Internal ribosome entry segment-mediated initiation of c-Myc protein synthesis following genotoxic stress

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Initiation of translation of the proto-oncogene c-*myc* can occur by either the cap-dependent scanning mechanism or by internal ribosome entry. The latter mechanism requires a complex RNA structural element that is located in the 5′ untranslated region of c-*myc*, termed an internal ribosome entry segment (IRES). Recent work has shown that IRESs are used to maintain protein expression under conditions when cap-dependent translation initiation is compromised; for example, during mitosis, apoptosis and under conditions of cell stress, such as hypoxia or heat shock. Induction of genotoxic stress also results in a large reduction in global protein synthesis rates and therefore we investigated whether the c-*myc* IRES was active following DNA damage. As expected, in cells treated with either ethylmethane sulphonate or mitomycin C there was a large reduction in protein synthesis, although this was brought about by two different mechanisms. However, in each case the c-*myc* IRES was active and c-Myc protein expression was maintained. Finally we showed that the proteins required for this process are downstream of the p38 mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated protein kinase (ERK)/MEK(MAPK/ERK kinase) signalling pathways, since pre-treatment of cells with inhibitors of these pathways before DNA damage is initiated inhibits both c-*myc* IRES activity and expression of c-Myc protein.

Key words: c-*myc* gene, DNA damage, internal ribosome entry segment, translation initiation.

INTRODUCTION

c-Myc is a nuclear phosphoprotein that, in conjunction with its binding partner Max, binds to E boxes [1,2]. Myc–Max heterodimers are potent activators of transcription, although the full complement of target genes with which c-Myc interacts have yet to be described [3]. The c-Myc protein plays a critical role in both cell proliferation and cell death and it is not surprising therefore that de-regulation of Myc expression is associated with a wide range of tumour types, particularly those of B-cell origin [4,5]. The regulation of c-*myc* is complex and involves changes in rates of transcription and alteration in stabilities of both the RNA and protein [6–8]. There is also overwhelming evidence that control of c-*myc* translation is important to the regulation of expression of c-Myc protein [9–13]. Most of the control of translation occurs at the initiation phase and this is the rate-limiting step of this process [14]. There are two mechanisms that are used by eukaryotic cells to initiate translation, the cap-dependent scanning mechanism and internal ribosome entry, and the data suggest that c-Myc protein synthesis can be initiated by both mechanisms [15].

The cap-dependent scanning mechanism of translation is used by the majority of mRNAs to initiate translation and requires the binding of the eukaryotic initiation factor (eIF) 4F complex (comprised of the cap-binding proteins eIF4E, eIF4G and eIF4A) to the mRNA, and recruitment of the 40 S ribosome [14]. The whole complex then scans to the first AUG codon in good Kozak consensus context. Global protein synthesis via this mechanism is a tightly regulated process and it is controlled by changes in the phosphorylation states of the eIFs and their binding partners, e.g. the cap-binding protein (eIF4E) and its partners eIF4E-binding protein (4E-BP) 1 and 2 [16]. It has been shown that up-regulation of c-*myc* translation initiation by cap-dependent scanning can occur via activation of the FK506-binding protein (FKBP) rapamycin-associating protein/mammalian target of rapamycin (FRAP/mTOR) signalling pathway [9]. This leads to increased phosphorylation of 4E-BP1 and thus a decrease in its association with eIF4E and an increase in its association with the eIF4G component of the eIF4F initiation complex [16].

For internal ribosome entry to occur a large complex structural element, an internal ribosome entry segment (IRES), is formed in the 5′-untranslated region (5′ UTR) of the mRNA. IRESs were first identified in picornavirus RNAs [17], but there are now many examples of cellular mRNAs that contain IRESs. In general these have been identified in genes whose proteins are associated with either cell growth or cell death, e.g. apoptotic protease activating factor-1 (Apaf-1) [18], X-linked inhibitor of apoptosis (XIAP) 1 [19], ornithine decarboxylase [20], vascular endothelial growth factor (VEGF) [21,22], fibroblast growth factor 2 [23] and c-*myc* [12–13]. The c-*myc* IRES is located in a 340 nt region downstream of the P2 promoter [12]. The fact that 75–90 % of c-*myc* transcripts are transcribed from this promoter would suggest that the majority of c-*myc* mRNAs in the cell have the potential to initiate translation by internal ribosome entry. Thus this is likely to be an important mechanism for c-*myc* regulation.

It appears that cellular IRESs are required to maintain the expression of critical proteins in situations where cap-dependent translation is reduced. These include: following heat shock, during mitosis, during apoptosis and cell stress, e.g. hypoxia. Ac-

Abbreviations used: eIF, eukaryotic initiation factor; 4E-BP, eIF4E-binding protein; EMS, ethylmethane sulphonate; MMC, mitomycin C; 5′ UTR, 5′-untranslated region; IRES, internal ribosome entry segment; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal-regulated protein kinase; MEK, MAPK/ERK kinase; FRAP/mTOR, FK506-binding protein rapamycin-associating protein/mammalian target of rapamycin; Apaf-1, apoptotic protease activating factor-1; XIAP, X-linked inhibitor of apoptosis; HRI, haem-regulated inhibitor; PKR, double-stranded-RNA-activated protein kinase; GCN, general control non-derepressible.

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cordingly, the IRES identified in ornithine decarboxylase [24] has been shown to be cell-cycle dependent, the VEGF IRES [22] is functional following oxidative damage and the XIAP [25], c-*myc* [11] and death-associated protein 5 (DAP5) [26] IRESs function during apoptosis. There are other situations where c-Myc protein levels have been shown to be increased [27] under circumstances in which protein synthesis levels are reduced, e.g. following DNA damage [28]. For example, treatment of B-cells with dimethylsulphate, ethylmethane sulphonate (EMS) or mitomycin C (MMC) causes a dose- and time-dependent induction of c-Myc [27], whereas it has been shown that treatment of cells with MMC causes an inhibition of global translation rates [28].

In this article we show that following treatment of cells with EMS or MMC there is inhibition of cap-dependent translation. Given that c-Myc protein synthesis can be initiated by cap-dependent scanning or by internal ribosome entry we investigated whether the c-*myc* IRES was able to function under these conditions. Our data suggest that the c-*myc* IRES is required to maintain c-*myc* expression following DNA damage and that proteins responsible for this process lie downstream of the p38 mitogen-activated protein kinase (MAPK) and/or extracellular-signal-regulated protein kinase (ERK)/MEK(MAPK/ERK kinase) signalling pathways.

