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Selective activation of human heat shock gene transcription by nitrosourea antitumor drugs mediated by isocyanate-induced damage and activation of heat shock transcription factor

(HSP70/HSP90/protein damage/transcriptional control)

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ABSTRACT **Treatment of cultured human tumor cells with the chloroethylnitrosourea antitumor drug 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) selectively induces transcription and protein synthesis of a subset of the human heat shock or stress-induced genes (HSP90 and HSP70) with little effect on other stress genes or on expression of the c-*fos*, c-*myc*, or β-actin genes. The active component of BCNU and related compounds appears to be the isocyanate moiety that causes carbamoylation of proteins and nucleic acids. Transcriptional activation of the human HSP70 gene by BCNU is dependent on the heat shock element and correlates with the level of heat shock transcription factor and its binding to the heat shock element *in vivo*. Unlike activation by heat or heavy metals, BCNU-mediated activation is strongly dependent upon new protein synthesis. This suggests that BCNU-induced, isocyanate-mediated damage to newly synthesized protein(s) may be responsible for activation of the heat shock transcription factor and increased transcription of the HSP90 and HSP70 genes.**

The chloroethylnitrosoureas (CENUs) are a class of antineoplastic agents used in clinical treatment of various tumors. These compounds are not biologically active in their parent state. They are chemically unstable in aqueous solution at physiological temperature and pH, undergoing spontaneous base-catalyzed decomposition to yield two bioactive species: a chloroethyldiazonium ion that alkylates and crosslinks and an isocyanate with carbamoylating activity (1). Interaction of these reactive species with cellular macromolecules is thought to initiate the events leading to the observed antitumor activity and cellular toxicity. DNA alkylation by the chloroethyldiazonium ion may generate single-strand breaks (2), which can subsequently react to produce DNA/DNA (2) or DNA/protein crosslinks (3). The role of isocyanate carbamoylation to overall cytotoxicity is not clearly established. However, a secondary role due to inhibitory effects on DNA polymerase activity (4), the repair of DNA strand breaks (5), RNA synthesis and processing (4), and regeneration of intracellular glutathione (6) has been demonstrated. The observed antitumor efficacy is most likely due to alkylating activity since nitrosoureas such as chlorozotocin, which lack carbamoylating activity, still retain efficacy.

The cytotoxic effects of antitumor compounds might be expected to perturb cellular homeostasis and activate expression of the heat shock or stress-induced proteins (HSPs). The stress proteins are induced in response to a wide range of external stresses, including heat shock, heavy metals, amino acid analogues, oxidizing agents, and teratogens (7–9). Given the wide range of conditions that cause the increased expression of stress proteins, we examined the effect of a broad range of antitumor drugs on cultured human cells. Although we had expected that most, if not all, cytotoxic antitumor drugs would induce stress proteins, we found that only the CENUs were potent activators of the human heat shock gene expression (10). In this study, we describe the molecular basis for alteration of HSP70 gene expression by this class of antineoplastic compounds and provide evidence that isocyanate-induced damage causes activation of heat shock transcription factor (HSF) and a subset of genes regulated by this transcription factor.

MATERIALS AND METHODS

HeLa and HT-29 cells were maintained in Dulbecco's modification of Eagle's medium (DMEM) containing 10% bovine calf serum. Cells in middle to late exponential growth were treated with drugs as described (10). 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU; Bristol-Meyers), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea [CCNU; National Cancer Institute (NCI)], 1-(2-chloroethyl)-3-(methylcyclohexyl)-1-nitrosourea (Me-CCNU; NCI), and 1,3-bis(*trans*-4-hydroxycyclohexyl)-1-nitrosourea (BHCNU; NCI) were dissolved in absolute ethanol, and ethyl, chloroethyl, and cyclohexyl isocyanate (Sigma) were dissolved in anhydrous dimethyl sulfoxide. All drugs were prepared at 100× concentration immediately before use. Cells were washed with phosphate-buffered saline (PBS) after drug treatment and pulse-labeled with [^{3}H]leucine (10 μCi/ml; 1 Ci = 37 GBq) in leucine-free medium for 1 hr at 37°C. Equal amounts of trichloroacetic acid-precipitable radioactivity were analyzed by two-dimensional (2D) gel electrophoresis (11).

