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A single nucleotide change in the c-*myc* internal ribosome entry segment leads to enhanced binding of a group of protein factors

Fiona E. M. Paulin[1], Stephen A. Chappell and Anne E. Willis*

Department of Biochemistry, University of Leicester, University Road, Leicester LE1 7RH, UK and
[1]Department of Anatomy and Physiology, Medical Science Institute, University of Dundee, Dundee DD1 4HN, UK

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ABSTRACT

A 340 nucleotide section of the c-*myc* 5′ untranslated region (UTR) contains an internal ribosome entry segment. We have described previously a mutation in this region of RNA in cell lines derived from patients with multiple myeloma (MM) which exhibit increased expression of c-*myc* protein by an aberrant translational mechanism. In this study we show by electrophoretic mobility shift assays (EMSA), north-western blotting and UV cross-linking that radiolabelled c-*myc* 5′ UTR RNA transcripts which harbour the mutation cause enhanced binding of cellular proteins. In addition, we also demonstrate that an MM derived cell line possesses an altered repertoire of RNA binding proteins. Our data suggest that the deregulated expression of c-*myc* in MM could result both from the effect of the mutation and the additional proteins which are present in these cell types.

INTRODUCTION

The c-*myc* protein is a key regulator in the processes of cellular proliferation, growth cycle progression and differentiation (1). A variety of different growth stimuli induce c-*myc* expression within the first 2 h of G_1 and this induction is sufficient to cause quiescent cells to enter S phase (2,3). In contrast, c-*myc* expression is down-regulated upon growth arrest and inhibition of c-*myc* expression using antisense oligonucleotides will prevent mitogen-treated cells from entering S phase (3). The c-*myc* protein functions as a transcription factor and along with its binding partner, Max, forms heterodimers which bind to a hexanucleotide sequence (CACGTG) known as an E box (4). Potential c-*myc*/Max target sequences include p53, eIF2α, orinithine decarboxylase, eIF4E, CAD, uracil DNA glycosylase, α prothymosin and MrDb (5).

c-Myc protein levels are regulated by a wide range of different mechanisms. These include changes in the rate of transcription, occurring at both the initiation and elongation phases (6–8), alterations in the stability of the mRNA (9,10), changes in the half-life of the protein (11) and translational control mechanisms (12–16). In common with many other mRNAs encoding growth-related proteins, the 5′ untranslated region (UTR) of c-*myc*, which is encoded by exon 1 (3), is long and highly structured. Such structured 5′ UTRs can regulate translation by three major methods: (i) they can inhibit the normal scanning method of translation initiation [involving the binding of the eukaryotic initiation factor (eIF) eIF4E to the 7 methyl G cap, the helicase eIF4A and eIF4G (see 17 for review)]; (ii) they can contain upstream open reading frames (uORFs) which can cause translational inhibition (18); or (iii) they can contain internal ribosome entry segments (IRESs) and so direct cap-independent translation initiation (19–21). The c-*myc* 5′ UTR encoded by exon I was proposed originally by Saito *et al*. as a modulator of the translational efficiency (22) and, more recently, it has been shown that c-*myc* 5′ UTR contains an IRES which is capable of directing internal initiation of protein synthesis (15,16). The IRES lies 390 nucleotides upstream of the AUG translation start codon (16) thus meaning that c-*myc* transcripts which initiate from the major promoters P1 and P2, giving rise to 10–25 and 75–95% of cellular transcripts respectively (3), will contain the IRES. To date, very few eukaryotic IRESes have been identified and the situations where they are required remain elusive with c-*myc* providing the first example of a proto-oncogene which can be regulated in this manner (15,16). However, it should be noted that c-*myc* can additionally be translated in a cap-dependent manner since cells over-expressing the cap binding protein eIF4E, also display enhanced expression of c-*myc* (23).

Several forms of human neoplasia are associated with the overexpression of the endogenous c-*myc* gene. This can occur by gene amplification (24,25) and chromosomal translocations, e.g. in Burkitt's lymphoma involving the c-*myc* locus on human chromosome 8 to any one of the immunoglobulin loci on chromosome 2, 14 or 22 (26,27). Two situations of aberrant translational control of c-*myc* have also been described (28,29). In patients with multiple myeloma (MM), a disorder characterised by expansion of a plasma cell type in the bone marrow and osteolysis (30), a 20-fold increase in the amount of c-*myc* protein occurs by an aberrant translational mechanism (29). It appears that the increase in the c-*myc* protein levels in these cells is, in part, due to a 3.4-fold increase in the degree of association of the *c-myc* mRNA with the polysomes. This occurs without an

*To whom correspondence should be addressed. Tel: +44 116 2523363; Fax: +44 116 2523369; Email: aew5@le.ac.uk

increase in polysome size thus suggesting that there is an enhancement in the degree of mobilisation of this message from inactive mRNPs to polysomes rather than an alteration in the reinitiation rate (29). A consistent point mutation (C→T transition at 2756) was identified in all MM cell lines examined, which lies within the region subsequently shown by deletion analysis to contain the IRES (16).

