Nitrogen mustard inhibits transcription and translation in a cell free system

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Received May 12, 1995; Revised and Accepted August 6, 1995

ABSTRACT

Nitrogen mustard and its derivatives such as cyclophosphamide, chlorambucil and melphalan are widely used anti-cancer agents, despite their non-specific reaction mechanism. In this study, the effect of alkylation by nitrogen mustard of DNA and RNA (coding for a single protein) was investigated using both a translation system and a coupled transcription/translation system. When alkylated DNA was used as the template for coupled transcription and translation, a single translation product corresponding to the 62 kDa luciferase protein was synthesised. Production of the translated product encoded by this template was inhibited by mustard concentrations as low as 10 nM, and 50% inhibition occurred with 30 nM mustard. A primer extension assay employed to verify alkylation sites on the DNA revealed that all guanine residues on the DNA template are susceptible to alkylation by nitrogen mustard. Similarly, when alkylated RNA was used as the template for protein synthesis, the amount of the 62 kDa luciferase protein decreased with increasing mustard concentration and a range of truncated polypeptides was synthesised. Under these conditions 50% inhibition of translation occurred with ~300 nM mustard (i.e. ~10 times that required for similar inhibition using an alkylated DNA template). Furthermore, a gel mobility shift assay revealed that mustard alkylation of the RNA template results in the formation of a more stable retarded RNA complex. The functional activity of the luciferase protein decreased with alkylation of both the DNA and RNA templates, with a half-life of loss of activity of 1.1 h for DNA exposed to 50 nM mustard, and 0.5 h for RNA exposed to 50 μ M mustard. The data presented support the notion that DNA is a critical molecule in the mode of action of mustards.

INTRODUCTION

Nitrogen and sulfur mustards and a number of their derivatives are potent alkylating agents (1). Nitrogen mustard (mechlorethamine, HN2) was the first clinically useful anticancer agent (2)

and derivatives such as phenylalanine mustard (melphalan), chlorambucil and cyclophosphamide are amongst the most widely used antitumor drugs (3), despite their non-specific reaction mechanism. In aqueous conditions these compounds spontaneously form reactive aziridinium ions (4) that can covalently bind to nucleophilic sites within proteins, nucleic acids and other biomolecules.

There is a substantial body of evidence which suggests the involvement of DNA in the mechanism of action of this group of compounds (5–8). Nitrogen mustards alkylate DNA preferentially at the N-7 position of guanine and N-3 position of adenine, resulting in the formation of monoadducts, as well as intrastrand and interstrand crosslinks (9–11). Although the relationship of these lesions to their cytotoxicity is unclear, there is some evidence that inhibition of DNA replication caused by DNA interstrand crosslinks is of particular importance (12).

Earlier work demonstrated that a higher concentration of sulfur mustard was required to inhibit RNA and protein synthesis than was required to inhibit DNA synthesis (13,14). Similar conclusions were also drawn from other studies using human and rat keratinocytes in cell culture (15,16). These studies therefore suggest that DNA is the most sensitive nucleic acid target of mustards. However, all these studies were performed *in situ* and involved measurement of the incorporation of DNA, RNA and protein precursors. The measurement of total precursor incorporation does not reveal the presence of truncated products, nor the functionality of the resultant DNA, RNA and protein. Furthermore, because mustards can also react with other cellular components such as glutathione and protein thiols, is not possible to define unambiguously the most critical site of action of the mustards, and their mode of action therefore remains unclear. It has also been demonstrated that prokaryotic RNA polymerase is able to insert an incorrect ribonucleotide opposite abasic and alkylated/depurinated sites on the DNA template (17,18). Therefore, a full length transcript could have a high amount of precursor incorporated but subsequent translation of the incorrect message may result in a protein of diminished activity.

In cells there are many repair mechanisms that correct damaged or modified bases in DNA (19). The importance of these repair mechanisms is that they ensure that genetic information is correctly copied and also that the correct information is transcribed and subsequently translated into proteins that play crucial roles in virtually all cellular processes. There is no known

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mechanism for the repair of RNA damaged post-transcriptionally and such damage may therefore lead to impairment of subsequent molecular processes (i.e. alkylation of RNA may lead to impairment of RNA splicing and translation). There are currently no reports in the literature regarding the effects of nitrogen mustards at the molecular level on the translation of proteins from either alkylated DNA or alkylated RNA templates. In the present study we have used a rabbit reticulocyte lysate in both a coupled transcription/translation system and a translation system to study the effect of nitrogen mustard alkylation of DNA and RNA on the translated protein. We report here that at the DNA level, nanomolar concentrations of nitrogen mustard inhibited formation of the full-length translated protein while at the RNA level higher concentrations of mustard were required to inhibit translation of the full length luciferase protein and generate simultaneously a range of truncated polypeptides.

