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Lp(a) plasma levels are largely determined by the apolipoprotein(a) [apo(a)] component, and differ across ethnicity (Ordovas, J. M., 2004). Although a number of polymorphisms in the apo(a) gene have been identified but apo(a) genetic regulation is not fully understood. Alleles (PNR, 4-12) have been found in the upstream regulatory region of apo(a) gene (Rubin, J., 2008). Pyrimidine/purine- (Pyr/Pur) mirror DNA sequences adopt an intramolecular triplex conformation (Kato, M., 2002) and provide target site for protein to recognize a specific binding site to inhibit or regulate gene expression, and also involved in triplex DNA mediated homologous recombination (Datta, H.J., 2001).

The intramolecular triple-helical DNA structure provides important signal for control of gene expression and requires homopyrimidine-homopurine mirror repeats to form H-DNA structures (Mirkin, S. M., 1987). Pu/Py mirror repeats which have tendency to form DNA intramolecular structures undergo modest energy level to provide stable DNA structure (Patel, P. H., 2004). The formation of triplex requires melting of DNA at the centre of mirror repeat and is directly proportional to the A+T content of the centre (Shimizu, M., 1989). We have found no significant difference in heterologous as well as homologous PNR repeats in different mammalian cell lines which were derived from different human being. We have chosen PNR10, PNR9 and PNR8 repeats for study, highly studied in human being especially in adolescent and children (Nascimento, H., 2009). Our hypothesis was that PNR10 might be more stable in forming a triplex than PNR8 because of greater relaxation of negative supercoiling (Sinden, R. R., 1994).

To analyze the emphasis of DNA structural differences on apo(a) gene regulation, we used most commonly occurring pentanucleotide repetitive
sequences \((TAAAA)_n=10,9,8\) in human apo(a) gene promoter upstream region (1.4kb), occurs in homologous and heterologous alleles. Topographical studies on the APS-mica sheet by AFM, thus, confirmed that plasmids containing PNR9 and PNR10 showed kink formation, a characteristic feature of intra-molecular triplex DNA (Tiner, W. J., 2001; and Kato, M., 2002), when compared either with control plasmid (plasmid without insert) or plasmid containing PNR8. Triple stranded DNA were earlier seen in samples of poly(dA).poly(dT) and poly(dG).poly(dC) (Hansma, H., 1996) and direct imaging of H-DNA formed by mirror-repeated Pu-Py repeats in supercoiled state was reported by AFM images, revealed a clear protrusion with a different thickness than that of the DNA duplex (Tiner, W. J., 2001).

Intramolecular triplex structure could be formed where either the purine or pyrimidine single strand roll back to form hoogsteen bond with adenine or thymine, A.T* A leaving single stranded oligopyrimidine S1 nuclease hypersensitive site. We have shown by S1 nuclease analysis that plasmids containing Pu:Py rich PNR10 and PNR9 sequences with mirror repeat form triplex structure. Mirror repeated Pu:Py tracts that form H-DNA occur as frequently as 1 in 50000 bp of human genomic DNA (Schroth, G. P., 1995) and might adopt an intramolecular triplex conformation (Kato, M., 2002) to lead cellular process. The S1 sensitive site was more sensitive to S1 than the endogenous sites in the parent plasmid. Analysis of the S1 nuclease activity of triple DNA structure formed in plasmid pUC19-10/9/8 supports the conclusion that single stranded selective targeted sites was formed due to triplex DNA structure formation and was supercoiled independently. Furthermore, the S1 nuclease did not affect the control (plasmid without insert) and pUC19- PNR8 supercoiled plasmid when tested at
similar concentration and under similar conditions. On the other hand, PNR8 which contains one repeat less, and was shown to be responsible for atherosclerosis across population studies, did not exist as triplex structure.

