Transformation of a novel direct-repeat repressor element into a promoter and enhancer by multimerisation

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ABSTRACT

Studies on the regulation of interferon (IFN) responsive genes have mainly been centred on the highly conserved IFN stimulated response elements (ISREs) which can mediate type I and II IFN inducibility. To date little is known about other functional *cis*-acting regulatory motifs in IFN responsive genes. We report here on the identification of a repressor element in the human MxA gene defined to a 19 base pair (bp) region which houses a 9 bp direct repeat. DNA-specific protein binding on this element is not affected by IFN treatment and is distinct from ISRE binding proteins. Remarkably, contrary to expectations, when the repressor element is multimerised and spliced, in either orientation, to a reporter gene it behaves like a functional, constitutive promoter. Positioning the multimerised element in front of the SV40 enhancerless promoter also led to enhanced expression. The same protein(s) seem to bind to both the single repressor element and its multimerised form. This discovery of phenotypic reversal on a repressor element via multimerisation may have important implications *in vivo*.

INTRODUCTION

The human MxA gene is one of several Mx genes that have recently been isolated from a number of different species (1,2). They are interferon (IFN)-inducible and some Mx genes have been shown to confer resistance against certain viruses. In particular, the human MxA gene is induced primarily by type I IFNs and has been shown *in vitro* to mediate the abrogation of influenza and vesicular stomatitis virus infections (3,4). Mx proteins have putative GTPase activity and are considered to belong to an increasingly important, new family of GTP-binding proteins. These are associated with intracellular transport and endocytosis such as the *Drosophila shibire* gene, yeast protein sorting gene VPS1 and rat dynamin-1 gene (5–8).

In elucidating the mechanisms of IFN actions, several IFN responsive genes have been isolated. Comparisons of their upstream sequences revealed the presence of a conserved motif termed IFN stimulated response element (ISRE) which can confer type I and II IFN inducibility and shows resemblance to the virus-responsive elements of IFN- α and - β promoters (9–11). The

MxA promoter has three such ISRE homologous motifs (12). Amongst the several factors that bind to the ISRE, a multimeric protein complex, ISGF3 (also known as E factor), has been implicated to be the transcriptional activating factor upon IFN stimulation (13–16). Not surprisingly, the focus of study on the regulation of IFN responsive genes has been directed at this ISRE region. Whilst the ISRE is an important basic IFN response motif, the observed differential responses in the induction of genes by type I and II IFNs and differences in tissue specificity of expression would suggest the presence of additional regulatory motifs and the associated *trans*-acting factors (9,17). Little data has been reported on the identification of other functional motifs in the regulatory sequence of IFN responsive genes. There is some evidence to suggest a repressor element located in the human 2',5'-oligo(A) synthetase promoter (18,19). Therefore, an investigation was conducted into the possible presence of other regulatory motifs in the human MxA promoter. We report here on the identification of a repressor element defined to a 19 base pair (bp) region which contains a 9 bp direct repeat. Surprisingly, multimerisation of this element can reverse its phenotypic effect and convert it into a different functional element that has both enhancer and promoter properties which may have significant implications in the study of mutagenesis.

MATERIALS AND METHODS

Cell culture

Human embryonic lung cells L132 (ATCC no. CCL 5) were maintained in M199 with 10% foetal calf serum, 2mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 2.5 μ g/ml amphotericin B, at 37°C under humidified atmosphere of 95% air and 5%CO₂ (Flow). Unless specified otherwise, induction with human type I IFN (Sigma, 5 $\times$ 10⁶ IU/mg) was performed overnight (15 h) at 2,000 IU/ml.

Plasmid construction

p1.1SalICAT is a construct where the 1.1kb *Sal* I MxA promoter fragment was cloned, in the correct orientation, into the *Sal* I site of the promoterless CAT vector, pCAT-Basic (Promega) as described in (12). A series of deletion constructs based on this p1.1SalICAT was created, by restriction digests and religation as indicated in Fig. 1, which comprised of p0.6aCAT, p0.6bCAT, p0.4aCAT and p0.4bCAT.