In this work we have investigated the effect that this mutation has on the binding of protein factors to the c-*myc* 5′ UTR and show that there is enhanced binding of proteins to RNAs harbouring the mutation. In addition, we demonstrate that an MM-derived cell line expresses a different repertoire of proteins which bind to this region.

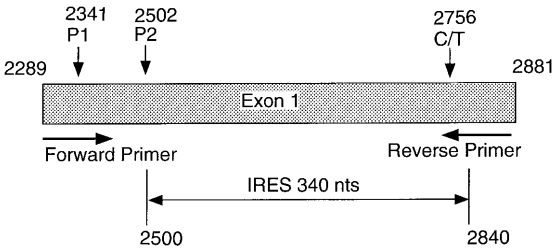

Figure 1. The 600 bp section of the *c-myc* 5′ UTR was obtained by PCR. The position of the IRES and the mutation are shown. This fragment was subsequently subcloned into the vector Bluescript SK+.

MATERIALS AND METHODS

Cell culture

The cell lines GM2132 (derived from an MM patient) and GM03201 (lymphoblastoid cell line derived from a healthy individual) were purchased from the American Type Culture Collection. Cells were grown in Roswell Park Memorial Institute medium (RPMI, GIBCO) containing 15% foetal calf serum in a 5% CO_2 humidified atmosphere.

Polymerase chain reaction

Primers 5′-GCCGGATCCCCGGCCCCTTTATAATGCGAG-3′ and 5′-GTGGAATTCCTTTGGTTTTTTTCCCCAAACTACC-CGAAA-3′, designed to contain restriction sites for *Bam*HI and *Eco*RI, were synthesised and used to amplify c-*myc* exon 1 +/– the mutation (2289–2881; numbering as in 31). PCR reactions contained 10 µl PCR buffer (Advanced Biosystems), 10 µl $MgCl_2$ (25 mM), 1 µl dNTPs (10 mM of each dATP, dCTP, dTTP, dGTP), 1 U of *Taq* DNA polymerase (Advanced Biosystems), 0.7 µg DNA and 20 µM of each oligonucleotide. The PCR reactions were carried out in a Perkin Elmer Cetus DNA thermal cycler at 94°C for 3 min followed by 37 cycles of (94°C for 2 min, 63°C for 3 min, 72°C for 2 min) and 72°C for 10 min. The resulting fragments (Fig. 1) were digested with *Bam*HI and *Eco*RI and ligated into pBluescript II SK+ (Stratagene) which had also been restricted with these enzymes and the ligation mixture transfected into competent JM109 cells. The plasmid pJHRV was a kind gift from Dr R. Jackson, University of Cambridge, and the plasmid pBluescript II SK+ containing a portion of the chicken GAPDH gene was a gift from Prof. D. Critchely, University of Leicester.

In vitro RNA synthesis

Vectors were linearised downstream of inserts, phenol and chloroform extracted, and then ethanol precipitated. An aliquot of 1 µg of restricted template was incubated with 1 µl of each rNTP (10 mM) (rATP, rGTP, rCTP, rUTP), 1 µl 0.75 M DTT, 1 µl RNasin ribonuclease inhibitor (Promega), 5 µl 5× transcription buffer (Stratagene) and 10 U of T3 RNA polymerase at 37°C for 1 h. DNA templates were removed by addition of 10 U of RNase free DNase for 20 min at 37°C, and after phenol and chloroform extractions precipitated using 0.1 vol 3 M NaOAc (pH 5.2) and 2.5 vol ethanol. Radiolabelled transcripts were synthesised as above (for the UV cross-linking analysis additionally 1 mM 4-thio UTP was also included), and either 5 µl 800 Ci/mmol, 10 mCi/ml [α-^{32}P]rCTP or rUTP in place of the appropriate unlabelled nucleotide. Unincorporated nucleotides were removed over a 1 ml Sephadex G-50 column. Transcript concentrations were determined by Cerenkov scintillation counting or by A_{260} values.