PNR9 and PNR10 sequences, in luciferase reporter assay, showed repression of transcription in a cell specific manner when it was compared with PNR8. Earlier studies showed that intramolecular triplex formation inhibited the transcription of human c-myc gene (Kim, H. G., 1998) and triplex DNA-mediated downregulation of Ets2 expression resulted in growth inhibition and apoptosis in human prostrate cancer cells (Carbone, G. M., 2004). Further, Py.Pu tract containing a 26bp quasi mirror repeats that underwent structural transition in the promoter of Na, K-ATPase α2 gene was reported (Potaman, V. N., 1996). TFO's have been shown to alter gene expression during transcription process by interfering either with the binding of transcription factors or providing cushion at binding site for proper binding (Hewett, P. W., 2006 and Svinarchuk, F., 1997). Triplex-forming oligonucleotides(TFO), binding to major groove ligands constitute an interesting DNA sequence-specific tool that can be used to target cleaving or cross-linking agents, transcription factors or nucleases to a chosen site on the DNA (Duca, M., 2008). Triple-helix target sites over represented in the human genome (Goni, J. R., 2004, Goni, J. R., 2006) are not directly targeted by transcription factors. In luciferase assay, when a 26 mer PNR-TFO having phosphorothioate backbone was utilized along with plasmids containing PNR8, -9, and -10, for transfection, interestingly, repression in luciferase activity was observed exclusively with PNR-8. The results suggested that PNR-TFO was able to bind to the target sequence of PNR-8 that did not exist in triplex form. Either it restricts accessibility or provides space to regulatory proteins combined with TFO on
endogenous gene promoter among synchronized cell population which might have contributed to the apparently reduced endogenous gene expression.

In *in vitro* experiments, mismatched oligonucleotides (M2, M3, M4, M5, poly A and poly T) were unable to form triplex structure with targeted site indicating that triplex mediated mechanism did not affect non-targeted gene, though they have same target at apo(a) promoter upstream. There was no interference in cell cycle due to apo(a)-TFO, vanished any false conclusion extracted through experiments. However, published data provided convincing evidence of triplex formation at extra chromosomal and chromosomal sites in cells by covalent cross linking of target DNA induced by TFOs (Wang, G., 1996, Giovannangeli, C., 1997, Oh, D.H., 1999, Vasquez, K. M., 2000 and Faria, M., 2000). Changes in chromatin structure have previously been correlated to differential gene expression by identification of regions of DNAse 1 sensitivity and hypersensitivity (Elgin, S.C.R., 1981, Weintraub, H., 1976 and Garel, A., 1976). If the third strand spans more than 10 bp then it will have to thread between the protein surface and the duplex to continually access the DNA major groove. Previous work has shown that triplex formation alters nucleosomal arrangement and functions as a nucleosomal barrier (Westin, L., 1995 and Espinas, 1996) whereas triplex formation might be possible on sequences such as polydA-polydT which do not easily wrap around the protein surface (Brown, P.M., 1998).

The ability of DNA sequences to adopt a particular conformation in response to their environment is mostly studied by triplex forming oligonucleotides. Further, PNR10 and PNR9 that naturally existed in triplex forms were not targeted by 26-mer PNR-TFO. In a similar manner, biotinylated-TFO was utilized in specifically targeting PNR8 sequences in chromatin (Ye, Z., 2007),
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thus, suggesting the natural existence of PNR9 and PNR10 in triplex form in chromatin. Our data suggested intermolecular triplex formation inside chromatin, nuclei and genome of the cells by apo(a)-TFO and also supports the conclusion that intermolecular triplex formation was inhibited by intramolecular formed triplex structure. It was proposed that TTS are not directly targeted by transcription factors, they may be important for gene functionality by acting as spacing fragment to help the correct positioning of transcription factors (Goni, J. R., 2006). In luciferase assay, the observed discrepancy with polymorphic PNR sequences (with different repeat numbers) in controlling gene repression might be due to their differential binding to yet unidentified transcription factors. These finding truly indicate that not only mirror repeat sequences but other factors such as the flanking regions, external environment, presence and absence of protein etc. also affect the structure formation.