Multimerisation of the 40 bp RE was accomplished by annealing two overlapping complementary oligonucleotides of the sequences, 5'-GAGAACCTGC GTCTCCCGCG AGTT-CCCGCG AGGCAAGTGC-3' and 5'-TCTCGCACTT GCC-TCGCGGG AACTCGCGGG AGACGCAGGT-3', followed by a phosphorylation reaction and a 3h ligation. Multimers of different sizes that were generated were then cloned into the appropriate pCAT-Basic vector or pBluescriptIIISK+ vector (pBS, Stratagene) and pCAT-Promoter vector (Promega) which contains the SV40 promoter without an enhancer. A shorter version of the 40 bp RE (rRE) was similarly constructed with two oligonucleotides, 19 bases in length that spanned the 9 bp repeat region, whose sequences are 5'-TCCCGCGAGT T-CCCGCGAG-3' and 5'-GGGACTCGCG GGAAGTTCG-3'. The size of each multimer and its orientation in the vector were determined by sequencing (Sequenase v2.0 kit, USB).

Transfections and CAT assays

L132 cells were plated onto 90mm culture dishes and grew to 70% confluence for transfections by calcium phosphate co-precipitation method as described in (20). Except otherwise stated, 20 μ g of test chloramphenicol acetyltransferase (CAT) plasmid and 10 μ g of reference pCH110 (Pharmacia), a β -galactosidase (β -gal) reporter gene were used per transfection. Harvested cells were resuspended in 220 μ l 0.25M Tris-HCl (pH7.8) and lysates were prepared for CAT assays as in (20). A 40 μ l aliquot derived from each transfection was used for β -gal assay to normalise the amount of lysate used in each set of CAT reactions. CAT conversion was quantified by a Chromscan densitometer (Joyce-Loebl).

Bandshift assays

L132 cells were grown in 135mm culture dishes, five at a time until confluent. Where appropriate, they were subjected to IFN treatment for a specified period before harvest. Nuclear lysates were prepared as described in (21). Typically, each harvest yielded about 1.5mg of proteins as determined by the Bio-Rad protein assay kit. Reagents for bandshift assays were from the Bandshift Kit (Pharmacia). 4 μ g of nuclear lysate and the equivalent of 30,000 cpm of probe were used per reaction. Unless otherwise stated, the protocol was as indicated in the kit. The asymmetric 40mer RE duplex and two multimers derived from the shorter 19mers were labelled by filling-in reactions with [α - 32 P]d CTP (>3,000Ci/mMol, Amersham) using Klenow. The ISRE duplex used in the competition assay had the sense sequence 5'-GGTCTGTGAGTTTC ATTTCTTCGCGGCG-3'.

RESULTS

Identification of repressor element (RE)

The 1.1 kb MxA promoter fragment in p1.1SalICAT contains 3 ISRE-like motifs (Figure 1A). Previous work on this construct had shown that it is tightly regulated by type I IFNs (12). Without exogenous induction its basal expression is negligible. In order to assess the functional significance of the 3 ISRE motifs, a series of deletion constructs was created which were p0.6aCAT, p0.6bCAT, p0.4aCAT and p0.4bCAT (Figure 1A). They were transfected into L132 cells and subsequently assayed for CAT activity. The results are summarised in Figure 1B. All constructs were inducible by type I IFNs even when only one proximal ISRE was present. However, the most salient finding was the high basal CAT expression with p0.4aCAT and p0.4bCAT. Comparative

sequence analysis between p0.6bCAT and p0.4aCAT suggested the high constitutive expression in the latter could be attributed to deletions made to either end of the promoter (-125 to -165 or 305 to 340). Interestingly, within the 40 bp of -125 to -165