Cell extract (CE) preparation

Approximately 60–90 × 10^6 cells were harvested by centrifugation at 1000 r.p.m. for 5 min and washed in phosphate buffered saline. Cell pellets were resuspended in 600 µl lysis buffer, (0.5% NP-40, 300 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES pH 7.4, 10% aprotinin, leupeptin and *N*-∝-*p*-Tosyl-L-lysine chloromethyl ketone at 1 µg/ml and nuclei removed by subsequent centrifugation at 13 000 r.p.m. for 10 min.

Electrophoretic mobility shift assays (EMSAs)

Approximately 8.6 × 10^{-8} nmol labelled transcript (23 000 c.p.m.) were incubated in a 10 µl buffer mix containing 5 µl 5× transcription buffer (200 mM Tris–HCl pH 8.0, 40 mM $MgCl_2$, 10 mM spermidine, 250 mM NaCl), 0.75 µl DTT (1 M), 1.5 µl tRNA (10 mg/ml), 1 µl rATP (10 mM), 1 µl glycerol and 0.17 µl vanadyl ribonucleoside complexes (10 mM). Nuclear free CEs were diluted in 0.01% DEPC-treated sterile, distilled water to a final volume of 20 µl and then incubated with the transcript mix at room temperature for 10 min. An aliquot of 3 µl 10× TBE loading buffer was added and samples loaded directly onto 0.7% agarose gels prepared using 1× TBE filter sterilised buffer. Samples were electrophoresed at 90 V for 2 h in 1× TBE filter sterilised buffer. Gels were dried under vacuum at 60°C for 2 h and exposed to Fuji-RX X-ray film at –70°C for 4–20 h.

North-western blotting

CEs were were separated by SDS–PAGE on either 7 or 10% gels. Proteins immobilised on nitrocellulose were allowed to renature by incubating the membranes in 5% BSA dissolved in 10–15 ml D67NP-40 solution [65% (v/v) D-Base (100 mM KCl, 0.2 mM K-EDTA, pH 8.0), 6.7 mM TEA pH 7.9, 170 mM DTT, 0.05% NP-40 filter sterilised] containing 1 mg/ml alkali denatured salmon testes DNA and 0.02 mg/ml yeast tRNA to block non-specific protein and nucleic acid binding sites. After at least 1 h incubation at room temperature, ^{32}P-labelled RNA transcripts were added and then incubated for a further 1 h at 30°C.

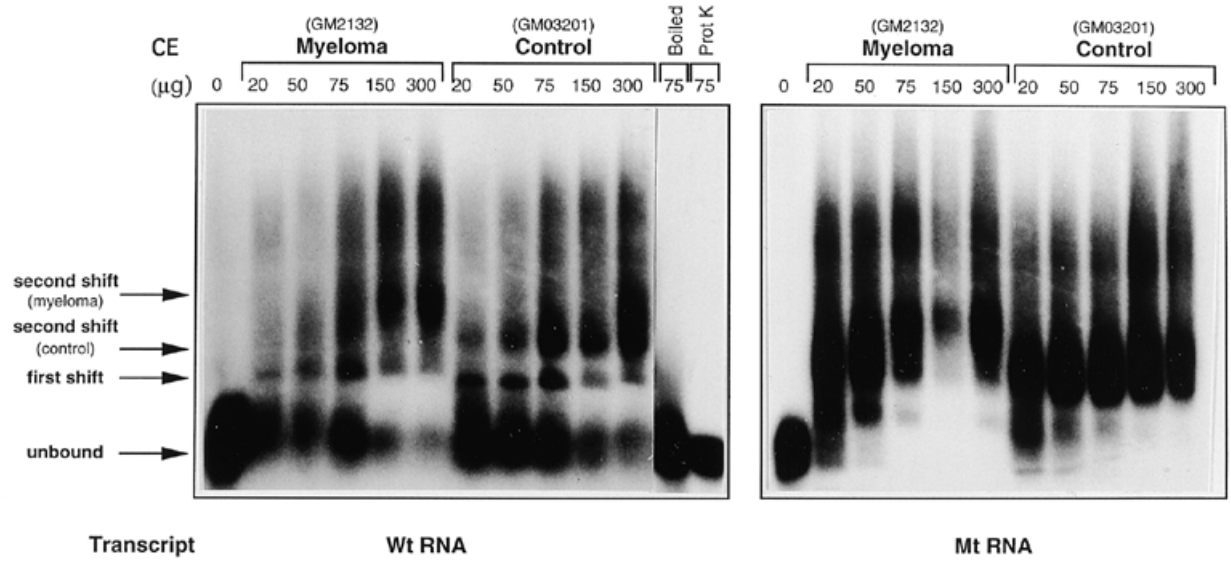

Figure 2. EMSAs showing CE titrations using either MM (GM2132) or control (GM03201) CEs. Equivalent amounts of either Wt, Mt *c-myc* 5′ UTR RNA, transcripts were incubated with CEs as indicated.