MATERIALS AND METHODS

Materials

Nitrogen mustard (mechlorethamine, HN2) and cycloheximide were purchased from Sigma-Aldrich Chemical Company Inc., Australia. Rabbit reticulocyte lysate coupled transcription/translation and translation systems, luciferase assay reagent, pPOLY(A)-luc DNA and *fmol* DNA sequencing system were purchased from Promega, WI. SP6 RNA polymerase, ribonuclease inhibitor (human placenta), BSA and ultra pure ribonucleotides were from Pharmacia, WI. [α - 32 P]dATP and [α - 32 P]ATP were purchased from Amersham, UK. [35 S]methionine was from Dupont, Australia. Restriction enzymes were obtained from either New England Biolabs, CT or Boehringer-Mannheim, Germany. Urea, bisacrylamide, acrylamide, ammonium persulfate, TEMED and DTT were obtained from BioRad, MA as electrophoresis purity reagents. All other chemicals were of analytical grade and all solutions were prepared using distilled, deionised and filtered water from a Milli-Q four stage water purification system (Millipore, MA).

Templates for protein synthesis

DNA template. The pPOLY(A)-luc plasmid DNA containing the luciferase gene sequence was transformed into the HB101 vector using the CaCl₂ technique and the plasmid DNA extracted and purified according to published procedures (20).

RNA template. The pPOLY(A)-luc plasmid was linearised and transcribed *in vitro* to produce RNA containing the luciferase sequence. Transcription was carried out in a 500 μ l reaction mixture containing 20 mM HEPES-KOH pH 7.5, 16 mM MgCl₂, 2 mM spermidine, 40 mM DTT, 3 mM of each of the four ribonucleotide triphosphates, 1 U RNase inhibitor, 5 U inorganic phosphatase, 50 μ g of linearised plasmid DNA and 2.5 U SP6 RNA polymerase. The mixture was incubated at 37°C for 4 h. RNase free DNase (100 U) was then added and incubation continued for a further 15 min. The transcribed RNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with ethanol, dried and then resuspended in nuclease free water. The concentration and purity of the RNA was measured using a Cary 118 spectrophotometer.

Alkylation of templates

DNA and RNA samples were reacted with appropriate concentrations of nitrogen mustard in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) at 37°C for 60 min (or for 0–6 h for time course studies). Unreacted mustard was removed by ethanol precipitation of the nucleic acids. The precipitated material was washed with 80% ethanol, dried and then resuspended in nuclease free water.

Protein synthesis

Protein synthesis was carried out *in vitro* using a translation system, as well as a coupled transcription and translation system. For coupled transcription and translation, the reaction mixture of 25 μ l contained 12.5 μ l rabbit reticulocyte lysate, 1 μ l of Promega transcription/translation buffer, 50 μ M amino acids mixture (minus methionine), 40 μ Ci [35 S]methionine, 1 U RNase inhibitor, 1 U SP6 RNA polymerase and 3 μ g of pPOLY(A)-luc plasmid DNA. For translation of the RNA transcript, the reaction mixture of 25 μ l contained 17 μ l of rabbit reticulocyte lysate, 50 mM of each amino acid (minus methionine), 40 μ Ci [35 S]methionine, 1 U RNase inhibitor and 5 μ g of RNA template. Both reaction mixtures were incubated at 30°C for 120 min and then stored at -20°C until required for post-translational analysis.

Denaturing gel analysis of translated products

A 5 μ l aliquot of the translation reaction was added to 20 μ l of SDS sample buffer [2% (w/v) SDS, 0.2% (v/v) glycerol, 5% (w/v) β -mercaptoethanol and 0.375 M Tris-HCl pH 8.8] and denatured at 100°C for 2 min prior to loading on a 12% SDS-polyacrylamide gel. Electrophoresis was carried out as previously described (21).