A

B

Figure 1. Deletion analysis of MxA promoter. (A) The 1.1kb *Sal* I (sl) promoter insert in p1.1SalICAT which contained all 3 ISREs was dissected by restriction enzymes: p0.6aCAT had the most 5' ISRE removed at the *Apa* I (a) site; p0.6bCAT was identical to p0.6aCAT except for a further deletion at its 3' end *Ava* I (av) site; p0.4aCAT contained a 0.4kb *Pst* I fragment which housed the 2 proximal ISREs and p0.4bCAT with 1 proximal ISRE was a slightly shortened version of p0.4aCAT whose 5' end was removed at the *Dde* I (d) site. (B) Typical CAT activity results expressed as percentage conversion of the above constructs: p1.1SalICAT (1), p0.6aCAT (2), p0.6bCAT (3), p0.4aCAT (4) and p0.4bCAT (5). All were inducible with type I IFNs even when only one proximal ISRE was present. However, the most salient finding was the high basal CAT expression with p0.4aCAT and p0.4bCAT. Comparative

region is a 9 bp direct repeat sequence (5'-GAGAACCTGCG-TCTCCCGCGAG T TCCCGCGAGGCAAGTGC-3', ref. 12).

In order to evaluate the possible repressor function of this 40 bp element, putatively termed repressor element (RE), it was reconstituted back into p0.4aCAT. Several sizes of tandem repeats were made and inserted, in the correct orientation, into the *Sph* I site of p0.4aCAT located just 5' to the existing 0.4 kb *Pst* I MxA fragment (Figure 2A). With the re-introduction

of just half an RE into p0.4aCAT, there was a slight reduction in constitutive expression from 59% to 26%, but with a complete RE, a more dramatic decline to 14% in basal expression was found (Figure 2A, lanes 1 to 6).

Relationship between RE and ISRE

Although high constitutive expression was observed in constructs that were devoid of the RE i.e. p0.4aCAT and p0.4bCAT, they remained distinctly inducible by type I IFNs (Figure 1B). Bandshift assays were then performed with the 40mer RE as probe on previously uninduced and IFN-induced L132 nuclear lysates to determine if its observed repressor function was also associated with protein(s) binding. Specific protein binding was indeed found (Figure 3A and B). Regardless of IFN status of the nuclear lysates used, two distinct bands were discernible. Though binding to the labelled RE could be competed out with a 50 or more fold excess of unlabelled RE, a 50 fold surplus of unlabelled ISRE (12) however, had no apparent competitive effect.

Multimerisation of RE in its MxA promoter

When multimers of RE were inserted immediately 5' to the 0.4kb *Pst* I MxA promoter fragment of p0.4aCAT, CAT expression once again showed high basal levels (Figure 2A, lanes 7 to 10). The surprising observation was that the constitutive levels associated with these multimerised RE constructs were much higher than the original p0.4aCAT without the RE. To determine if this enhanced derepression exhibited by RE multimers could be a case of trans-acting factor(s) being sequestered away from its main active site, co-transfection experiments were performed (Figure 2B). A tetrameric RE was cloned into the pBS vector and co-transfected with either p0.6bCAT or pMx1CAT (12). Tetrameric RE *in trans* did not have any apparent effect on p0.6bCAT and pMx1CAT (Figure 2B, lanes 1, 2, 5 to 10). The latter 2 constructs, however clearly remained inducible with IFNs.

RE multimers show enhancer and promoter characteristics

To investigate the possibility that RE multimers could have enhancer-like characteristics, several multimeric forms were cloned into the *Bgl* II site of pCAT-Promoter (Promega). pCAT-Promoter contains an enhancerless SV40 promoter. It was found that multimers greater than 2 REs were able to enhance, by several fold, the basal expression of pCAT-Promoter (Figure 4, lanes 1 and 2). Monomeric RE showed no apparent enhancer activity (data not shown).

Surprisingly, when different RE multimers alone were cloned into the promoterless pCAT-Basic, it was found that they were also able to drive the CAT reporter gene. The levels of expression were comparable to pCAT-Control (Promega), a control CAT vector driven by an SV40 promoter with an enhancer (Figure 4, lanes 3 to 8). In addition, the RE multimers could behave like functional promoters in either orientation. IFNs had no apparent effect on their expression. To attempt to narrow down the critical sequence within this 40 bp RE responsible for the unexpected enhancer and promoter effects, a shorter RE (rRE) was synthesised and multimerised. This 19mer rRE, taken from its parent 40mer RE, contained only the 9 bp direct repeat. This reduced version would eliminate any fortuitous functional sequence that might have been accidentally created at the junctions during the oligomerisation of the original RE.