EXPERIMENTAL

Cell culture and transient transfections

The cell lines GM00637 and HeLa were obtained from the Human Genetic Mutant Cell Repository, Camden, NJ, U.S.A. Cells were maintained at 37 °C in 95 % air/5 % CO_2 in Dulbecco's modified Eagle's medium containing 10 % fetal calf serum (Gibco-BRL).

Cells were treated with either MMC or EMS at a range of doses for 24 h or 4 h before harvesting. For treatment with signalling inhibitors, cells were pre-incubated in serum-free media containing either rapamycin (20 nM), PD90859 (30 μM) or SB203580 (30 μM) or an equivalent dilution of solvent (DMSO) for 30 min before the addition of the DNA-damaging agent.

Cell viability

Cell viability was determined using propidium iodide staining. Cells (2×10^5 per time point) were washed three times in PBS and then fixed in a solution of 1:1 methanol/acetone for 10 min. After five washes in PBS, the cells were incubated with 900 μl of PBS and 100 μl of propidium iodide (50 μg/ml) at 37 °C for 30 min before fluorescence analysis. Apoptosis was assessed by determining the percentage of cells with condensed nuclei relative to the total population of nuclei counted.

Calcium phosphate-mediated DNA transfection of mammalian cells was performed essentially as described in [29] with minor modifications [15]. Alternatively cells were transfected using FuGene6 (Roche) following the manufacturer's instructions. All transfections were performed in triplicate on at least three independent occasions.

The activity of firefly and *Renilla* luciferases in lysates prepared from transfected cells were measured using a dual-luciferase reporter assay system (Promega) and light emission was measured over 10 s using an OPTOCOMP I luminometer. The activity of β-galactosidase in lysates prepared from cells transfected with pcDNA3.1/HISB/LacZ (Invitrogen) was measured using a Galactolight plus assay system (Tropix).

Determination of protein synthesis and DNA synthesis

For determination of protein and DNA synthesis following DNA damage, cells were seeded at a concentration of 5×10^5/ml (in triplicate), then incubated with/without the damaging agent in the presence of either [^{35}S]methionine or [^{3}H]thymidine. At the end of the labelling periods cells were washed twice in PBS and then re-suspended in a final volume of 1 ml of PBS. Cell suspensions were then spotted in triplicates of 20 μl on to a piece of 3MM filter paper (Whatman) divided into squares, and the filter paper dried. Filters were washed for 3×15 min in 5 % trichloroacetic acid and then for 15 min in methanol before drying and cutting into squares. Radioactivity was measured by scintillation counting.

SDS/PAGE and Western blotting

In each case Western-blot analysis of cell samples was performed on at least three independent occasions. For analysis of c-*myc*, α-actin, eIF4E and eIF2α proteins, cell pellets were solubilized in electrophoresis buffer [50 mM Tris/HCl (pH 6.8)/4 % SDS/10 % 2-mercaptoethanol/1 mM EDTA/10 % glycerol/0.01 % Bromophenol Blue) by sonication. Cell extracts (10^6 cells/lane) were then analysed by SDS/PAGE on 7.5 % or 10 % 16 cm gels (Bio-Rad) and the proteins were transferred on to nitrocellulose (Schleicher and Schuell) by electroblotting in transfer buffer [0.2 M glycine/20 mM Tris/20 % (v/v) methanol] for 1.5 h at 85 V. For analysis of the phosphorylation states of the 4E-BP1 and p70 S6 kinase proteins, cell pellets were solubilized in extraction buffer (50 mM sodium β-glycerophosphate, pH 7.4/0.5 mM sodium orthovanadate/1 mM EDTA/1 mM EGTA/1 mM dithiothreitol/1 % Triton X-100/10 % glycerol) with 1 μM microcystin, 10 % aprotinin, 0.1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml tosyl-lysylchloromethane ('TLCK') added immediately before use. Cell extracts were centrifuged at 16000 ***g*** for 5 mins to pellet cell debris and 0.5×10^6 to 1×10^6 cell equivalents of extract mixed with an equal volume of $2 \times$ electrophoresis buffer was boiled and analysed by SDS/PAGE. p70 S6 kinase proteins were analysed using 7.5 % polyacrylamide gels and transferred as described above.

For analysis of 4E-BP1, samples were resolved on 15 % polyacrylamide gels, transferred for 2 h on to a PVDF membrane (Gelman Sciences) and the membrane fixed in 0.05 % glutaraldehyde in Tris-buffered saline containing 0.1 % Tween-20 (TBS-T) for 30 min. Equal loading of protein was determined on all blots by staining with Ponceau S. Blots were blocked by incubation in 5 % skimmed milk in TBS-T for 1–2 h and then probed with the relevant antibodies for 1 h at room temperature. c-Myc protein was detected using the mouse monoclonal antibody 9E10 (generated by Dr T. Harrison, University of Leicester, Leicester, U.K.) at 1:400 dilution and α-actin proteins were detected using a mouse monoclonal antibody (Sigma) at 1:10000. Rabbit polyclonal antibodies, used to detect eIF4E, phosphorylated eIF2α, eIF2α, p70 S6 kinase and 4E-BP1, were gifts from Dr S. Morley (University of Sussex, Brighton, U.K.; eIF4E), Dr N. Redpath (University of Leicester; p70 S6 kinase), Professor R. Denton (University of Bristol, Bristol, U.K; 4E-BP1) and Professor C. Proud (University of Dundee, Dundee, U.K.; eIF2α), and were used at dilutions of 1:7000, 1:2000, 1:1000, 1:2000 and 1:2000, respectively. Blots were then incubated with peroxidase-conjugated secondary antibodies raised against mouse or rabbit immunoglobulins and developed using the chemiluminescence reagent 'Illumin 8' (generated by Dr M. Murray, Department of Genetics, University of Leicester, Leicester, U.K.).

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Immunoprecipitations

Cells were labelled and immunoprecipitations were performed as described previously [30]. Briefly, 2×10^6 cells, either untreated or 4 or 24 h after the addition of 20 mM EMS (GM00637 cells) or 20 μM MMC (HeLa cells), were labelled with 250 μCi of [^{35}S]methionine in 1 ml of methionine-free medium for 30 min. After addition of fresh complete medium, cell samples were harvested at 0, 20, 40 and 60 min. Cells were solubilized in Ab buffer [30] and disrupted by passing through a syringe attached to a 21-gauge needle. The samples were pre-cleared by the incubation for 1 h at 4 °C with mouse IgG and Protein A/G–agarose (Santa Cruz Biotechnology). Myc proteins were immunoprecipitated overnight at 4 °C using a Myc monoclonal antibody C-33 (Santa Cruz Biotechnology). Samples were subjected to SDS/PAGE and the amount of radiolabel incorporated was visualized on a PhosphorImager (Molecular Dynamics). Experiments were performed on three independent occasions.