Luciferase chemiluminescence assay

A 5 μ l aliquot of the translation mixture was added to 50 μ l of the luciferase assay reagent [20 mM tricine, 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 mM DTT, 270 μ M coenzyme A, 470 μ M luciferin, 530 μ M ATP and 1.07 mM (MgCO₃)₄Mg(OH)₂·5H₂O]. This reaction mixture was immediately placed in luminometer (Model L-3000, New Brunswick Scientific Corporation Inc.) and the intensity of emitted light was measured.

Primer extension

Unlabelled plasmid DNA (pPOLY(A)-luc) encoding the luciferase gene was alkylated with various concentrations of mustard at 37°C for 60 min. Alkylation sites were mapped with *Taq* polymerase using a Promega Corporation *fmol* DNA sequencing system. A 20mer oligonucleotide primer was synthesised using an Applied Biosystems Model 381A DNA synthesiser. The primer was complementary to the -87 to -67 region relative to the +1 site of the luciferase mRNA. The sequencing reactions were carried out according to the Promega protocol. Control and alkylation site mappings were carried out with nucleotide mixes containing a 3:1 ratio of 7'-deaza-dGTP/dGTP using a Perkin-Elmer/Cetus thermal cycler. Approximately 500 fmol of the pPOLY(A)-luc DNA, 5 pmol of the primer and 20 μ Ci [α - 32 P]dATP were used for each cycling reaction (total volume of 6 μ l). The cycling conditions involved a 5 min incubation at 95°C, followed by 30 cycles at 95°C for 30 s, 60°C for 30 s and

70°C for 1 min, followed by a 5 min extension at 70°C. After the completion of cycling, the samples were cooled to 4°C and 3 μ l of the stop solution (10 mM NaOH, 95% formamide, 0.05% each of bromophenol blue and xylene cyanol) was added to each sample. The samples were then heated at 90°C for 5 min, cooled rapidly in ice and loaded onto a 12% denaturing polyacrylamide gel containing 7.5 M urea. Electrophoresis was carried out at 2000 V in TBE buffer for 3 h. The gel was then fixed in 10% (v/v) each of methanol and acetic acid prior to drying using a BioRad Gel Drier.

Gel mobility shift assay

Radiolabelled RNA encoding the luciferase gene was synthesised essentially as described above for protein synthesis except that the amount of ATP varied. The reaction mixture contained 50 μ Ci of [α -³²P]ATP and 0.5 mM ATP. Alkylated and non-alkylated RNA aliquots (20 000 c.p.m.) were incubated with 15 μ l of the rabbit reticulocyte lysate and 1 U RNase inhibitor for 120 min at 30°C. To inhibit incorporation of amino acids, 50 mM of cycloheximide (inhibitor of peptidyl transferase) was added to the reaction mixture. A 5 μ l aliquot of this mixture was loaded on a 5% non-denaturing polyacrylamide submarine gel containing 100 μ M MgCl₂. Electrophoresis was carried out at 100 V in TBE buffer containing 100 μ M MgCl₂ for 3 h at 4°C. The gel was then fixed in 10% acetic acid prior to drying using a BioRad Gel Drier.

Gel analysis and quantitation

Gel analyses were performed using the Molecular Dynamics Model 400B PhosphorImager with quantitation carried out using Molecular Dynamics ImageQuant software (Molecular Dynamics, CA).

RESULTS

The effects of mustard alkylation on protein synthesis were investigated in two stages. Firstly, the effect of mustard alkylation of DNA and RNA templates was assessed by measuring the amount and activity of the translated protein. The second stage assessed the location of alkylation sites using a primer extension assay and measured the effects of RNA alkylation on translation initiation and elongation.

Protein synthesis from alkylated DNA and RNA

Samples of DNA encoding the luciferase gene were alkylated with a range of nitrogen mustard concentrations and then subjected to coupled transcription and translation to determine the effect of alkylation of DNA on the transcribed and subsequently translated gene product. Only one translated product was observed (Fig. 1A) corresponding to the full length 62 kDa luciferase protein. The amount of the protein decreased with increasing mustard concentration (Fig. 1B), with protein synthesis almost completely inhibited at 1.0 μ M mustard. A mustard concentration of 30 nM was required to reduce the amount of luciferase protein to half that synthesised using an untreated template.

