HeLa cells were dosed with the FITC-conjugate of a polyamide designed to bind to the HRE of VEGF (5'-TACGTG-3') and found by confocal microscopy to localize to the nucleus. VEGF mRNA levels in cells dosed with this FITC-polyamide conjugate and later treated with desferoxamine (DFO) to induce hypoxia-regulated gene expression were lower than VEGF mRNA levels, indicating that this FITC-polyamide conjugate was not only localizing in the nucleus, but was able to regulate gene expression levels of a medically relevant endogenous inducible gene. This initial work has since been validated by polyamide-regulated expression of prostate specific antigen (PSA) upon dihydrotestosterone (DHT) induction in LNCaP (prostate cancer) cells.[60]

Summary:

Although it has been shown that a wide array of polyamide core-dye conjugates are able to localize to the nucleus of a range of cells, the widespread use of dye conjugates is problematic due to the high cost and instability of fluorescent dyes. The thrust of the work presented in this thesis deals with efforts to identify structural modifications to polyamides that facilitate their biological activity, are chemically stable, and do not interfere with binding affinities and specificities. As one of the aims of this work was to avoid pricey fluorescent dyes, such as FITC, detecting cell uptake and nuclear localization by confocal microscopy was not an option. The recently published result demonstrating VEGF mRNA regulation in HeLa cells was instead adopted as a biological readout of polyamide biological activity.

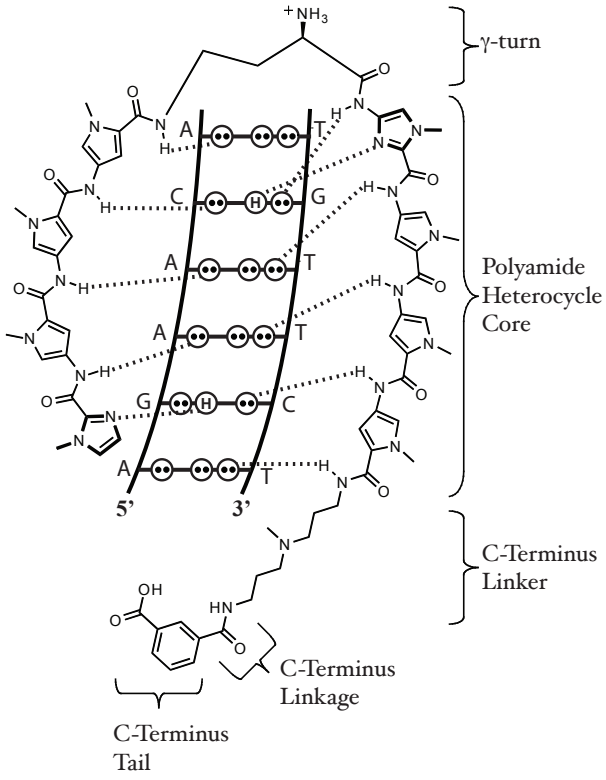

Figure 1.11. Schematic illustration of the hydrogen-bonding pattern mediating DNA recognition by a polyamide. The focus of work to identify structural modifications to improve polyamide biological activity discussed in this thesis, namely the C-Terminus linker, C-terminus linkage and C-terminus tail, are labeled.

Structural modifications to polyamides to effect cell uptake can be made at the C-terminal tail, off the amine of chiral turns, or potentially on heterocycles modified with an internal linker group. As illustrated in figure 1.11, modifications to the C-terminal portion of polyamides can be introduced at a number of positions, including the C-terminal tail

group, the C-terminal linker, or the linkage between tail and linker. Chapter 2 describes initial successes in finding a suitable FITC-replacement, while Chapter 3 deals with further applications of one of these novel tail groups, isophthalic acid (IPA). In addition, microarray data are presented that show that polyamides affect a fairly modest number of genes. Chapter 4 describes the effects of modifications made to the linker and linkage group, as well as attempts to discover the structural basis for the observed cellular uptake of FITC- and IPA-polyamide conjugates through synthesis of a focused library of tail derivatives.

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