Isoelectric focusing

To determine the phosphorylation states of eIF4E, non-phosphorylated and phosphorylated forms of the protein were separated using one-dimensional isoelectric focusing. Cell pellets were lysed in extraction buffer at a concentration of 2×10^7 cells/ml and prepared as for analysis by Western blotting. Sample (20 μl) was mixed with the appropriate volume of 7 × isoelectric focusing sample buffer (described in [9]) and urea was added to a final concentration of 9 M. Isoelectric focusing was performed essentially as described [9], using a Bio-Rad minigel apparatus with ampholytes in the pH range 3–10 (Bio-Rad), and 0.01 M glutamic acid and 0.05 M histidine at the anode and cathode, respectively. Focused gels were then Western blotted for 30 min and probed for eIF4E as described above.

Northern-blot analysis

Total cellular RNA was prepared and analysed by Northern blotting exactly as described previously [9]. DNA probes used for the detection of c-Myc and GAPDH mRNA species were also as described in [9].

RESULTS

Exposure of cells to EMS and MMC causes an inhibition of DNA and protein synthesis

The cell lines GM00637 and HeLa were treated with EMS or MMC, and this caused protein synthesis rates to decrease in a time- and dose-dependent manner (Figures 1A–1D). The reduction in global protein synthesis differed between the cell lines. Consequently there was greater inhibition of HeLa cells with MMC than with EMS and in GM00637 cells the converse was true (results not shown). Exposure of GM00637 cells to 20 mM EMS caused a reduction in protein synthesis of 80 % and exposure of HeLa cells to 20 μM MMC caused a similar reduction (Figures 1A and 1B). Using these drug doses we found that there was a reduction in protein synthesis in GM00637 cells 4 h after treatment and in HeLa cells 24 h after treatment (Figures 1C and 1D). Interestingly, the reduction in protein synthesis occurred at times where the cells would be repairing the damage caused. Thus DNA damage caused by EMS is repaired more rapidly than intra-strand cross-links formed by MMC [27]. There was also a large decrease in radioactive thymidine incorporation showing that DNA synthesis was inhibited and, as expected, we found an induction of p53, indicative that DNA damage was occurring (Figures 1A and 1B, and results not shown). At the doses of EMS or MMC used, cells were not apoptotic after treatment (4 and 24 h respectively), and 95 % of the cells were still viable and did not contain condensed nuclei, as judged by propidium iodide staining with the highest doses shown (Figures 1E and 1F).

We have shown previously that in B-cells following genotoxic stress c-Myc protein is still synthesized [27]. To test whether this was also occurring in these cell types Western blotting and Northern analysis of c-*myc* was carried out.

c-Myc protein is still synthesised following treatment of cells with EMS and MMC

The c-Myc protein has a short half-life of around 20–40 min [30] and it is poorly translated by the cap-dependent scanning mechanism of translation due to its long highly structured 5′ UTR [9,15]. Thus as general protein-synthesis rates declined by more than 80 % following exposure of cells to the damaging agents it might be expected that there would be a reduction in the level of c-Myc. To investigate c-Myc expression, GM00637 cells and HeLa cells were treated with EMS and MMC respectively, nuclei were harvested, proteins were separated by PAGE and the gels immunoblotted. The blots were probed using the c-*myc*-specific antibody 9E10 and re-probed with anti-laminin antibodies as a loading control (Figure 2). In both cases, treatment of cells caused a small, but reproducible, induction in the level of the c-Myc protein (Figure 2). A similar induction of c-Myc protein was observed previously following treatment of B-cells with EMS and MMC, although the doses that elicited the maximal response were lower, probably reflecting the different sensitivities of these two cell types to these drugs [27].

To determine whether there was an alteration in the half-life of the c-Myc protein following DNA damage, pulse–chase immunoprecipitation analysis was performed. Thus HeLa and GM00637 cells were treated with MMC and EMS for 4 or 24 h respectively before radiolabelling with [^{35}S]methionine for 30 min followed by the addition of an excess of unlabelled methionine (Figure 3). Samples were taken at the time points shown and c-Myc protein was immunoprecipitated. In the HeLa clls the half-life of the c-Myc protein was approx. 30 min and in GM00637 cells the half-life was slightly longer, 40 min. No differences were observed in the presence of the DNA-damaging agent. Thus an increase in half-life cannot account for the presence of c-Myc protein under conditions where there is a decrease in overall translation rates.

To investigate whether a translational response or a transcriptional response was occurring, parallel samples were analysed by Northern blotting. Total RNA was isolated and separated by agarose-gel electrophoresis and probed with a c-Myc-specific probe. GAPDH was used as a loading control (Figure 4). There was no increase in c-Myc RNA following treatment of cells with either compound and indeed at the higher doses used there was a small reduction in c-Myc RNA levels (Figure 4). Thus in the absence of a transcriptional response we investigated whether a translational response could be used for the maintenance of c-Myc expression.

There is an inhibition of the FRAP/mTOR signalling pathway by treatment of cells with EMS

We have shown previously [9] that an increase in the cap-dependent scanning mechanism of c-Myc-protein translation initiation occurs via signalling through the FRAP/mTOR pathway. Thus the levels and phosphorylation states of proteins in this pathway were determined to see whether this correlated with the increased expression of c-*myc*. Samples were treated with the doses of EMS shown (Figure 5), harvested after 4 h, and the