Mustard was also reacted with the RNA template, and the alkylated template was translated using a rabbit reticulocyte lysate translation system. Figure 2A shows an SDS-polyacrylamide gel analysis of the luciferase gene products and quantita-

Figure 1. Coupled transcription and translation of the luciferase protein. (A) Autoradiogram of 12% SDS-PAGE analysis of the [³⁵S]methionine labelled 62 kDa luciferase protein synthesised from a DNA template. Aliquots of 3 μ g DNA were reacted with 0.01, 0.05, 0.1, 0.5, 1.0 and 10.0 μ M mustard and then subjected to a TNT coupled transcription and translation system at 30°C for 2 h. Untreated DNA template is labelled as control. (B) Quantitation of the amount of [³⁵S]methionine labelled protein. The relative intensity of the labelled protein is shown as a function of mustard concentration.

tion of the products is shown in Figure 2B. The amount of the 62 kDa luciferase protein decreased with increasing mustard concentration and synthesis was completely inhibited at 200 μ M mustard, with ~0.3 μ M mustard resulting in a 50% decrease in the amount of full-length protein (Fig. 2B, panel A). As well as a decrease in full length protein, five major truncated polypeptides (or groups of polypeptides) of ~45, 36, 28, 22 and 19 kDa were also observed, with the polypeptide(s) of ~19 kDa increasing with increasing mustard concentration (Fig. 2B, panel A insert). The 19 kDa product(s) was the only translated product observed at 200 μ M mustard.

In addition to producing truncated products, mustard alkylation of RNA may also interfere with initiation of translation. To assess whether the truncated polypeptides represent the full extent of the mustard-induced impairment of the translation process, it was therefore necessary to establish if the same number of polypeptides was initiated after each drug treatment. Since the amount of incorporated [³⁵S]methionine increased with polypeptide chain length, the mole-fraction of each polypeptide in each lane was calculated by normalising the radioactivity with respect to the number of labelled amino acids incorporated into each polypeptide. As shown in panel B of Figure 2B, the total number of polypeptide chains in each lane was essentially constant over the range of the mustard concentrations used to pre-treat the RNA. Therefore, the same number of polypeptides was initiated in the presence and absence of mustard. However, alkylation of the

Figure 2. Translation of the luciferase protein. (A) Autoradiogram of a 12% SDS-PAGE analysis of the [35 S]methionine labelled translated products from the RNA template. Aliquots of 5 μ g RNA were reacted with 0.5, 5.0, 50 and 200 μ M mustard and then subjected to a rabbit reticulocyte lysate translation system at 30°C for 2 h. Untreated RNA template is labelled as control. Approximate molecular weights of the translated products are shown on the right hand side of the autoradiogram. (B) Quantitation of the translated products. The intensity of the full length 62 kDa protein and the lower molecular weight truncated products (~19 kDa, insert) are shown in panel A. The relative number of all polypeptides chains in each lane is shown in panel B.

RNA template resulted in the production of truncated polypeptides in contrast to untreated RNA template where all initiated chains reached full-length (62 kDa)

Luciferase assay

The luciferase protein is monomeric and does not require further processing for functional activity. The activity of this protein could therefore be assayed directly from the translation mixture. An aliquot of the translation mixture was added to the luciferase reagent and the chemiluminescence intensity measured using a

Figure 3. Luciferase chemiluminescence assay. The effect of reaction time of mustard with the DNA (■) and RNA (●) templates on the activity of the luciferase protein is shown. Aliquots of DNA (3 μ g) were reacted with 50 nM mustard and RNA (5 μ g) were reacted with 50 μ M mustard at 37°C for a varying alkylation times between 0 and 6 h. The alkylated templates were then subjected to the appropriate translation system and incubated at 30°C for 2 h. Aliquots of 5 μ l of the reaction mixture were added to 50 μ l of the luciferase assay reagent and the intensity of emitted light measured using a luminometer.

luminometer. Figure 3 shows the intensity of chemiluminescence emitted as a function of reaction time of the DNA template with 50 nM mustard and of the RNA template with 50 μ M mustard. Because nitrogen mustard reacts rapidly with DNA (22) the zero time point values were determined using unalkylated DNA and RNA templates. The intensity of emitted light decreased with increasing reaction time for both cases and no enzymatic activity was detectable after a 4 h reaction time. The half-life loss of the luciferase activity was 0.5 and 1.1 h for the alkylated RNA and DNA templates, respectively, as determined by fitting to a single exponential decay model.