Cytoplasmic RNA was isolated from treated cells by lysis in a buffer containing 100 mM NaCl, 10 mM Tris·HCl (pH 8), 2 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 1% 2-mercaptoethanol. S1 nuclease assays were performed using a fragment of the human HSP70 gene that was 5′ end-labeled at the *Nco* I site and that protects a 514-nucleotide transcript (12, 13). For *in vitro* run-on transcription assays, nuclei were isolated from equal numbers of cells harvested at various times after treatment and the reactions were performed as described (14). Gel mobility shift assays were performed with whole cell extracts prepared from treated HeLa cells using 10 μg of extract and a ^{32}P-labeled

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Abbreviations: CENU, chloroethylnitrosourea; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; Me-CCNU, 1-(2-chloroethyl)-3-(methylcyclohexyl)-1-nitrosourea; BHCNU, 1,3-bis(*trans*-4-hydroxycyclohexyl)-1-nitrosourea; HSP, heat shock protein; HSF, heat shock transcription factor; HSE, heat shock element; 2D, two-dimensional.

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heat shock element (^{32}P-HSE) oligonucleotide containing four inverted HSE sequences (5′-CTAGAAGCTTCTAGAAGCTTCTAG-3′) (15, 16). HeLa cells were transiently transfected with HSP70 promoter construct vectors as described (12). The transfected cells were harvested either after treatment with BCNU or vehicle, as described, or after heat shock (42°C for 1 hr). Cytoplasmic RNA was isolated and analyzed by S1 nuclease protection assays for HSP-CAT mRNA (CAT = chloramphenicol acetyltransferase; ref. 12) and endogenous HSP70 mRNA. For genomic footprinting analysis, $\approx 2 \times 10^7$ control or cycloheximide-pretreated HeLa cells were treated with 300 μM BCNU, as described. As a positive control, an equivalent number of cells was subjected to heat shock at 42°C for 30 min and processed concurrently. Immediately following treatment, cells isolated by mild trypsinization were resuspended in DMEM at 20°C, and genomic DNA was isolated following a 5-min treatment with 0.2% dimethyl sulfate (17). The isolated, methylated DNA was then used for ligation-mediated PCR genomic footprinting (17, 18).

RESULTS

Selective Activation of HSP70 and HSP90 by Nitrosoureas. We examined the effects of BCNU on the pattern of gene expression in cultured human tumor cells by analyzing the pattern of protein synthesis by 2D gel electrophoresis. Examination of proteins synthesized after a 1-hr treatment with 300 μM BCNU revealed a significant induction of HSP70 synthesis and slight induction of HSP90, the constitutively expressed p72, and the glucose-regulated protein GRP78 (Fig. 1). Surprisingly, BCNU had little effect on the synthesis of other cellular proteins (Fig. 1).

To establish whether BCNU affected gene expression at the transcriptional level we used *in vitro* nuclear run-on assays to assess the transcription rates of selected genes, including HSP90, HSP70, HSP60, and HSP27 stress genes and the c-*fos*, c-*myc*, and β-actin genes (Fig. 2). Within 1–2 hr following BCNU treatment, transcription of the HSP70 gene increased 20-fold and returned to near basal levels by 6 hr. HSP70 mRNA levels increased with slightly delayed kinetics (Fig. 2*C*). Similar patterns but a lower level ($\approx$10-fold) of transcriptional induction were observed for the HSP90 gene. In contrast, BCNU treatment had less than a 2- to 3-fold effect on the transcription of HSP60 and HSP27 genes or on the HSP70-related genes GRP78 and p72.