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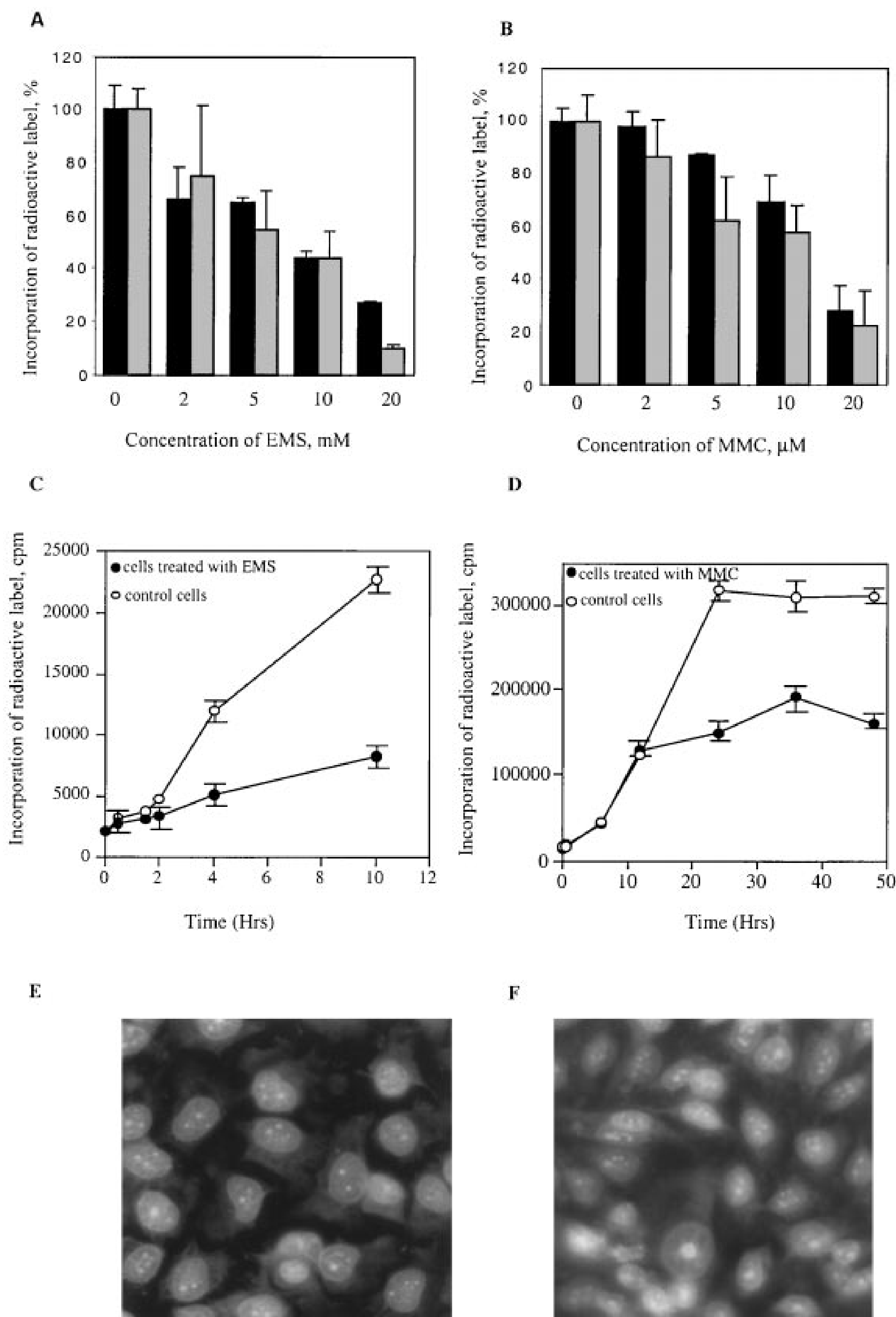

Figure 1 Inhibition of protein and DNA synthesis in GM00637 and HeLa cells following DNA damage

(**A**) GM00637 cells (in triplicate) or (**B**) HeLa cells (in triplicate) were treated with EMS and MMC, respectively, at the concentrations indicated, for 4 or 24 h respectively. Protein and DNA synthesis was estimated by determining the incorporation of [^{35}S]methionine (black bars) or [^{3}H]thymidine (grey bars). (**C**) GM00637 cells (in triplicate) or (**D**) HeLa cells (in triplicate) were treated with 20 mM EMS and 20 μm MMC, respectively, for the times indicated. Protein synthesis was estimated by determining the incorporation of [^{35}S]methionine. (**E**) GM00637 cells exposed to 20 mM EMS and (**F**) HeLa cells exposed to 20 μM MMC stained with propidium iodide.

proteins were separated by PAGE and immunoblotted. In agreement with the data shown in Figure 1, which show that global protein synthesis rates are decreased following exposure of cells to EMS, it can be seen that that the translational components of this pathway were inhibited following exposure to EMS. Thus there are decreases in phosphorylation of eIF4E (to about 60 % of its value in untreated cells, where almost 100 % of eIF4E appears to be phosphorylated; Figure 5), its binding partner 4E-BP1, albeit to a lesser extent, and in p70 S6 kinase (Figure 5). These changes would cause an increase in the association of eIF4E with its binding partner and a decrease in the eIF4E that was available to bind as part of the eIF4F complex to the 7 methyl G cap. There was no change detected in the levels of any of the proteins examined in these studies, even

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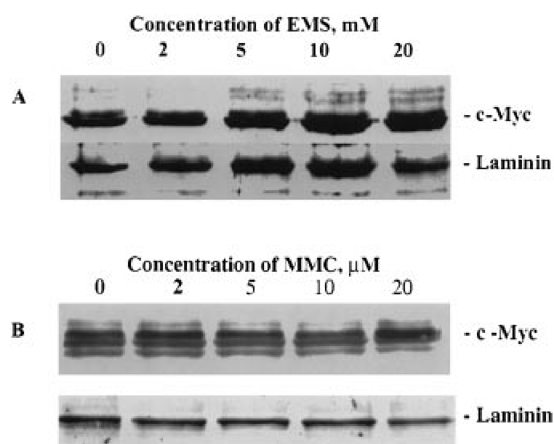

Figure 2 c-Myc protein levels were still maintained following DNA damage

GM00637 cells (**A**) or HeLa cells (**B**) were incubated with EMS and MMC for 4 and 24 h respectively. Nuclei were harvested from the cells, and lysates separated by SDS/PAGE and Western blotted. Blots were probed with anti-c-Myc antibodies and then stripped and re-probed with anti-laminin antibodies.

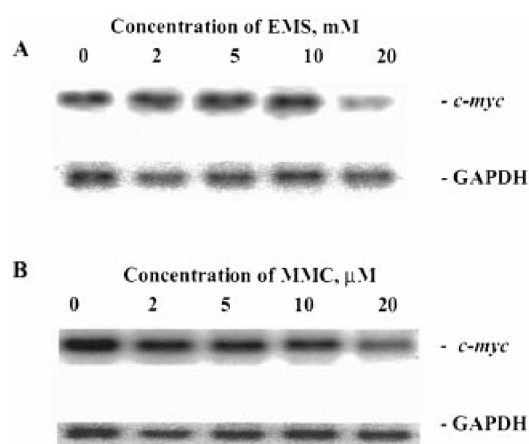

Figure 4 There was no increase in c-Myc mRNA levels following DNA damage

Total RNA was prepared after exposure of GM00637 cells to EMS (**A**) or HeLa cells to MMC (**B**). Samples were separated by agarose-gel electrophoresis and transferred to nitrocellulose. Blots were probed with either a c-Myc- or GAPDH-specific probe.

though there was a large decrease in protein-synthesis rates. This would suggest that they all have relatively long half-lives in these cells. These data are in agreement with those of Tee and Proud [28] who also showed a decrease in cap-dependent protein synthesis following DNA damage.