Triplexes formation in the cells led us to search nuclear protein which could recognize this DNA structure or PNR region. Acrydite-labeled PNR was utilized in order to identify a previously known oncoprotein Evi-1, identified through MATCH™ TRANSFEC 6.0 (http://www.gene-regulation.com/cgi-bin/pub/programs/) and has >80% homology at binding sites, from HepG2 cells that preferentially was shown to bind to triplex forming PNR9 and -10. As PNR-TFO target site directly overlaps with Evi-1 consensus binding sequence, we tried to establish the role of Evi-1, if any, in regard to TFO targeting in cells. Although Evi-1 failed to show any direct role upon PNR-mediated gene repression, its preferential binding to PNR10, both in vitro and in chromatin, suggests that it might have stabilized triplex-forming PNR sequences. Intramolecular or intermolecular triple helices could be recognized by specific proteins that stabilize
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triplex structures. However, triplex formation inside living cells and the gene regulation mechanism of TFO molecules still needs to clarification, as the data on the transcription dependence of triplex formation are inconsistent (Macris, M. A., 2003, Brunet. E., 2006 and Giovannangeli, C., 1997).

Apo(a) gene 1.4 kb promoter upstream contains multiple transcription factors binding sites (Sarita, N., 2004) and we have found few transcription factors binding on that region through DNA-protein complex mobility shift assay. Before drawing any conclusion about these transcription factors, it was necessary to identified it. So, these transcription factors were purified by magnetic beads (Dynal Beads) and analyzed by peptide fragmentation sequencing (LC-ESI-MS/MS). The peptide masses did not match with available peptide masses of protein, indicated presence of some novel proteins which have not been recognized till now. Identification of proteins binding specifically to particular nucleic acid structures can lead to comprehension of their role in vivo and contribute to the discovery of structure-related gene regulation. Several single-strand binding proteins specific for sequences involved in the formation of H-DNA have been described in a variety of biological systems (Kolluri, R., 1991; Michelotti, E. F., 1995 and Muraiso, T., 1992). The role of these proteins could be to induce or stabilize the H-DNA structure by trapping the single-stranded portion. Its biological function could be mediated by modulating the binding of transcription factors or by interacting with specific DNA-binding proteins. The protein described here could have an important function in the regulation of gene expression by stabilizing (Evi-1) an intramolecular triplex DNA in vivo and in particular it could enhance the formation of imperfect triplexes. It might also stabilize intermolecular triplexes. This latter activity could be important for the development of the anti-gene strategy
whereby an ODN forming a local triple helix can be used to modulate gene expression in a sequence-specific way (Thuong, N. T., 1993).

Our data suggested that triplex formation in the polymorphic pentanucleotide repeats combined with Evi-1 and other transcription factors act as apo(a) gene transcription repression in the cells. It was interesting to investigate the effect of such triple helix forming potential sequences, present of upstream of apo(a) gene, to understand how local supercoil induces DNA structure and influence gene expression in eukaryotic systems where DNA is wrapped around nucleosomes (Sarkar, S. P., 1992). Our results indicate that this TFO could act as a selective transcriptional repressor and it could be used as therapeutic tool to reduce over-expression of apo(a) gene. Together the data with Evi-1 suggested that not only the Evi-1 and TFO some other factors also have significant role in apo(a) gene expression. Analysis of the binding properties of the apo(a)-TFO showed that it bound to the target DNA with very high affinity and was affected by intramolecular triplex structure of DNA. Formation of triple helical structure by TFO provides recognizable structure to bind Evi-1 and other transcription factors and thus regulate gene expression.

In conclusion, we have discovered that PNR sequences with higher repeat numbers in apo(a) promoter form intramolecular triplex structure that has implications upon apo(a) gene regulation. However, lack of a single pentanucleotide repeat makes it unable to form intramolecular triplex and affect the regulation of apo(a) gene.
It could be overcome by introducing triplex forming oligonucleotide to make intermolecular triplex which also control the gene regulation. It was interesting to note a new role of Evi-1 as a triplex binding protein and stabilizing it, although its role was earlier shown to bind directly to GATA-2 promoter (Yuasa, H., 2005) and its role upon protecting cells from stress induced cell death (Kurokawa M, 2000). The novel protein may have played an important role in regulation and triplex binding. Further work required, especially on this protein, is to characterize its function, to investigate its triplex-binding properties and its potential biological functions.