An intronic polymorphic domain often associated with susceptibility to affective disorders has allele dependent differential enhancer activity in embryonic stem cells

C.E. Fiskerstrand, E.A. Lovejoy, J.P. Quinn*

Department of Veterinary Pathology, University of Edinburgh, Summerhall, Edinburgh EH9 1QH, UK

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Abstract Variable number tandem repeats (VNTR) within non-coding regions of a number of genes have been correlated with susceptibility to various disease states. In particular, a VNTR polymorphism of a 16 or 17 bp element within intron 2 of the human serotonin transporter gene has been correlated with a predisposition to affective disorders. We have demonstrated that this region will support differential levels of reporter gene expression in differentiating embryonic stem cells, this being dependent on the presence of 10 or 12 copies of the repeat. The VNTR domain can therefore act as a transcriptional regulator, a property which potentially contributes to disease susceptibility.

Key words: Serotonin transporter; Affective disorder; Enhancer; Gene promoter; VNTR polymorphism; Embryo stem cell

1. Introduction

Genetic factors have been implicated in the aaetiology of mental illness but identification of the gene(s) involved is very difficult because of the complex inheritance patterns of these disorders. Clinical abnormalities in monoamine metabolism, in particular serotonin (5-HT) and dopamine have been implicated in the pathophysiology of many CNS related disorders and the use of drugs that block the effects of the serotonin transporter (5-HTT) and the dopamine transporter (DAT1) have pointed to these being candidate genes involved in behavioural or mental disturbances [1,2]. Both 5-HTT and DAT1 genes contain polymorphic regions termed variable number tandem repeats (VNTRs) within non-coding regions but the role of these VNTRs is at present highly debatable [3–12].

A VNTR polymorphism within intron 2 of the human 5-HTT gene (STin2) comprises between 9 and 12 copies of a 16 or 17 base pair length element (Figure 1a), the number of repeat elements having been correlated with a predisposition to affective disorders and anxiety symptoms [3–5,13,14]. Because of the location of this polymorphism and the size of the individual repeat elements, we postulated that this domain might act as a transcriptional regulator.

A small number of publications have indicated that VNTRs representing susceptibility factors for various disorders or diseases can act as transcriptional regulators. These include a 5′ VNTR within the 5-HTT promoter and also the IDDM2 locus that encodes susceptibility to human type 1 diabetes [15]. In order to define the potential regulatory properties of the STin2 VNTR we therefore proposed to determine whether it could support reporter gene expression in cell line models and, if so, would the VNTR support differential reporter gene expression based on the copy number of the repeat. The latter would potentially correlate with predisposition to affective disorders by modulating expression of 5-HTT or an adjacent gene at this locus.

2. Materials and methods

2.1. Construction of reporter gene constructs

Genomic DNA from 9-copy (Stin2.9), 10-copy (STin2.10) and 12-copy (STin2.12) VNTR individuals was amplified by PCR using primers: Pr1 (+818 to +838) $^{5'}$GTCAGTATCACAGGCTGCGAG$^{3'}$ and Pr2 (+1120 to +1099) $^{5'}$TGTTCCTAGTCTTACGCCACTG$^{3'}$ (Figure 1a) of Battersby et al. [3]. Nucleotide numbering commences at the start of exon 1 and is consistent with the presence of STin2.12. PCR products were cloned into the pGL3 promoter vector (pGL3p, Promega) upstream of the SV40 promoter. A number of clones of each construct were sequenced in both directions to confirm the number of repeats, orientation and exact nucleotide sequence.

2.2. Growth and transfection of cells

Embryonic Stem (ES) cells, HM-1, were cultured as per Magin et al. [16] in the presence of LIF. Cells at 70–80% confluence in 24 well plates were transfected in triplicate with 1μg pGL3p vector or specific 5-HTT VNTR pGL3p construct using Lipofectin (Life Technologies) and following the manufacturer's directions. Cells were then transferred to specific medium +/− LIF and +/− retinoic acid (RA) for 64h (optimal for ES cells).

HeLa cells were cultured under standard conditions. 2×10^5 cells were incubated for 16h in medium containing 0.5% serum, transfected by electroporation with 5 to 20 μg DNA using the Equibio EasyJect at the optimal 250V and capacitance of 1500mF then incubated in complete medium for 48h (optimal for HeLa).

2.3. Analysis of reporter gene expression

Cells were washed with PBS, lysed with 250 μl Promega lysis buffer and supernatants assayed for luciferase activity using the Promega assay reagent and Life Sciences Labsystems Luminoskan RT. Results are quoted as expressed luciferase from each construct relative to baseline pGL3p expression (Relative Luciferase Expression).