During apoptosis, where there is also a large decrease in protein synthesis, many of the eukaryotic initiation factors are cleaved by the effector caspases [31–34]. The scaffold protein eIF4G I is particularly susceptible to caspase-mediated cleavage

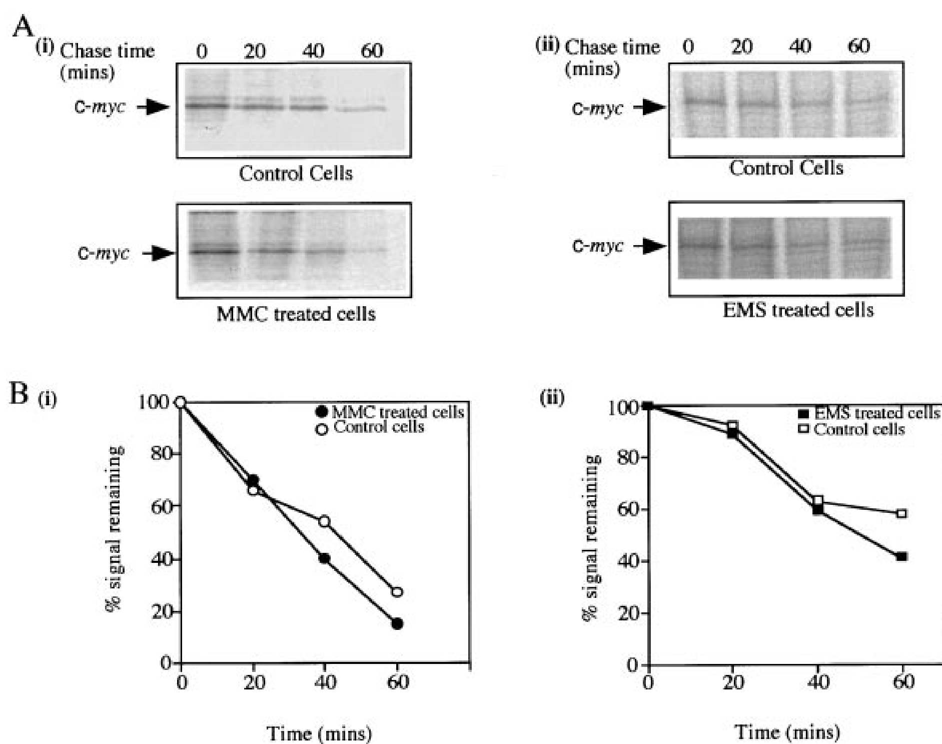

Figure 3 c-Myc protein stability is unaltered during genotoxic stress

Determination of the half-life of the c-Myc protein in untreated HeLa (i) and GM00637 (ii) cells and at 24 or 4 h post-addition of 20 μM MMC and 20 mM EMS respectively. Pulse–chase analysis and immunoprecipitation were performed as described in the Experimental section. Cells were labelled for 30 min and then harvested following the chase times indicated. (**A**) Samples were subjected to PAGE and the amount of radiolabel incorporated into each band was determined using a PhosphorImager. Representative gels of pulse–chase/immunoprecipitations are shown. (**B**) PhosphorImager analysis of the gels shown in (**A**). ○, Untreated cells; ●, cells incubated with the drugs shown.

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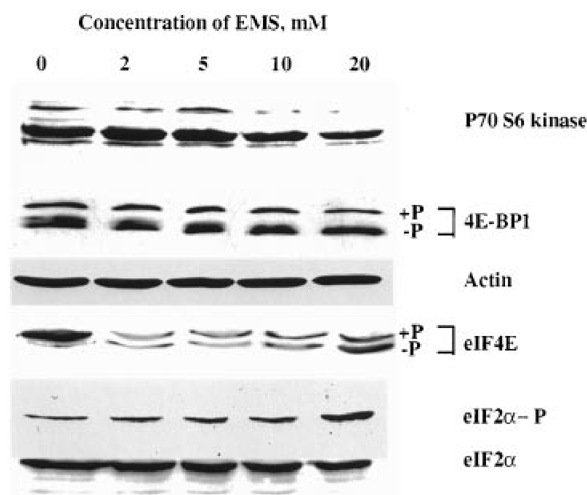

Figure 5 Exposure of GM00637 cells to EMS caused changes in phosphorylation states of eukaryotic initiation factors

GM00637 cells were treated with the doses of EMS shown for 4 h. Cell extracts (10^6 cells) were separated by SDS/PAGE, Western blotted and probed with antibodies against p70 S6 kinase and 4E-BP1, and stripped and reprobed with anti-actin antibodies as a loading control. The phosphorylated (+P) and dephosphorylated (−P1) forms of eIF4E, in extracts prepared in the presence of phosphatase inhibitors, were separated by isoelectric focusing and then Western blotted and detected as for SDS/PAGE. Cell samples were also harvested, separated by SDS PAGE and electroblotted for the phosphorylated form of eIF2α (eIF2α-P). This blot was then stripped and re-probed for eIF2α expression.

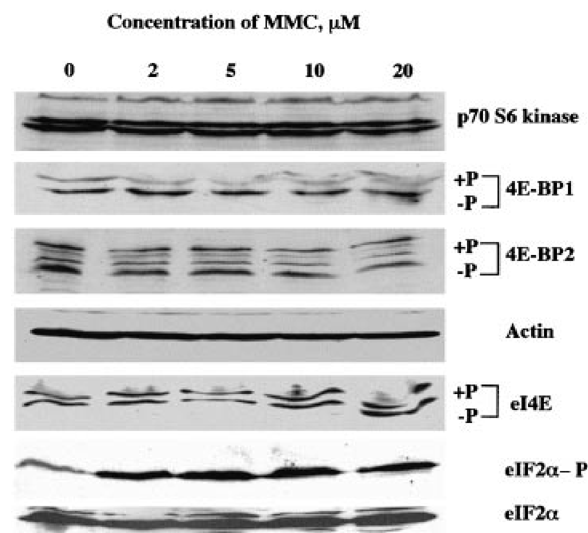

Figure 6 Exposure of HeLa cells to MMC caused a large increase in the phosphorylation of eIF2α

HeLa cells were treated with the doses of MMC shown for 24 h. Cells extracts (10^6 cells) were separated by SDS/PAGE Western blotted and probed with anti-p70 S6 kinase antibodies and 4E-BP1 and 2 antibodies. Blots were stripped and probed with antibodies against actin as a loading control. The phosphorylated (+P) and dephosphorylated (−P) forms of eIF4E, in extracts prepared in the presence of phosphatase inhibitors, were separated by isoelectric focusing and then Western blotted and detected as for SDS/PAGE. Cell samples were also harvested, separated by SDS PAGE and electroblotted for the phosphorylated form of eIF2α (eIF2α-P). This blot was then stripped and re-probed for eIF2α.