Membranes were washed in D67NP-40 solution and exposed directly to Fuji-RX X-ray film for 1–40 h at room temperature.

UV cross-linking assay

Radiolabelled RNA transcript (2.4 nmol; 4.5×10^5 c.p.m.) was incubated with 20 μg of CE in a 30 μl buffer mix (containing 10 mM HEPES pH 7.4, 3 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 1 mM ATP, 6% glycerol) in the presence or absence of unlabelled competitor transcripts, for 10 min at room temperature in a 96-well microtitre plate (Falcon). The samples were then incubated for a further 10 min with heparin at a concentration of 0.2 mg/ml. Samples were UV-irradiated on ice for a period of 30 min using a 312 nm UV light source. RNase A (0.2 mg/ml) was added to each of the samples and incubated at 37°C for 30 min to allow degradation of any unprotected RNA species. An equal volume of 2× SDS sample buffer was added to the samples prior to separation by SDS–PAGE (7.5% gels). Gels were then stained using Coomassie blue, dried, and results visualised on a Molecular Dynamics PhosphorImager.

RESULTS

Investigation of the affinity of cytoplasmic proteins for the Wt and Mt c-*myc* 5′ UTR RNA transcripts

To analyse whether proteins were capable of binding to the *c-myc* 5′ UTR, EMSAs were performed whereby radiolabelled transcripts were incubated in the presence of cellular proteins. Nuclear free CEs were prepared from both an MM cell line, GM2132, and a control lymphoblastoid cell line, GM03201, which were found to be the most representative from each class as determined by FACS analysis (data not shown). The GM2132 cell line additionally has been shown to contain the highest level of *c-myc* protein of all the myeloma cell lines and hence could potentially contain enhanced levels of any putative *trans*-regulatory factors (29). Radiolabelled transcripts containing the Mt and Wt *c-myc* 5′ UTR (Fig. 1) sequences were incubated with increasing concentrations of GM2132 CE or GM03201 CE in standard buffer conditions for 10 min. Samples were then separated by agarose gel electrophoresis, the gels dried and visualised by autoradiography. The results of these EMSAs are shown (Fig. 2).

In each case, the addition of increasing amounts of CE resulted in the presence of less unbound transcript which was consequently associated with the elevated signal in higher molecular weight complexes. By comparing the electrophoretic mobility shift patterns obtained with the GM2132 CEs (MM) with those from the GM03201 CEs (control) it can be seen that the second shift is higher with the GM2132 CE than the GM03201 CE. This would tend to suggest that in the MM cell line there is an altered repertoire of RNA binding proteins.

The electrophoretic mobility shift patterns obtained with the Wt and Mt c-*myc* 5′ UTR transcripts were very similar indicating that both RNA species probably bind the same proteins. However, for any given CE concentration, incubated with the same concentration of radiolabelled RNA, a higher proportion of the Mt transcript was present in a bound state compared to the equivalent Wt transcript. Thus, it appears that there is enhanced binding of proteins to the mutant transcript.

Identification of specific c-*myc* 5′ UTR–protein complexes

Two different techniques were employed in order to examine further the proteins which bind to the c-*myc* 5′ UTR: north-western blotting and UV cross-linking.

North-western blotting

Varying concentrations of nuclear free CEs from both the control (GM03201) and myeloma (GM2132) cell lines were size fractionated by SDS–PAGE and electroblotted on to nitrocellulose membranes. Proteins were allowed to partially renature on the membranes and then probed using radiolabelled transcripts. The results obtained using the Mt and Wt *c-myc* 5′ UTR, HRV-IRES