Primer extension

Because coupled transcription and translation of the gene product was inhibited at very low concentrations of mustard (nanomolar amounts), it was necessary to verify that the DNA was indeed alkylated at these concentrations and to establish the preferred alkylation sites. A primer extension assay was therefore employed to detect alkylation sites on the DNA template. The plasmid DNA encoding the luciferase gene was alkylated with various concentrations of mustard for 60 min at 37°C prior to analysis. Mustard-induced blockages were evident after treatment of the DNA with 0.1 and 0.5 μ M nitrogen mustard. Figure 4A shows mustard-induced blockage of progression of *Taq* polymerase along the DNA template. With the alkylated DNA, *Taq* polymerase was blocked at sites corresponding to mustard induced adducts. Figure 4B shows the relative intensity and location of the blockage sites on the template DNA. Of the 130 bp sequenced 36 blockages were detected of which 35 were located at guanine residues. At 200 μ M mustard the *Taq* polymerase activity was almost totally inhibited. With the unalkylated template, the *Taq* polymerase was able to extend fully to yield the full-length product.

Formation of gel-retarded complexes

A gel mobility shift assay was used to examine the ability of ribosomes (and/or proteins) to bind to alkylated RNA. Figure 5

Figure 4. Alkylation sites on the DNA template. (A) Autoradiogram of a 12% polyacrylamide gel showing the effect of mustard alkylation on the processivity of *Taq* polymerase. Aliquots of the pPOLY(A)-luc DNA were alkylated with 0.1, 0.5 and 200.0 μ M mustard and then subjected to 30 cycles of denaturation, primer annealing and extension. The lanes labelled dG, dC, dA and dT are the sequencing lanes. Untreated DNA is denoted as CONTROL. The length of the newly synthesised DNA strand is numbered with respect to the +1 start of RNA synthesis. (B) Quantitation of the alkylation sites. The location and relative intensities of the *Taq* polymerase blockage sites [0.5 μ M lane of (A)] are shown with respect to the sequence of the template DNA strand, numbered with respect to the +1 site of the transcribed message. The position of the start codon is underlined.

shows the effect of alkylating RNA with 100 μ M mustard on the formation of a retarded complex. With non-alkylated RNA in the presence of cycloheximide (lane 3), a stable complex was formed (cycloheximide was included to inhibit the activity of peptidyl transferase). This complex was not apparent when the reaction was performed in the absence of cycloheximide (lane 4) indicating that the complex had dissociated following translation of the RNA. In the presence of both mustard and cycloheximide (lane 5), the retarded complex was also formed. However, when alkylated RNA was incubated with the lysate in the absence of cycloheximide (lane 6), this complex was still apparent.

DISCUSSION

Transcription and translation

Alkylation of the DNA template may have a number of effects on transcriptional processes. Firstly, it has been shown using prokaryotic *in vitro* transcription systems that alkylation of a DNA template by nitrogen mustard causes termination of transcription, resulting in truncated RNA molecules and a decreased amount of full-length RNA (23,24). These transcriptional blockages occur at all alkylated guanine sites on the template strand (23) and therefore presumably at all sites of interstrand crosslinking (although this has yet to be confirmed). Secondly, it has also been demonstrated that alkylation of the

promoter region increases the stability of the initiated ternary transcription complex and results in decreased elongation activity of the RNA polymerase (25). In addition, since a number of potential 5'-GNC DNA interstrand crosslinking sites (26) exist in the promoter region of the DNA used in this study, alkylation of these sites could result in impairment of formation of the open complex. All of these documented effects would result in a decrease of transcriptional activity and, consequently, a decreased amount of full-length RNA transcript. Because alkylated DNA gives rise to the formation of truncated transcripts, it follows that truncated polypeptides should also be formed.