[32–34] and thus to test whether eIF4G was cleaved immunoblotting was also performed. However, the eIF4G was full length and there was no evidence of cleavage (results not shown).

Inhibition of cap-dependent translation in HeLa cells is caused by eIF2α phosphorylation

The expression and phosphorylation states of the eIFs were also analysed in HeLa cells treated with MMC. Surprisingly, in this case no change in the phosphorylation states of eIF4E or 4E-BP1 or 2 were found (Figure 6). The central component in the ternary complex, eIF2, is phosphorylated at Ser-51 of the α-subunit in response to stress by the kinases haem-regulated inhibitor (HRI) and double-stranded-RNA-activated protein kinase (PKR) [14]. This prevents recycling of eIF2 between successive rounds of initiation and inhibits ternary complex formation [35]. Thus the phosphorylation states of eIF2 was also determined. There was no change in the overall level of eIF2α; however, there was an increase in the phosphorylation of this protein following exposure of HeLa cells to the lowest dose of MMC and this would contribute significantly to the decrease in protein synthesis observed (Figures 1 and 5). A small increase in eIF2α phosphorylation was also observed at the highest dose of EMS used (Figure 5). These data would imply that alternative mechanisms are used by these two cell types to decrease translation initiation.

c-Myc IRES is functional following treatment of cells with EMS and MMC

As cap-dependent translation is inhibited following genotoxic stress, the initiation of c-Myc protein synthesis could be occurring by internal ribosome entry. To test whether the expression of c-*myc* was mediated via the IRES following DNA damage, cells were transfected with the dicistronic plasmid pRMF, which contains the c-*myc* IRES between *Renilla* and firefly luciferase genes (Figure 7A). Cells were co-transfected with a plasmid that expressed β-galactosidase as a control. Cells were then treated with increasing doses of EMS or MMC, samples harvested and assayed for luciferase activity. It can be seen that there was an increase in firefly luciferase relative to *Renilla* luciferase which reflected the reduction in global protein rates that was observed (Figures 1 and 7). There was an 11-fold increase in the relative level of firefly luciferase following treatment of cells with 20 mM EMS. This would imply that that the c-*myc* IRES was used to initiate protein synthesis under these conditions, whereas the cap-dependent initiation of translation of *Renilla* luciferase was inhibited (Figure 7B, panels i and ii). These results are not due to a difference in the stabilities of the luciferases in these cells since we find that the protein half-lives of firefly and *Renilla* luciferases are similar, between 2.5 and 3 h (results not shown). In the case of MMC there was a smaller, but again dose-dependent, increase in the expression of the firefly (IRES-mediated) luciferase synthesis by 2.5-fold at a concentration of 10 μM (Figure 7C, panels i and ii).

To determine whether the use of internal ribosome entry following damage to DNA was a more general phenomenon cells were transfected with the plasmid pRAF that contains the Apaf-1 IRES [18]. Cells were then treated with the concentrations of MMC shown in Figure 7(D), harvested and luciferase activities determined. The Apaf-1 IRES was also used under these conditions and hence at 10 μM MMC the firefly luciferase activity was increased 3-fold relative to the expression of *Renilla* luciferase (Figure 7D, panels i and ii). Similar results with the Apaf-1 IRES were obtained following treatment of cells with EMS (results not shown). Thus these data suggest that, following DNA damage, internal ribosome entry is used as a mechanism for initiation of

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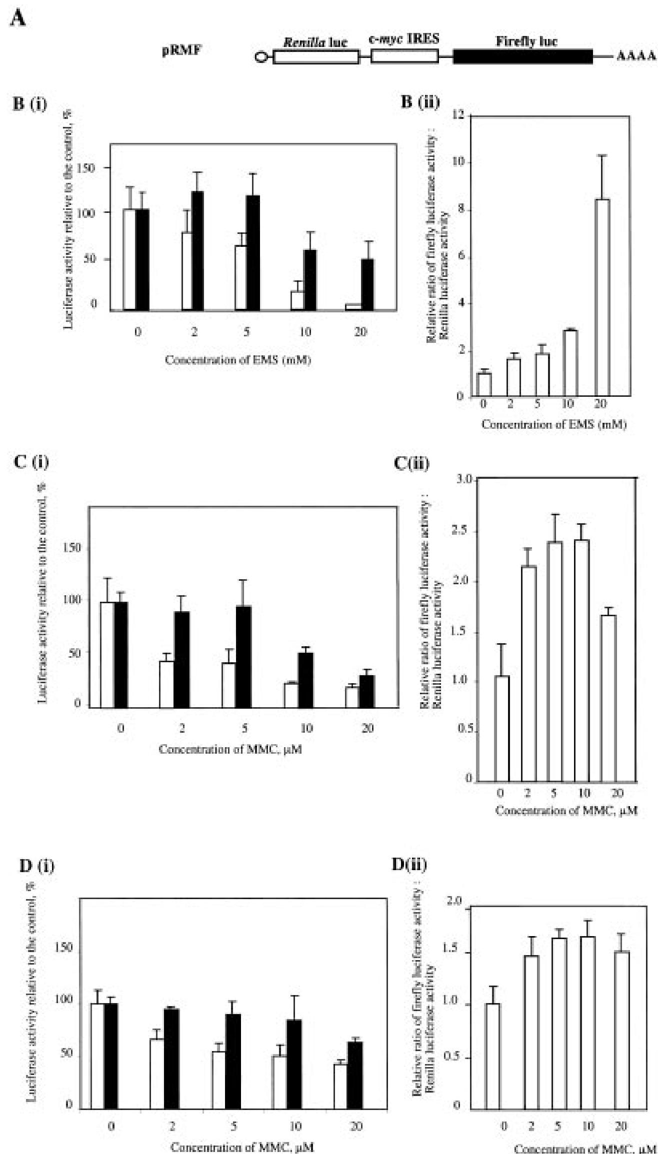

Figure 7 The c-*myc* IRES was still active following genotoxic stress

HeLa or GM00637 cells were transfected (in triplicate) with pRMF (**A**) and a transfection control pcDNA3.1/HisB/LacZ. Following DNA damage induced with either (**B**) EMS (GM00637) or (**C**) MMC (HeLa) cells were harvested and lysed. Cells were also transfected with the Apaf-1 IRES and then treated with MMC (**D**). Lysates were assayed for *Renilla* and firefly luciferase and β-galactosidase activities. The activities of firefly (black bars) and *Renilla* (white bars) luciferase relative to the control untreated sample, normalized to β-galactosidase activity, are shown in (**B**)–(**D**), panels (i). The relative ratio of firefly luciferase:*Renilla* luciferase is shown in (**B**)–(**D**), panels (ii).