Various multimers of rRE were cloned into pCAT-Basic in either orientation. CAT assays from these constructs showed rRE

Figure 2. Reconstitution of the putative repressor element (RE) into p0.4aCAT. (A) Restoration of RE resulted in dramatic reduction of basal expression from 58% to 14% (lanes 2 and 6) but RE multimers led to a greater elevation of uninduced (-) CAT expression (lanes 8 and 10). All constructs remained IFN inducible (+). Each CAT reaction was normalised to 0.2A₄₂₀/1.5h of β -gal activity and incubated for 1.5 h. (B) The enhanced basal expression associated with the presence of RE multimers *in cis* was absent *in trans*, in relation to the MxA promoter. a = p0.6aCAT; b = p0.6bCAT; c = pMx1CAT; a+ = p0.6aCAT and (RE)₄ cloned into pBS; c+ = pMx1CAT and (RE)₄ cloned into pBS. p0.6aCAT, p0.6bCAT and pMx1CAT as described earlier, showed little constitutive expression (-) but responded to IFN induction (+) (lanes 1 to 4, 7 and 8). Each transfection consisted of 20 μ g CAT construct, 20 μ g pBS with or without an (RE)₄ insert and 8 μ g pCH110. Each CAT reaction was normalised to 0.4A₄₂₀/2h of β -gal activity and incubated for 1.5 h. The induced expression of p0.6aCAT (lanes 1 and 5) was uncharacteristically lower than previous CAT assay results.

multimers were still able to show promoter activity (Figure 4, lanes 11 to 13). In addition, there was an apparent direct correlation between the number of rRE and the amount of CAT expression.

Bandshift assays performed with a 2.5 times oligomer of the rRE as probe revealed the presence of several specific bands, depending on the concentration of NP-40 used. In any case, varying excess amounts of unlabelled (rRE)_{2.5} and RE monomer were able to compete out all specific binding of the (rRE)_{2.5} probe (Figure 3C).

DISCUSSION

Repressor element in the IFN-inducible MxA gene

The ISRE motif has been the single most important regulatory element identified in the study of IFN inducible gene expression. It is becoming clear that different ISREs exhibit different affinities even for the same ISGFs (IFN stimulated gene factors). This may, in part, account for the variation in IFN induced expression of different genes *in vitro* and *in vivo* (16). However, the ISRE *per se* is unlikely to be the only element that is responsible for all the expression behaviour of any particular IFN responsive gene. Flanking sequences are shown to determine the specificity of expression to a particular type of IFN (9). Recently, Lew *et al.* (22) reported the identification of a repressor element termed, IFN-gamma activation site (GAS), whose guanylate-binding protein gene is type I and II IFN-inducible. Chernajovsky and

Kirby-Sanders (23) also described the presence of a possible repressor element at position -450 of the type I IFN inducible human 6-16 promoter. Apart from such reports, little has been published about the identification of other regulatory elements in IFN responsive genes. This contrasts with other cytokine-inducible genes such as the rat 1-acid glycoprotein promoter, where a number of different elements are recognised (24). Here, we provided evidence for a repressor element (RE) in close proximity to 2 ISREs in the human MxA promoter. Deletion of 40 bp (from -125 to -165) from the MxA regulatory sequence resulted in an abrupt rise in constitutive expression suggesting that the gene was under active repression in the resting state. This high basal level of expression could be reduced from 58% to 14% when the RE was reconstituted as a single copy (Figure 2A). A possible explanation that repression was not completely restored to its original 1% level might be the introduction of base mismatches at the reconstituted site. We then showed that this RE was capable of binding to specific protein(s), regardless of the IFN status, which was not competed out by an excess ISRE, suggesting that DNA-binding proteins of the RE and ISRE are different. Furthermore, bandshift results suggest that the 19 bp core sequence is the protein(s) binding site and that a multimeric protein complex is involved in DNA binding (Figure 3C). A repressor element physically distinct from the ISREs would offer greater flexibility and better fine tuning of expression. It would suggest a scenario where MxA expression is the net result of an active IFN induction step via the ISRE(s)