In the present work, a decreasing amount of full length protein was observed after alkylation of DNA by low levels of mustard in the coupled transcription and translation system but this was not accompanied by detectable levels of truncated polypeptides. It therefore seems likely that either the translation machinery only recognises and processes the full-length RNA transcript, or that the incomplete transcripts are rapidly degraded. The protection of mRNA from the action of nucleases as it is synthesised and enters the translation machinery is achieved, at least in part, by its association with sequence-specific RNA binding proteins and the presence of a poly(A) tail (27). The role of the poly(A) tail in the stability of mRNA is a well documented phenomenon and several pathways of degradation of deadenylated RNA have been reported: 3'-5' exonucleolytic degradation (27); 5'-3' exonucleolytic decay; sequence specific internal cleavage (28). The

Figure 5. Effect of mustard alkylation on the formation of retarded RNA complex. The autoradiogram of a 5% non-denaturing polyacrylamide gel mobility shift assay of the formation of the labelled RNA and ribosomes (and/or specific proteins). The retarded RNA complexes were formed by incubating labelled RNA with the rabbit reticulocyte at 30°C for 2 h. Lanes 1 and 2 represent unalkylated and alkylated RNA respectively with no lysate added. Lanes 3–6 represent RNA incubated with rabbit reticulocyte lysate with either alkylated RNA (lanes 5 and 6) or unalkylated RNA (lanes 3 and 4). Cycloheximide was present in the reaction mixtures of lanes 3 and 5.

poly(A) sequence is also known to play a significant role in both the initiation and efficiency of translation and this has been the subject of recent reviews (29,30). Munroe and Jacobson (30) suggested that the poly(A) tail and the poly(A) binding protein are necessary for the formation of the 80 S initiation complex and act to enhance translation. Transcripts lacking either the RNA binding protein recognition sequences or the poly(A) sequence would therefore be expected to be rapidly either degraded or inefficiently translated.

Translation

There were two major effects of mustard alkylation of the RNA template on translation. The amount of the 62 kDa luciferase protein decreased with increasing mustard concentration and truncated polypeptides of discrete lengths were also synthesised.

The fact that the number of polypeptide chains initiated from an alkylated RNA template remained constant (Fig. 2B, panel B), irrespective of prior treatment with differing levels of mustard, shows unequivocally that all polypeptide chains are either terminated at alkylation sites, or else reach full-length. This therefore suggests that mustard alkylation of the RNA template does not affect the initiation of translation in this system. The decrease of full-length polypeptides with increasing mustard levels is therefore completely accounted for by an increasing level of truncated polypeptides. The '19 kDa' truncated product presumably represents a range of polypeptides of ~19 kDa. The amount of these truncated products increased dramatically with increasing mustard level (Fig. 2B, panel A insert) and therefore there was a concomitant decrease in the number of ribosomes reaching the alkylation sites which give rise to the longer

truncated products. Mustard effects at these sites are therefore less apparent because of the reduced amount of ribosome reaching these sites. Truncated polypeptides presumably arise from termination of translation elongation at major mustard alkylation sites (monoadducts or intrastrand crosslinks) or alternatively, at secondary structure sites which are stabilised by mustard alkylation.

If the truncated polypeptides arise from blockage of ribosomes at alkylated sites on the RNA, this implies that there are specific sequences on the RNA which are particularly susceptible to alkylation. The sequence specificity of mustard alkylation of RNA is presently not known. However, since DNA is known to be preferentially alkylated at runs of contiguous guanine residues (9–11) and alkylguanine derivatives are the major product of RNA alkylation by sulfur mustard (31), the luciferase RNA was analysed in terms of the occurrence of G_n sequences ($n = 2-4$). However, there was only a limited correlation between the length of the truncated polypeptide and the length of the RNA message up to the potential alkylation site. Shooter and co-workers (31) were also unable to detect any correlation between the relative number of alkylguanine derivatives (in a RNA-containing bacteriophage $\mu 2$) and plaque forming ability, and have suggested that other minor alkylation products may be responsible for inactivation of the phage. Furthermore, the response of the ribosome to alkylation sites on RNA is currently not known. The ribosome may readthrough some sites and the truncated polypeptides may reflect a subpopulation of the total alkylation sites on the RNA.