Carbamoylation via the Isocyanate Moiety Is the Inducer of Stress Protein Expression. The CENUs such as BCNU, CCNU, and Me-CCNU produce a plethora of covalent modifications to intracellular constituents. Since induction of the stress response has been linked to protein damage (19, 20), we examined whether the signal for CENU induction of HSP70 was due to isocyanate-mediated protein modification. Cells were treated with equimolar doses of these CENUs and with BHCNU, which retains high carbamoylating activity and no alkylating or crosslinking activity. Maximal levels of HSP70 mRNA were reached at 3–5 hr after a 1-hr treatment with each of these drugs. Shown in Fig. 3*A* is the S1 nuclease analysis of HSP70 mRNA levels 4 hr after treatment. Treatment with BCNU, CCNU, Me-CCNU, or BHCNU induced high levels of HSP70 mRNA. To obtain support for the hypothesis that the isocyanate moiety generated by the aqueous decomposition of these compounds was responsible for activation of the stress response, cells were treated with chloroethyl or cyclohexyl isocyanate, the carbamoylating moieties generated from BCNU or CCNU, respectively, or with ethyl isocyanate. Cytoplasmic RNA was isolated from cells at 4 hr after a 1-hr treatment and analyzed by S1 nuclease protection. As shown in Fig. 3, treatment with the isocyanates induced HSP70 mRNA to levels similar to that obtained with the parental drugs.

Nitrosourea Activation of HSP70 Transcription Is HSE Mediated. To identify sequences in the HSP70 promoter required for BCNU mediated-transcriptional activation, HeLa cells were transiently transfected with a human HSP70-CAT construct (LSNWT) that contains HSP70 promoter sequences (−188 to +150) fused to the CAT gene (12). The transfected cells were subjected to heat shock or BCNU, and relative endogenous HSP70 and CAT mRNA levels were measured by S1 nuclease analysis. HSP-CAT mRNA and endogenous HSP70 mRNA were induced by BCNU (Fig. 4*A*). A more detailed localization of the BCNU-responsive cis elements in the HSP70 promoter was obtained using a promoter with a mutation in the HSE and a construct retaining basal promoter sequences (−74 to +1). The results of these studies (data not shown) indicated that an intact HSE was necessary and that the region from −74 to +1 was insufficient for BCNU induction.

A second approach to directly identify the cis-acting element(s) in the HSP70 promoter that responded to BCNU treatment used the *in vivo* genomic footprinting method. We have previously used the ligation-mediated PCR genomic footprinting method to identify factors bound *in vivo* to the HSP70 promoter and to demonstrate the inducible binding of HSF with specific guanine residues in the HSE (18). Heat shock results in dimethyl sulfate hypersensitivities in the

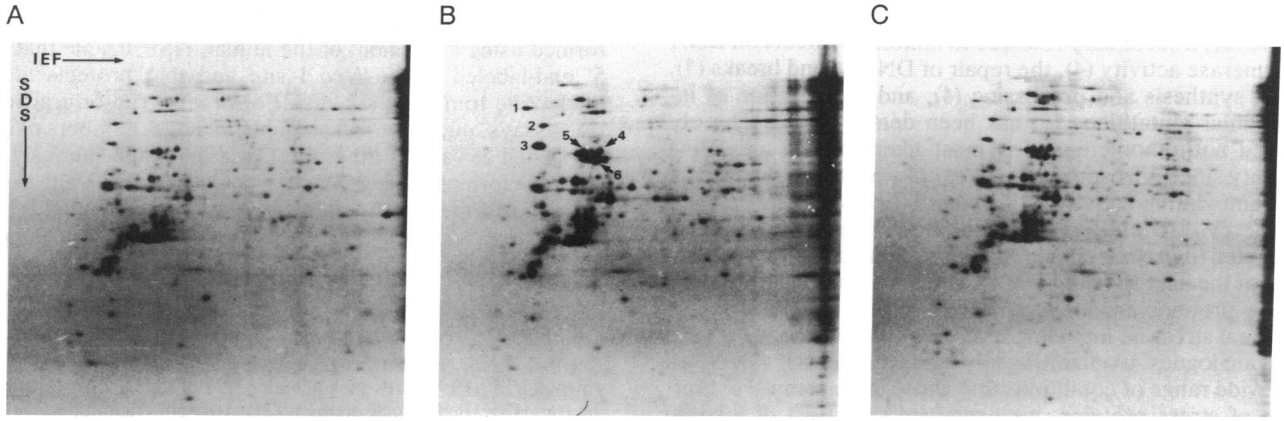

FIG. 1. Patterns of protein synthesis following heat shock or BCNU treatment analyzed by 2D electrophoresis. HT-29 cells were treated for 1 hr with either ethanol (control, *A*), heat shock at 42°C (*B*), or 300 μM BCNU (*C*). Cells were pulse-labeled with [^{3}H]leucine (10 μCi/ml) immediately after heat shock or 3 hr after BCNU treatment. Proteins are identified in the autoradiograms as follows: HSP100 (1), HSP90 (2), GRP78 (3), p75 (4), p72 (5), HSP70 (6). IEF, isoelectric focusing.