Figure 3. Protein(s) binding to RE and rRE. (A) Bandshift assays were performed with the [α-³²P]dCTP labelled, double stranded 40mer RE as probe on nuclear lysates derived from untreated or IFN-induced L132 cells (as indicated). Lane 1 = probe without lysate; lane 2 = probe and nuclear lysate; lane 3 = as in 2 and specific competitor in 100 fold excess (unlabelled probe); lane 4 = as in 2 and 50 fold excess of non-specific competitor (1kb ladder DNA marker, Gibco-BRL). Regardless of IFN treatment (see also panel B), 2 specific bands (sb) were detected which was only competed out by unlabelled probe (lane 3). nb = non-specific binding. Note that a 50 fold excess of RE (specific competitor) was also sufficient to compete out the labelled probe and that the faint band seen below nb was not a consistent feature in other bandshift assays (data not shown). (B) Binding pattern with the 40mer RE as probe was the same regardless of the IFN status of nuclear lysates used which was not competed out with an 50 fold excess of double stranded ISRE. Lane 1 = probe without lysate; lane 2 = probe with nuclear lysate from cells incubated with IFN for 15 h; lane 3 = as in lane 2 and 50 fold excess of ISRE; lane 4 = as in lane 3 except that the nuclear lysate was from cells incubated with IFN for only 2 h; lane 5 = as in lane 3 except that the nuclear lysate was from uninduced cells; lane 6 and 7 = as in lane 2 with 50 and 100 fold excess of unlabelled RE respectively. Abbreviations as in (A). (C) Bandshift assays were performed with an (rRE)_{2.5} as probe in the presence of different amounts of non-specific competitors at >0.1% NP-40. Lane 1 = probe without lysate; lane 2 = probe with uninduced nuclear lysate; lane 3 = as in 2 and 100 fold excess of non-specific competitor; lanes 4, 5 and 6 as in lane 2 with 100 fold, 50 fold and 20 fold excess of specific (rRE)_{2.5} competitor respectively; lanes 7, 8, 9 and 10 as in lane 2 with 120 fold, 100 fold, 50 fold and 20 fold excess of endogenous 40mer RE respectively. sb indicates specific bands. Two other specific bands, as indicated by smaller arrows, are only detectable where [NP-40] > 0.1%. The different banding patterns seen above suggests the involvement of a multimeric protein complex which may have undergone differing degree of dissociations.

with a possible derepression event at the RE. The bandshift pattern did not show any apparent change regardless of the IFN induction status of the lysates. At present the full explanation for this observation is not known. It may be that repression is mediated via a second protein(s) which interacts with the RE binding protein(s) and possibly, given the close proximity to the ISREs, with other interferon-stimulated gene factors (ISGFs) as well.

The identification of a discrete RE in an IFN inducible gene has given a different approach in the study of IFN inducible gene regulation. Investigation into the protein factor(s) associated with the RE could complement well with the current work, performed in several laboratories, on the various ISGFs that bind to the ISRE.

Phenotypic reversal from repressor to enhancer/promoter by multimerisation—implications on mutagenesis *in vivo*

While examining the repressor element in the MxA promoter, we came across an unexpected phenomenon when this RE was multimerised. Instead of repression, its functional phenotype was completely reversed. Tandem repeats of greater than 2 RE inserted in front of the MxA or SV40 promoter, in either orientation, conferred enhanced expression. The RE multimer could also behave like an independent, constitutive promoter when cloned into a promoterless CAT vector and is functional not only in human L132 cells but in rat L6 cells (data not shown) as well as in fish muscles *in vivo* (25). It is unlikely that this promoter feature was the result of a fortuitous creation of functional junction sequences as multimerisation of only the core 19 bp monomer (rRE), which contained the 9 bp direct repeat, behaved in a similar way. In addition, there was a direct correlation in the number of repeats and the level of CAT expression. Experiments are underway to determine the importance of spacing between the two 9 bp repeats. Great care must be taken in the interpretation of this rather surprising finding. The behaviour of the artificially created multimer might bear no

functional, mechanistic relation to the RE monomer in the endogenous MxA gene. The demonstration that specific protein(s) bound to the (rRE)_{2.5} element could be competed out by unlabelled RE (40mer) would suggest the two elements share common DNA-binding protein(s). The phenotypic divergence of these two elements may rest in differences in complexities of subsequent protein-protein interactions. Nonetheless, we have demonstrated a phenomenon *in vitro*, of phenotypic reversal by oligomerisation of a discrete motif.