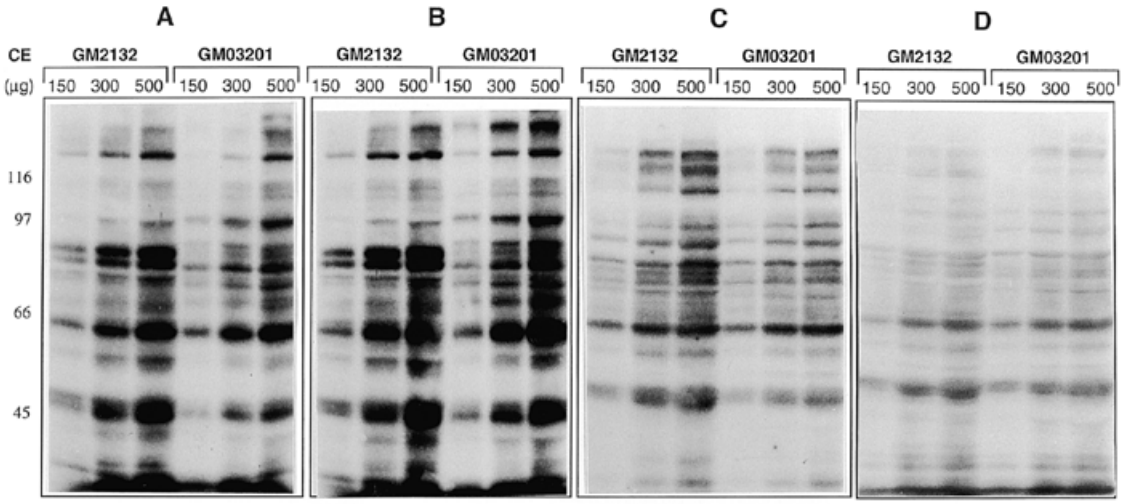

Figure 3. North-western analysis of MM (GM2132) and control (GM03201) CEs. Different concentrations of CEs were separated by SDS–PAGE, blotted on to nitrocellulose and probed using equivalent amounts of radiolabelled RNA transcripts. (**A**) Wt *c-myc* 5′ UTR transcript. (**B**) Mt *c-myc* 5′ UTR transcript. (**C**) HRV-IRES transcript. (**D**) GAPDH transcript.

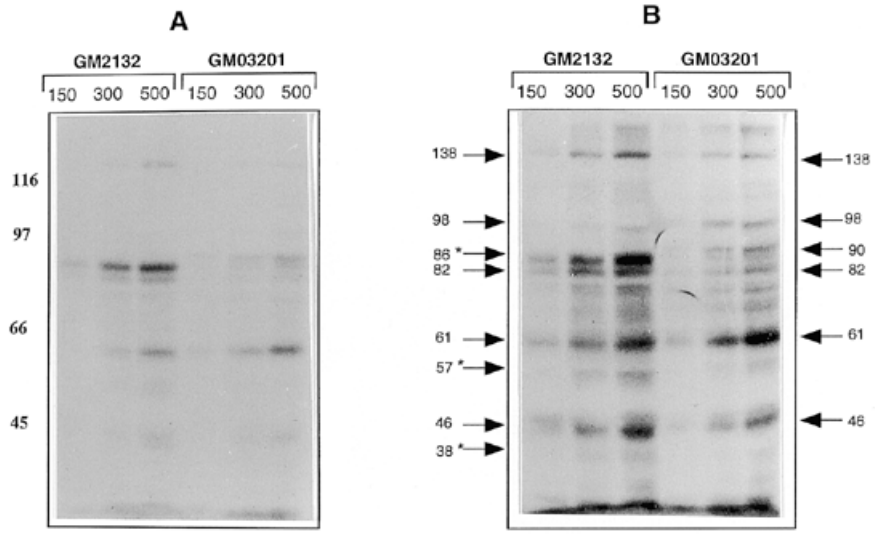

Figure 4. North-western analysis of myeloma and control CEs in the presence of non-specific inhibitors. (**A**) Wt *c-myc* 5′ UTR transcript with 2 μg of unlabelled GAPDH transcript as non-specific competitor RNA. (**B**) Mt *c-myc* 5′ UTR transcript with 2 μg of unlabelled GAPDH transcript as non-specific competitor RNA. Proteins which are present at higher levels in MM extracts (GM2132) are marked (*).

and the GAPDH transcripts are shown (Fig. 3). In addition, north-western analysis using the Wt and Mt *c-myc* 5′ UTR transcripts was performed in the presence of an excess of unlabelled GAPDH transcript acting as a non-specific competitor (Fig. 4A and B).