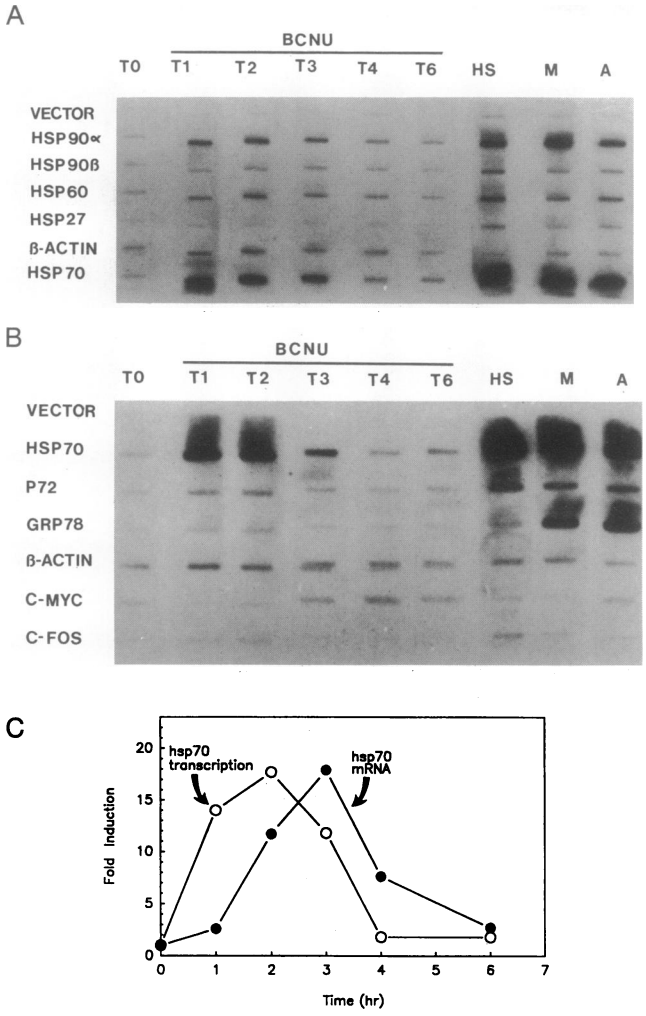

FIG. 2. *In vitro* transcription in isolated HeLa cell nuclei following BCNU treatment. Nuclei were isolated over the course of 6 hr after treatment (T6) with 300 μM BCNU and *in vitro* transcription reactions were performed. Nuclei of cells subjected to heat shock at 42°C for 30 min (lanes HS) or treated with 30 μM $CdSO_4$ (lanes M) or 5 mM azetidine (lanes A) for 4 hr were concurrently isolated. The ^{32}P-labeled RNAs were hybridized to filter-bound DNAs of members of various stress gene families (*A*) and members of the HSP70 stress gene family and growth-regulated genes (*B*). (*C*) Quantitation of HSP70 transcription rates and mRNA levels (not shown) induced by 300 μM BCNU as measured by scanning densitometry.