3. Results

3.1. STin2 VNTR sequences

Stin2.9, STin2.10 and STin2.12 PCR products were cloned into the vector pGL3p and a number of clones of each sequenced. The 12-copy VNTR repeat elements have been numbered in two ways in Figure 1b in order to define both the

*Corresponding author. Fax: (44) (131) 650 6511.
E-mail: j.quinn@ed.ac.uk

Abbreviations: 5-HTT, Serotonin transporter; Stin2, Serotonin transporter intron 2; VNTR, variable number tandem repeat; RA, retinoic acid; ES, embryonic stem cells

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position of the element within the VNTR and it's individual sequence, ie. RP1 to RP12 identify the position and lower case letters, a-g, identify the sequence of each unique repeat element. Differences between these elements are only seen at specific positions ie. residues 6, 10, 11, 12, 14 and 15. In addition, only C/T and G/A substitutions are seen. Relative to STin2.12, STin2.10 is missing d and g element sequences which comprise RP9 and RP10. Repeated attempts to clone the 9-copy STin2.9 VNTR produced unstable constructs which were therefore not suitable for use in the current transfection experiments.

3.2. *Embryonic stem cells, but not HeLa cells, support differential levels of reporter gene expression from the 10- and 12-copy STin2 VNTRs*

The potential for the two VNTRs to support reporter gene expression was assessed by transfecting luciferase expression constructs containing STin2.10 and STin2.12 in triplicate into ES cells, which were subsequently incubated in the presence or absence of LIF, and into HeLa cells. Data from duplicate ES cell transfection experiments is presented in Figure 2 as luciferase expression relative to that from the pGL3p vector. Reporter gene expression was dramatically different depending on whether or not LIF was present in the growth medium post transfection. In the presence of LIF, STin2.10 showed some reduction in reporter gene expression (relative expression = 0.31 ± 0.02) and STin2.12 had no significant effect (relative expression = 1.14 ± 0.15). Withdrawal of LIF however, resulted in dramatically increased expression from both constructs. ie. STin2.10 increased the relative expression by 5.77 (± 1.54) fold and STin2.12 by 167.85(± 10.35) fold over pGL3p. In contrast to ES cells, neither VNTR exhibited any influence on reporter gene expression in HeLa cells (data not shown).

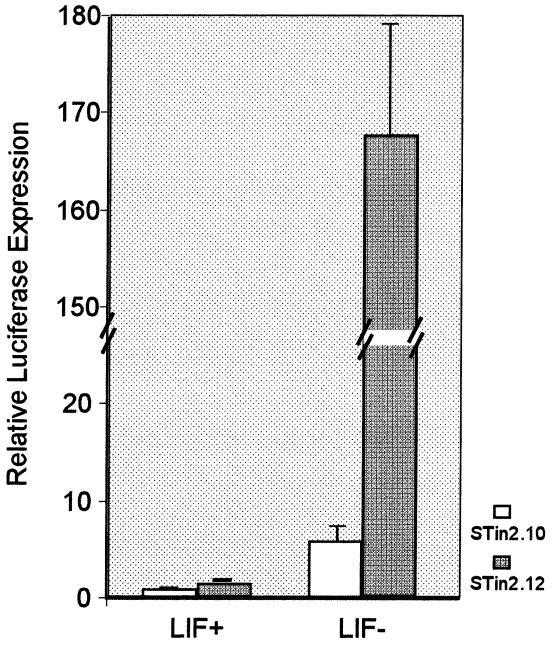
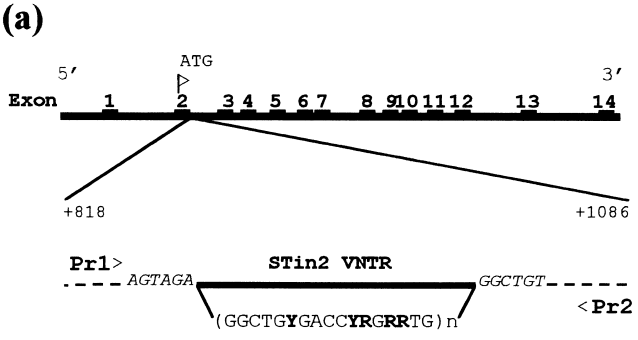


Fig. 2. ES cells support differential STin2.10 and STin2.12 reporter gene expression. Transfected ES cells were incubated for 64h in medium ± LIF. Reporter gene expression from triplicate STin2.10 and STin2.12 transfections is presented as luciferase expression relative to baseline pGL3p. Data represents the combined results of two experiments.