For each RNA transcript, a large number of RNA binding proteins were observed and although each one gave rise to a characteristic pattern of proteins, many were common to all of the transcripts. This suggests that they either represent non-specific RNA-binding proteins or bind RNA simply as a result of their high levels of expression. The latter is particularly evident at low protein concentrations. A number of differences were seen between the two cell lines; however, in general, the majority of these were not transcript specific implying that the myeloma cell lines have an altered spectrum of general RNA protein binding factors (Fig. 3A–D).

The use of unlabelled GAPDH competitor (Fig. 4A and B) significantly reduced the number of proteins binding to both the *c-myc* 5′ UTR transcripts although the effect was most marked for the Wt transcripts. Again this implies that the Mt transcript has a greater affinity for protein binding which is consistent with the EMSA data.

Predominantly, Wt *c-myc* 5′ UTR binding proteins, with molecular weights of 138, 82, 61 and 46 kDa were found in both GM2132 and GM03201 CEs, whilst proteins of 90 and 86 kDa appeared to be GM03201 CE and GM2132 CE specific respectively. Minimal binding to four other species of 79, 61, 76 and 70 kDa was also observed, the latter two of which were GM03201 CE specific.

All of the aforementioned proteins were also found to bind the Mt transcript, albeit with higher affinity. This non-specific elevation in binding capacity of the Mt transcript was also

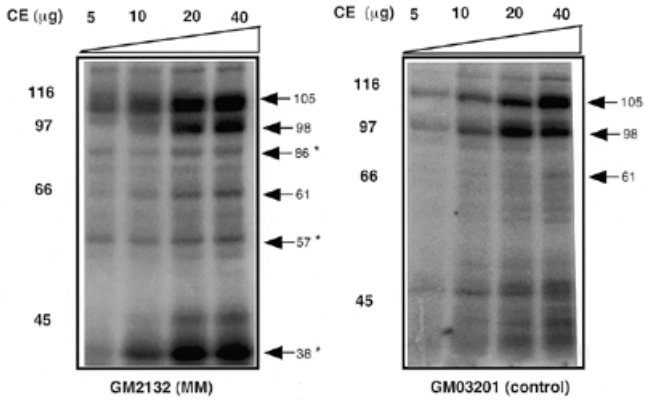

Figure 5. UV cross-linking analysis of MM (GM2132) and control (GM03201) CEs. Different concentrations of CEs were incubated with radiolabelled c-*myc* Wt transcripts, exposed to UV light and then treated with RNase A (as described in Materials and Methods). A phosphorimage of the SDS–PAGE gel is shown. Proteins which are present at higher levels in MM extracts (GM2132) are marked (*).

accompanied by the binding of four additional proteins of 160, 98, 57 and 38 kDa which were not initially detected with the Wt transcript. In addition, there appears to be an increased level of proteins of 57 and 38 kDa in the myeloma-derived cell line. Longer exposures of the north-western probed with the Wt transcript (data not shown), in conjunction with the singular transcript north-western data, showed that the Wt transcript is also capable of binding these factors although with significantly lower affinity.

UV cross-linking analysis

UV cross-linking was performed to verify that the proteins identified by the previous method were indeed capable of directly interacting with the *c-myc* 5′ UTR RNA. Accordingly, [^{32}P]CTP-radiolabelled Wt *c-myc* 5′ UTR transcripts were incubated with increasing amounts of GM2132 myeloma and GM03201 control CEs, proteins cross-linked to the RNA and unprotected RNA digested with RNase A. The proteins were then separated by SDS–PAGE and proteins binding RNA fragments visualised on a phosphorimager. Three major proteins were identified which interacted with the Wt c-*myc* 5′ UTR with molecular weights of ~105, 98 and 38 kDa (Fig. 5). It can be seen that the GM2132 myeloma cells contain an increased level of the 38 kDa protein (Fig. 5) and, in addition, proteins with molecular weights of ~86 and 57 kDa were present at higher levels or had enhanced RNA binding activity in the MM-derived cell lines (Fig. 5). Proteins with identical molecular weights were found to interact with the radiolabelled Mt c-*myc* sequence yet, as before, stronger binding was observed between this transcript and these protein factors when compared to the Wt sequence (data not shown).