The question that has arisen is whether this phenomenon, which is quite separate from the original finding of an endogenous RE in the MxA gene, has any relevance *in vivo*. We believe there may well be. The acquisition of a functional element through repetition of a relatively short sequence is quite well documented. Multimers of a consensus hexamer taken from the virus-inducible elements, in promoters of mouse and human—and β -IFNs, when inserted in front of an otherwise minimal, non functional promoter could mediate virus-inducible transcription (26). Repeats of this nature, which show homology to ISRE, appear also to be able to function as constitutive, silencing and IFN inducible elements depending on the context of the hexamer and the flanking sequences (10). Likewise, multimerisation of different enhancer elements, such as the Sph element, found within the SV40 enhancer can lead to the creation of new enhancers (27,28). Of particular interest is the pattern similarity between the 9 bp direct repeats in the RE of MxA and the Sph motifs I and II which are also composed of a direct imperfect repeat of 9 bp. Naturally occurring short tandem repeats, found in the U3 regions of many retroviruses, are functional enhancer elements (29,30). In humans, direct repeats of different sizes are widely distributed throughout the genome (e.g. 31 and refs. therein). Though their functions are often unclear, short duplications are present within some genes such as in the 6-16 IFN-inducible promoter and the placental protein 14 gene (32,33). Given the formation of functional elements through tandem repeats is a fairly well recognised occurrence, the enhancer/promoter phenomenon

Figure 4. The artificially created RE multimer exhibits apparent enhancer and promoter activities independent of its original MxA promoter. pCAT-Promoter vector (Promega) expression (lane 2) of 14% was increased to 58% when an (RE)₃ was inserted just 5' to its SV40 promoter at the *Bgl* II site, in the sense orientation (lane 1). No enhancer activity was seen when only an RE monomer was used (data not shown). An (RE)₃ was inserted into the *Pst* I site of pCAT-Basic (Promega), a promoterless CAT vector, in the sense (lanes 5 and 6) and reverse (lanes 7 and 8) direction. Their expression in the presence (lanes 4, 6 and 8) and absence of IFNs was compared to a positive control, pCAT-Control vector (Promega) (lanes 3 and 4). CAT expression as a result of (RE)₃ insertion, in either orientation (lanes 5 to 8), was comparably as high as the positive control (lane 3 and 4). Note however a slight drop in CAT expression in the presence of IFN (lane 6 and 8) as compared to untreated cells (lane 5 and 7) may be due to the indirect antiproliferative effects of IFNs. This effect may be less apparent with the strong SV40 promoter present in pCAT-Control (lanes 3 and 4). Lane 9 and 13 were pCAT-Basic controls and lane 10 was without transfected DNA. The original RE was reduced to a 19mer (rRE; see main text), multimerised and inserted into pCAT-basic. Lanes 11 and 12 showed rRE multimers of 8 and 2.5 repeats respectively, in the reverse orientation. These multimerised rREs remained active and showed dosage response. Each CAT reaction was normalised to 1.0A₄₂₀/2h of β -gal activity and incubated for 1 h.

associated with multimerisation of the RE in the MxA gene should not be too surprising. The novelty aspect is in the demonstration of phenotypic reversal in gene regulation. In a wider context, it is conceivable that mutagenesis by deletion, translocation or duplication can result in the coming together or creation of DNA repeats. This may lead to the formation of a functional regulatory element in the vicinity of an essential gene whose disruption can have profound adverse effects on the cell. No homology with the 19 bp, containing the 9 bp repeats, was found in the data bank suggesting its uniqueness. However, it is interesting to note that the 5' end of the human proto-oncogene c-abl contains one of the 9 bp repeats of the MxA RE (34). A recent report (35) on the *de novo* Alu insertional mutation that led to neurofibromatosis type1, suggests that Alu retrotransposition is an ongoing process in the human germ line. This example illustrates one plausible mechanism where direct repeat elements may be formed.

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