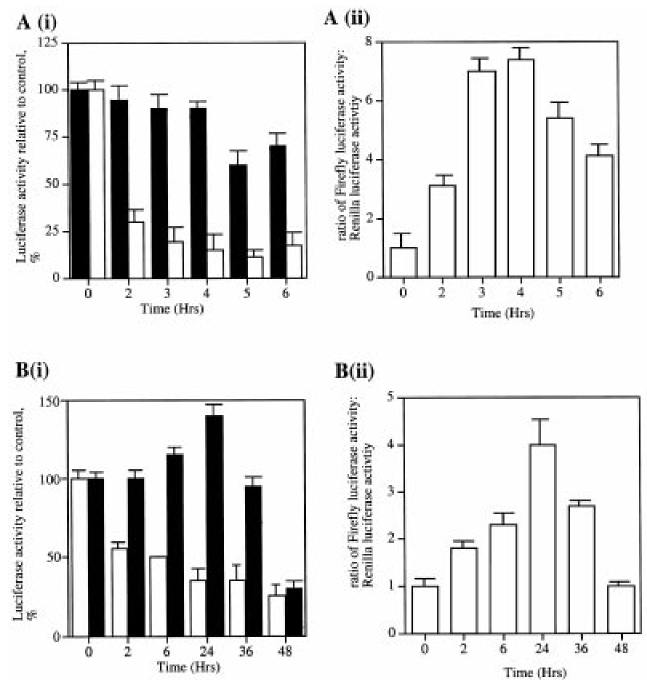

Figure 8 Time-dependent induction of c-*myc* IRES activity following genotoxic stress

HeLa or GM00637 cells were transfected (in triplicate) with pRMF and a transfection control pcDNA3.1/HisB/LacZ. GM00637 cells were treated with 10 mM EMS (**A**) and HeLa cells were treated with 10 μM MMC (**B**) and samples were harvested at the times shown. Lysates were assayed for *Renilla* and firefly luciferase and β-galactosidase activities. The activities of firefly (black bars) and *Renilla* (white bars) luciferase relative to the control untreated sample, normalized to β-galactosidase activity, are shown in panels (i). The relative ratio of firefly luciferase:*Renilla* luciferase is shown in panels (ii).

translation. In this regard, it was shown recently that the XIAP IRES was functional following exposure of cells to γ-irradiation [25].

Since there was a time-dependent decrease in protein synthesis (Figures 1C and 1D) following treatment of cells with either EMS or MMC, experiments were performed to establish whether the c-*myc* IRES activity correlated with the time-dependent decrease in protein synthesis. Cells were co-transfected with the dicistronic plasmid pRMF (see above and Figure 7A) and a plasmid that expressed β-galactosidase. HeLa or GM00637 cells were then treated with 10 μM MMC or 10 mM EMS respectively and samples were harvested at the time points shown in Figure 8. There was a maximal induction of the synthesis of firefly luciferase that was mediated by the c-*myc* IRES at 4 h following treatment of GM00637 cells with EMS or 24 h following treatment of HeLa cells with MMC (Figures 8A and 8B). This correlates with the times at which we observed a reduction in protein-synthesis rates with these agents (Figure 1).

Proteins that mediate internal c-*myc* ribosome entry lie downstream of p38 MAPK pathway

We have shown previously that during apoptosis the proteins responsible for maintenance of c-Myc expression via the IRES lie downstream of the p38 MAPK [11]. To investigate whether this was also the case following genotoxic stress, HeLa cells were transfected with the plasmid pRMF (Figure 9A). The cells were then treated with either rapamycin to block the FRAP/mTOR signalling pathway or either PD98059 or SB203580 to investigate the contribution of the ERK and p38 MAPK pathways respectively. The cells were then exposed to MMC to assess the effect of these agents on IRES-mediated initiation following DNA damage. Treatment of cells with rapamycin caused a decrease in the cap-dependent synthesis of the *Renilla* luciferase, but had only a small effect on the cap-independent translation of the firefly luciferase (Figure 9A). Rapamycin had no effect on

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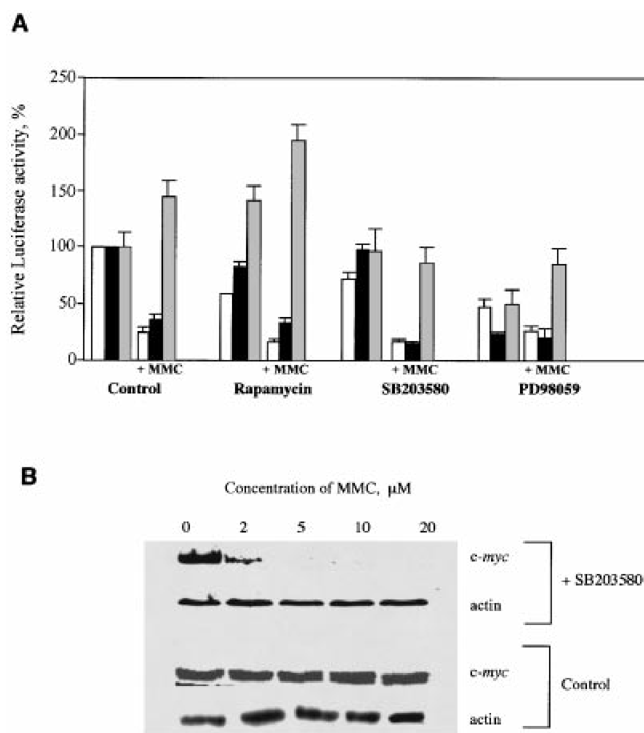

Figure 9 The proteins required for IRES-mediated translation lie downstream of the p38 and ERK MAPKs

(**A**) HeLa cells were co-transfected (in triplicate) with a dicistronic reporter plasmid containing the luciferase gene fused in-frame with the c-*myc* IRES (pRMF) and with a plasmid harbouring the gene for β-galactosidase (pCDNA3.1/HisB/Lacz) to act as a transfection control. Cells were then pre-incubated in serum-free media containing rapamycin (20 nM), PD90859 (30 μM), SB203580 (30 μM) or an equivalent dilution of solvent (DMSO) for 30 min before the addition of 20 μM MMC. After 24 h cells were harvested and lysed and subsequent lysates were assayed for the activities of *Renilla* or firefly luciferases and β-galactosidase. The activity of firefly and *Renilla* luciferases relative to the transfection control is shown. White bars, *Renilla* luciferase; black bars, firefly luciferase; grey bars, ratios of *Renilla*:firefly. (**B**) 10^6 cells were incubated with the doses of MMC shown with or without (Control) a 30 min pre-incubation with 30 μM SB203580. Samples were harvested and analysed by PAGE and Western blotting with an anti-c-Myc antibody and then stripped and re-probed with an anti-actin antibody.