UV competition assays

In order to investigate the specificity of the interactions of these proteins with the c-*myc* 5′ UTR, UV cross-linking analysis was performed using radiolabelled Wt transcripts in the presence of unlabelled Wt or Mt *c-myc* 5′ UTR, HRV-IRES and GAPDH transcripts. The amount of radioactivity in each RNA–protein complex was determined using a Molecular Dynamics PhosphorImager. In both cell lines, addition of an excess of unlabelled Wt/Mt *c-myc* 5′ UTR or HRV-IRES transcripts had an effect on the formation of the protein complexes when compared to GAPDH (Fig. 6A and B). As before, we observed much less of the 38 kDa protein in the control cell lines when compared to the myeloma cell lines (Fig. 6A and B). In addition, prior incubation of CEs with an excess of the unlabelled Mt *c-myc* 5′ UTR transcripts had, in general, a greater inhibitory effect on the proteins binding to the radiolabelled Wt transcripts than the unlabelled Wt-*myc* transcripts (Fig. 6A and B).

The Mt transcript competed with the radiolabelled Wt RNA for binding to the 105 and 98 kDa proteins with 38 and 18% respectively of the radiolabelled protein complexes remaining after preincubation with a 30-fold molar excess and 11 and 9 % remaining after preincubation with a 100 molar excess (Fig. 6A). In contrast, preincubation with unlabelled Wt only resulted in a reduction of the 105 or 98 kDa radioactive protein complexes of 52 and 54% at a 30 molar excess and 22 and 28% with a 100 molar excess (Fig. 6A). The HRV-IRES transcripts had a marked effect with the 98 kDa protein and a 100 molar excess ablated the binding of the Wt transcripts (Fig. 6A). However, excess unlabelled Mt, Wt or HRV-IRES transcripts competed equally for binding to the 38 kDa protein with the radiolabelled Wt RNA (Fig. 6A).

DISCUSSION

We have described previously a C→T mutation at position 2756 (a region which we have subsequently shown to contain an IRES) in cell lines derived from patients with MM which show an increased expression of the c-*myc* protein by translational mechanisms (29). To investigate the effect of this mutation on protein binding and to determine whether the MM cells lines contain an altered spectrum of RNA binding proteins, three complementary approaches were employed.

The data obtained from the EMSAs demonstrated that the RNA sequences harbouring the mutation were able to enhance the binding of all proteins, a feature which we observed using all three techniques.

North-western blotting identified a wide range of general RNA binding proteins since the majority of these proteins were also found to bind the GAPDH transcript. However, addition of GAPDH as a competitor significantly reduced the non-specific binding and allowed the identification of putative c-*myc* 5′ UTR-specific binding proteins. Both the Wt and Mt *c-myc* 5′ UTR transcripts displayed similar protein binding potential, as predicted by virtue of the fact that only 1 nt out of 600 was altered. However, again there was enhancement in RNA binding affinity to the *c-myc* Mt transcript compared to the Wt sequence. For example, the Mt transcript bound more strongly to proteins of 98 and 38 kDa and, in addition, bound two other proteins of 160 and 57 kDa. Some differences in general RNA binding factors were also detected between the myeloma GM2132 and control GM03201 CEs (Table 1). This could account for the slight discrepancies in the electrophoretic mobility shift patterns obtained and may reflect the altered differentiated states of these B-cell clones. Alternatively, the additional proteins observed in the myeloma CEs may be attributable to the malignant phenotype of these cells.