An alternative explanation for the cause of truncated polypeptides is that they may be due to regions of secondary structure of the RNA. Mustard alkylation may add stability to such structures. Such a mechanism for termination of translation has previously been reported (32–34). The relationship of secondary structure to RNA translation is complex and reflects a number of variables, including size, position and stability of the secondary structures, as well as the cellular environment and potential destabilisation of the duplex by a variety of translational processes (35). The ability of ribosomes (and/or specific proteins) to bind to alkylated RNA was probed by a gel mobility shift assay. Under the experimental conditions used, a stable complex was induced by alkylated RNA. A likely explanation for this observation is that when a ribosome complex reaches an alkylation site or stabilised secondary structure on the alkylated RNA molecule, the processivity is blocked and the ribosome remains associated with the RNA. The biological consequences of such trapping of ribosomes (and/or proteins) on alkylated RNA sites are yet to be determined but from the present data it is likely that this trapping results in termination of translation. It should be noted that while this interpretation is reasonable, it is completely speculative at this stage. Additional experiments are now in progress to confirm the nature of the retarded complexes.

There are conflicting reports regarding the effects of RNA modification on the translated product. Grunberger and co-workers (36) demonstrated that the binding of benzo[a]pyrene diolepoxide to mRNA blocks incorporation of amino acids into proteins but did not result in truncated polypeptides. They showed however that modification of the mRNA inhibits formation of the initiation complex, probably by disruption of the ordered secondary structure of the mRNA, making it less recognisable to ribosomes and initiation factors. In contrast, others have reported that benzo[a]pyrene diolepoxide alkylation of the RNA inhibits

Figure 6. Diagrammatic summary of the effects of alkylation of the DNA and RNA templates by nitrogen mustard on the translated products.

elongation of the translation complex, resulting in premature termination of the growing polypeptide chain (37). The present study has clearly demonstrated that translation of the alkylated RNA template is inhibited during the elongation phase (rather than at initiation) resulting in the formation of truncated polypeptides. However at this stage it is not possible to define the mechanism of induction of truncated polypeptides by nitrogen mustard and future work will now be directed towards elucidating the molecular processes involved in the formation of these truncated polypeptides.

Relative effects of DNA and RNA alkylation

Protein synthesis was inhibited by 50% when the DNA and RNA template were alkylated by 30 and 300 nM mustard, respectively. It therefore appears that the DNA template is some 10-fold more susceptible than RNA to the effects of alkylation by nitrogen mustard. This suggests that transcription is a more readily hindered process than translation and hence DNA is probably the more sensitive target for the mode of action of mustards. This conclusion is consistent with other studies involving mustard effects *in situ* (13–16). This may reflect either the differences in rates of mustard alkylation of the DNA and RNA templates (which are currently not known), or the differences in the response of RNA polymerase to alkylated DNA template compared to the response of ribosomes to an alkylated RNA template (or a combination of both effects). The greater reactivity of DNA arises from the double helical structure which results in a higher nucleophilicity for alkylation compared to that of single stranded RNA (38). The overall effect of alkylation of this preferred target site is a combination of inhibition of replication, inhibition of transcription and a consequential effect on translation

through the production of non-functional mRNA. Since these effects occur at biologically relevant concentrations (10 nM), it is likely that such processes also occur *in vivo*. These effects therefore have implications for both the toxic, mutagenic and carcinogenic properties, as well as anticancer activity of this group of compounds.

Overall, nitrogen mustard alkylation of the DNA and RNA templates has two different effects on the type and quantity of translated products and this is summarised diagrammatically in Figure 6. The alkylated DNA template yields a single polypeptide corresponding to the full-length protein. Previous studies have shown that transcription of mustard alkylated DNA in prokaryotic systems results in the formation of truncated RNA transcripts. If the same process occurs in eukaryotes, then it appears that these truncated transcripts are either degraded or are non-functional templates, with only full-length transcripts (with a poly(A) tail) being translated. When alkylated RNA was used as the template, a range of truncated polypeptides was formed in addition to the full-length product. Mustard alkylation therefore appears to terminate the translation elongation complex at alkylation sites, although the effect of RNA secondary structure cannot yet be completely discounted. Further studies are required to understand the possible implications of the effects of mustard binding to nucleic acids in terms of disruption of all aspects of gene expression. In particular, determination of the sequence specificity of mustard alkylation of RNA and the response of ribosomes to this alkylation, are issues which are currently being addressed. This information will greatly enhance understanding of the biological significance of mustard-induced truncated polypeptides.

ACKNOWLEDGEMENTS

This work was carried out with the support of the Australian Research Council (DRP) and a scholarship from the Australian Agency for International Development (AM).

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