IRES-mediated translation initiation and following treatment of cells with MMC there was the same relative increase in firefly luciferase activity as in the control cells. The p38 inhibitor SB203580 alone caused a small reduction in *Renilla* luciferase activity but, interestingly, pre-treatment with this drug abolished the IRES-dependent relative increase in firefly luciferase activity in the presence of the DNA-damaging agent (Figure 9A). Pre-treatment of HeLa cells with the MEK inhibitor PD98059 alone caused a large reduction in both cap-dependent and cap-independent translation and also abrogated the relative increase in firefly luciferase in the presence of MMC (Figure 9A). To determine the effect that pre-treatment of cells with the SB203580 inhibitor had on c-Myc protein expression, HeLa cells were incubated with this drug and then treated with MMC. Cells were harvested at the time points for Figure 9, samples separated by PAGE and the corresponding Western blots probed with anti-c-Myc antibody and then re-probed with anti-actin antibodies. In the presence of the SB203580 inhibitor there was a dramatic decrease in c-Myc expression (Figure 9B) which corresponded to the inhibition of the c-*myc* IRES activity (Figure 9A). Taken together these data would indicate that the proteins that are required for cap-independent translation lie downstream of the ERK and p38 MAPKs and not the FRAP/mTOR pathway.

DISCUSSION

Following DNA damage initiated by either MMC or EMS there was a large reduction in global protein-synthesis rates. Similar results have been observed with incubation of cells with etoposide [28]. Despite the decrease in global protein synthesis following DNA damage, we observed a small increase in the expression of c-Myc protein that is not due to changes in transcription or half-life of the protein (Figures 2–4). This is in agreement with a previous study where we showed that in B-cells there was a similar increase in c-Myc expression under these conditions [27]. As we have shown that initiation of c-Myc protein synthesis can occur by both cap-dependent and cap-independent mechanisms we investigated how the inhibition of protein synthesis was mediated. Treatment of GM00637 cells with EMS caused changes in the phosphorylation states of the canonical eukaryotic initiation factors and their binding partners (Figure 5). This would have the effect of decreasing the amount of eIF4F complex and therefore reducing protein synthesis. Thus it is unlikely that c-Myc synthesis is being initiated by the scanning mechanism under these conditions, given that it has a long structured 5′ UTR and is normally translationally repressed [9].

Treatment of HeLa cells with MMC had no effect on eIF4E, 4E-BP or p70 S6 kinase phosphorylation, but there was a large increase in the phosphorylation of eIF2α (Figure 6). This event would rapidly reduce the level of functional eIF2 and therefore result in a decrease in translation initiation [35]. The status of eIF2α phosphorylation is controlled by at least four kinases, namely GCN2 (general control non-derepressible 2) [36], PKR [37], HRI [38] and the pancreatic eIF2α kinase/PKR-like endoplasmic reticulum kinase (PEK/PERK) [39]. We do not know which of these kinases is responsible for the eIF2α phosphorylation, although the most likely candidate for this would be PKR since this protein is activated by pro-apoptotic stress [37]. There is evidence that suggests that the initiation of synthesis of specific proteins can occur under conditions of low functional eIF2 and that these mRNAs are less sensitive to changes in eIF2α phosphorylation e.g. GCN4 [36]. In addition, in Jurkat cells, after initiation of apoptosis via the Fas receptor, there is an increase in phosphorylation of eIF2α. It has been suggested that this may again allow synthesis of specific proteins that have a low requirement for eIF2 by increasing the effective concentration of the translation apparatus that is available for initiation [40]. Finally, activation of PKR, which causes an increase in eIF2α phosphorylation, augments the synthesis of Fas and Bax, although the mechanism by which this occurs is unknown [41]. Thus our data would suggest that initiation of synthesis of c-Myc also required low functional eIF2.

Our data show that following treatment of cells with DNA-damaging agents c-*myc*-IRES-driven translation still occurs, and we therefore propose that initiation of c-Myc synthesis occurs via internal ribosome entry (Figures 7 and 8). This would imply that IRESs are able to function under conditions where there is a low amount of functional eIF2 (Figure 6), although the mechanism(s) by which this occurs requires further investigation. In agreement with this it has been shown that the IRES found in the 5′ UTR of the cationic amino acid transporter cat-1 can also function under conditions of low eIF2α [42]. The Apaf-1 IRES was still able to initiate translation of firefly luciferase and this may reflect the need for the cell to quickly undergo apoptosis in situations when the cell is unable to repair severe DNA lesions. Given our

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data and work by others [19,25,43], we posit that a switch from cap-dependent to cap-independent translation following DNA damage is a general phenomenon.

The control of global protein-synthesis rates is a highly regulated process and the control of the activities of the components of translation initiation is brought about, for the most part, by changes in phosphorylation states. The kinases that interact with the canonical eukaryotic translation-initiation factors lie downstream of FRAP/mTOR, leading to phosphorylation of 4E-BP1/2 and S6 kinase [44]. In addition, the p38 and ERK pathways are also involved, as it has been shown that Mnk1 (MAPK-integrating kinase 1) phosphorylates eIF4E [45–47]. Specific inhibitors of these pathways show that the proteins responsible for IRES-mediated translation of c-*myc* lie downstream of the p38 and/or ERK MAPKs, but not FRAP/mTOR (Figure 9). This is similar to the situation that occurs during apoptosis, where we also found the c-*myc* IRES to be active [11], and it is known that exposure of cells to DNA-damaging agents similarly causes induction of the stress-activated kinases [35].

In conclusion, we have shown that c-Myc protein synthesis still occurs following DNA damage and our data suggest that its synthesis is initiated by the IRES. The role of c-Myc during DNA damage is not clear although it may be required for the transcription of genes required for the repair process.

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