(b)

Repeat No.	Element Identity	1 12 17
RP1	a	GGCTGTGACCCAGGGTG
RP2	b	GGCTGTGACCCGGAGTG
RP3	c	GGCTGTGACCCGGGGTG
RP4	d	GGCTGTGACCC GGGTG
RP5	e	GGCTGCGACCTGGGGTG
RP6	f	GGCTGTGACCTGGGATG
RP7	d	GGCTGTGACCC GGGTG
RP8	g	GGCTGTGACCTGGGGTG
[RP9	d	GGCTGTGACCC GGGTG]
[RP10	g	GGCTGTGACCTGGGGTG]
RP11	d	GGCTGTGACCC GGGTG
RP12	f	GGCTGTGACCTGGGATG

Fig. 1. Genomic location and sequence of the STin2 VNTR. The location of the intronic VNTR within intron 2 of 5-HTT [30] is shown with the consensus repeat element sequence indicated (Y = T/C, R = A/G). Pr1 and Pr2 were used to PCR the VNTR region. b) The STin2.12 VNTR sequence showing repeats RP1 through RP12. Unique elements are delineated 'a' to 'g'. Repeats RP9 and RP10 (bracketed) are not present in the STin2.10 VNTR.

Retinoic acid (RA) is used as a morphogen in the differentiation of ES cells [17]. RA, added at 10–500nM to both LIF+ and LIF− media post transfection, was capable of modulating reporter gene expression from our VNTR contructs both with and without LIF in the medium, Figure 3a, although LIF− effects were more dramatic than LIF+. The enhanced expression which was seen after withdrawal of LIF was progressively reduced by increasing concentrations of RA, STin2.12 expression being reduced by up to 60% and STin2.10 by up to 16% by 500nM RA.

4. Discussion

A genetic basis for the progression of, or predisposition to, affective disorders and other behaviour related traits is likely to be multifactorial. Although polymorphic regions composed of VNTRs are often associated with predisposition to a particular disease, these genetic markers have generally been used to predict individuals at risk and few attempts have been made to determine the mechanisms behind this correlation. The size of the individual repeat elements within the recently described STin2 VNTR polymorphism suggests potential for binding transcription factors which would implicate the VNTR as a transcriptional regulator of gene expression at this locus.

Our transient transfection studies have shown that the STin2 VNTRs can indeed act as transcriptional regulators

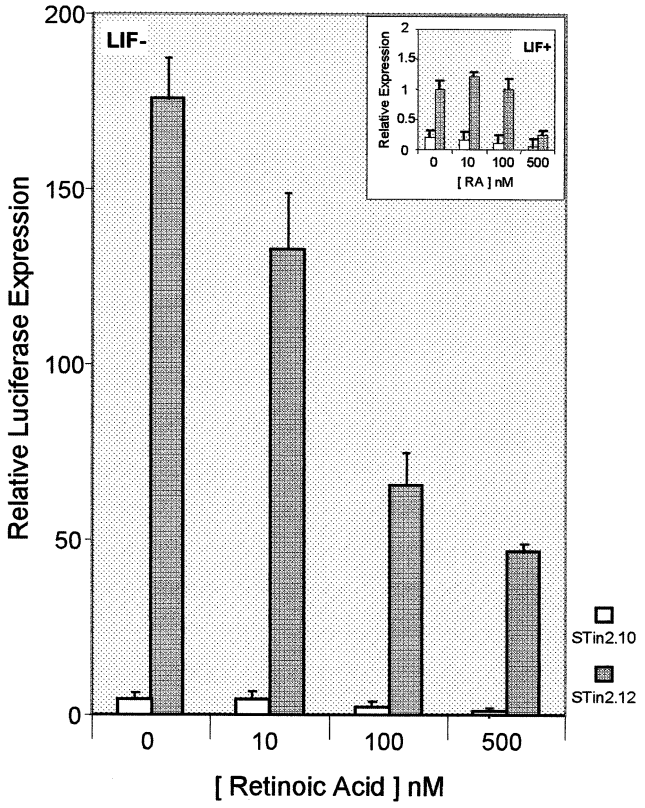


Fig. 3. Effect of retinoic acid on STin2 VNTR reporter gene expression in ES cells. Transfected ES cells were incubated ± LIFand plus RA at 0, 10, 100 or 500nM. Results are presented as relative luciferase expression from triplicate transfections. LIF+ data is shown as an insert within the LIF− figure.