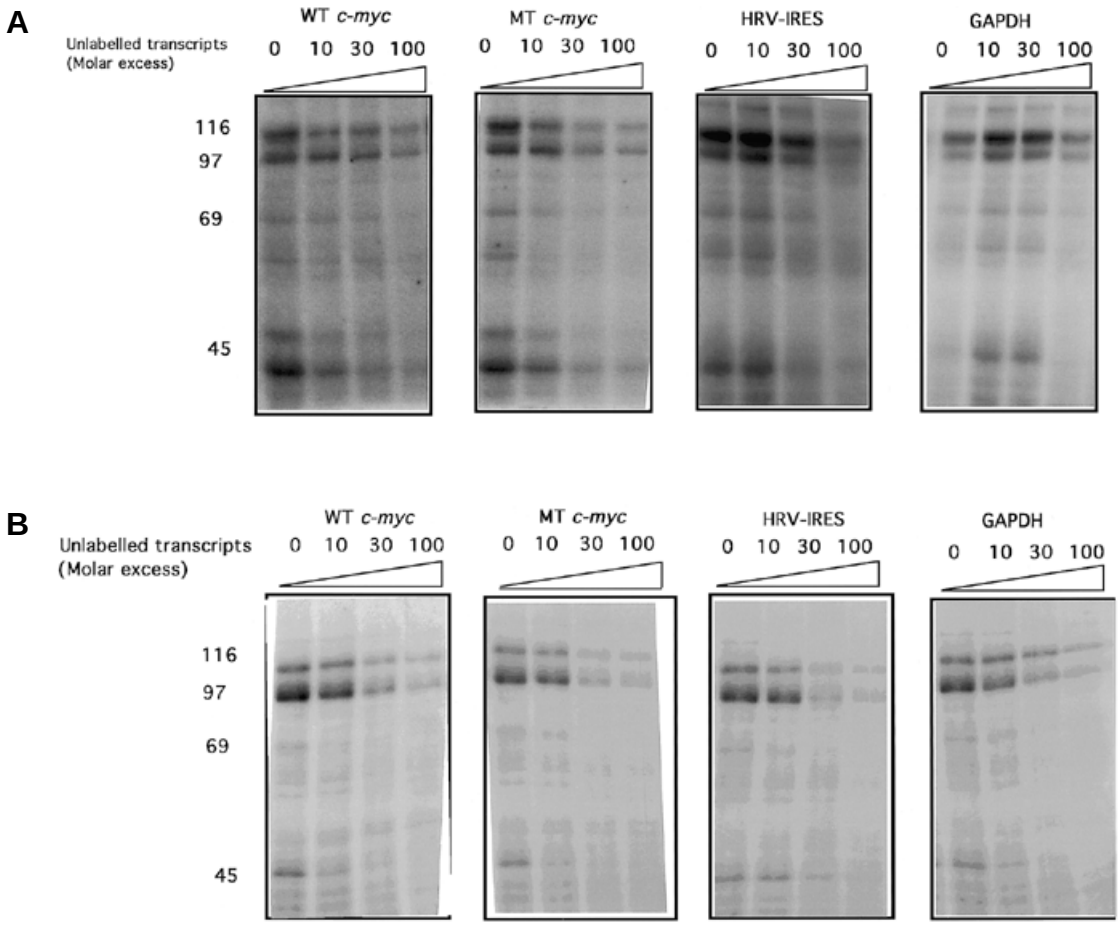

Figure 6. UV cross-linking competition assays of protein complex–c-*myc* 5′ UTR with unlabelled RNAs. Twenty µg of MM (GM2132) (**A**) or control (GM03201) (**B**) CEs were incubated with the radiolabelled Wt *c-myc* 5′ UTR with increasing molar excesses of competitor RNAs as shown. A phosphorimage of the SDS–PAGE is shown.

Table 1. Summary of the proteins derived from either control (GM03201) or MM cells (GM2132) which interact with the c-*myc* 5′ UTR RNA transcripts

Method	GM2132 c-*myc* 5′ UTR-specific binding proteins (kDa)	GM03201 c-*myc* 5′ UTR-specific binding proteins (kDa)	Proteins which show enhanced binding to the mutant sequence (kDa)	Summary of proteins binding to the c-*myc* 5′ UTR (kDa)
North-western blotting	86, 57, 38	90	160, 98, 57, 38	160, 138, 98, 61, 57, 38
UV cross-linking	86, 57, 38		105, 98	105, 98, 61, 57, 38

Proteins of 105, 98, 86, 61, 57 and 38 kDa were found to bind to both Mt and Wt-*c-myc* 5′ UTR transcripts in UV cross-linking experiments, although it could be seen that the myeloma cell lines had increased levels of the 86, 61, 55 and 38 kDa proteins (Table 1). Using excess unlabelled RNA transcripts we observed that the mutant sequences were able to compete more effectively than the Wt RNA for proteins with molecular weights of 105 and 98 kDa. Interestingly, proteins with molecular weights of 98 and 38 kDa have been shown previously to interact with the HRV-IRES and have been implicated in IRES function (32).

In conclusion, the north-western analysis and the UV cross-linking experiments imply that the C→T point mutation at 2756 is sufficient to enhance the interaction of a protein of 98 kDa to the *c-myc* 5′ UTR and our preliminary data suggest that this protein may bind around this 2756 site (F.E.M.Paulin and A.E.Willis, unpublished results). Several examples exist in viral systems which demonstrate that single mutations are capable of altering IRES function. For example, a single substitution in the FMDV-IRES has been shown to increase the degree of internal ribosome entry 1.5–5-fold (33). We speculate that this C→T mutation which we have found in cell lines derived from patients with MM combined with the altered protein repertoire could alter the initiation of translation through the IRES. However, the proof for this hypothesis awaits the purification of the factors involved and the investigation of their effects using functional assays.

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