and that activity can be cell-specific. ES cells are pluripotent cells derived from the inner cell mass of blastocysts. Culturing ES cells in the presence of LIF allows them to be maintained in an undifferentiated state for many passages in culture [18]. In this state ES cells do not support enhancer activity by either VNTR construct and STin2.10 appears to reduce expression from the SV40 minimal promoter to some extent. Withdrawal of LIF is sufficient to induce differentiation of ES cells into endoderm-like cells and morphologically distinguishable cell populations normally appear over a period of days [19]. Changes at the molecular level can however be seen as early as 36hrs after LIF withdrawal and these appear to be sufficient to permit enhancer activity of the STin2 VNTR domains when in the context of an SV40 minimal promoter-driven reporter gene construct. In contrast to results obtained from ES cells, no response to the presence of either VNTR in the constructs could be detected in HeLa cells. Although LIF- and LIF+ ES cells support such different levels of reporter gene expression, preliminary electrophoretic mobility shift analyses (EMSA) using oligonucleotides representing unique repeat elements have not shown any obvious correlation between novel factor binding and the activation described above. HeLa cell extracts on the other hand do appear to form far fewer complexes than ES cells. The mechanisms involved in transcriptional control by VNTRs are likely to be complex and the relationship between activation, or lack of, and the observed complexes is under investigation.

Under the conditions used in this study, STin2.12 acted as a significantly stronger enhancer than STin2.10 in differentiating ES cells. Although it appears that the presence of the two additional repeat elements is enough to increase the enhancer potential of the VNTR, the presence of a number of distinct elements within the VNTRs highlights the potential for variations within the VNTR, as distinct from just copy number. Repeated motifs can be identified within the VNTR which resemble known transcription factor binding sites. Transient transfection data leads us to believe that the VNTR contains both positive and negative regulatory motifs.

In the context of the whole 5-HTT promoter, the VNTR may affect both distribution and rate of transcription. It has in fact now been demonstrated that both STin2 VNTRs exhibit highly restrictive patterns of expression in transgenic mouse embryos when in the context of a reporter gene construct under the control of the β-globin minimal promoter. [MacKenzie et al., submitted]. Expression of the marker gene in embryos transgenic for either of the constructs was consistent except within the rostral hindbrain where STin2.12 drove higher expression of the marker gene than STin2.10. This observation is in agreement with our ES cell transfection data which also shows STin2.12 to be the stronger regulator. Interestingly, the rostral hindbrain has been shown to express 5-HTT mRNA [20] and is associated with the development of rostral serotonergic neuronal clusters [21]. Our observation that RA can modulate the activity of the STin2 VNTR constructs is therefore interesting because the developing embryonic nervous system is also known to be sensitive to RA [22]. In vitro, the combined effects of removal of LIF and addition of RA can induce ES cells to differentiate into neuron-like cells through some as yet unknown mechanism [23]. Although potential retinoic acid response element motifs [24] are found within the VNTR ie. $^{5'}$AGTAGA $^{3'}$ immediately 5′ of the VNTR (Figure 1a) and an inverted motif $^{5'}$TGACCC$^{3'}$ in the middle of a number of the repeats, we have no evidence for direct RA interaction with the VNTR.

As the STin2 VNTR region is correlated with a predisposition to affective disorders, understanding the regulatory role of the VNTR will have direct clinical relevance to our understanding of the aaetiology of the disorders and suggest novel strategies for the treatment of these conditions via manipulation of 5-HTT gene expression. More generally it demonstrates that polymorphic regions, previously only used as genetic markers, may have a function in regulating specific gene expression. We view the STin2 VNTR as distinct from the trinucleotide repeats, such as the CAG/CTG elements previously associated with schizophrenia which, although having variable copy number, are unlikely to have sufficient sequence information in each repeat to specify a sequence-specific transcription factor binding site. A precedent for VNTRs to act as transcriptional enhancers has been demonstrated within other genes, e.g the 5′ promoter polymorphism in the 5-HTT gene itself [25] and a locus within the IDDM2 gene [15,26–28]. Our results suggest that many previously identified polymorphic VNTR regions with known disease associations and in which the repeat elements are of sufficient length to contain transcription factor binding motifs, should be re-evaluated to determine whether they could act as transcriptional regulators.

In conclusion, we have shown that the Serotonin transporter intron 2 VNTR can act as a transcriptional regulator with potential for defining cell-specific differential gene expression. The mechanism whereby Stin2.10 and Stin2.12 exert distinct

effects is unclear, however, as seen in figure 1, individual repeat elements show sequence variations. In principle, one or two nucleotide changes in a consensus sequence can either specify for a specific transcription factor complex or can alter binding affinity [29]. It is conceivable therefore that both the number of repeats comprising the VNTR and the sequence of individual elements within it are likely to be major determinants of VNTR activity. The relative importance of each element within the VNTR is currently being analysed and the functional significance is being determined both in vitro and by transgenic analysis in vivo.

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