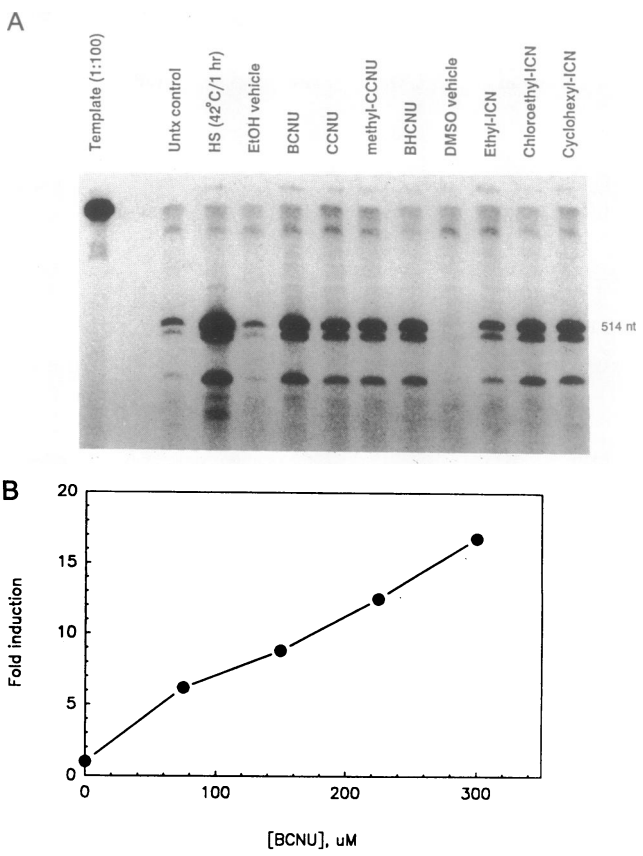

FIG. 3. HSP70 mRNA levels in HeLa cells treated with nitrosoureas or isocyanates (ICNs). (*A*) S1 nuclease analysis of cytoplasmic RNA isolated from untreated cells (Untx), from cells subjected to heat shock at 42°C for 1 hr (HS), or from cells 4 hr after treatment with vehicle [ethanol, dimethyl sulfoxide (DMSO)] or 300 μM drug. The size of the correctly initiated HSP70 transcript is 514 nucleotides (nt). (*B*) HSP70 mRNA dose–response to BCNU treatment in HeLa cells. Relative HSP70 mRNA levels were assessed 4 hr after treatment by slot blot hybridization to a ^{32}P-labeled HSP70 gene-specific probe.

guanine at position −95 of the noncoding strand and protections of the guanine residues at −96 and −97 (Fig. 4*B*). The analysis of genomic DNA from BCNU-treated cells revealed nearly identical protections as the DNA from cells exposed to heat shock, consistent with the corresponding increase in HSP70 transcription rate.

To examine the role of *de novo* protein synthesis in these interactions, cells were treated with 100 μg of cycloheximide per ml for 30 min prior to, during, and subsequent to BCNU treatment. This dose of cycloheximide was sufficient to inhibit >98% of protein synthesis, yet the genomic footprint of cycloheximide-treated cells prior to BCNU was identical to untreated cells. These results suggest that concurrent protein synthesis is necessary for BCNU-mediated HSF–HSE interactions.

Nitrosoureas Induce HSF. The results of transfection of heat shock promoter vectors and *in vivo* footprinting suggest that BCNU activates HSF. To directly demonstrate this, we examined the levels of HSF in whole cell extracts from BCNU-treated cells that bound to a synthetic oligonucleotide containing the consensus HSE binding site using the gel mobility shift assay (15). As shown in Fig. 5, within 1 hr after drug treatment, high levels of HSF were induced and maintained for up to 6 hr. By comparison, the levels of HSF induced in BCNU-treated cells were slightly lower than in heat shock, cadmium sulfate-treated, or proline analogue-treated cells.

The results of the gel shift analysis and *in vivo* footprint studies indicate that the ability of BCNU to induce *in vivo* binding to the HSF requires concurrent protein synthesis. Therefore, we examined whether pretreatment with cycloheximide affected BCNU induction of HSF. HeLa cells treated with BCNU in the presence of 100 μg of cycloheximide per ml for 30 min prior to, during, and subsequent to the treatment were assayed for HSF levels. As shown in Fig. 6, cycloheximide pretreatment reduced the level of BCNU-induced HSF by 90%, suggesting that the mechanism of BCNU induction of HSF is dependent on nascent protein synthesis. Therefore, the mechanism of BCNU activation of HSF may involve the isocyanate-mediated carbamoylation of nascent protein molecules.

DISCUSSION

The CENUs are a class of pharmacologically reactive molecules capable of alkylation, crosslinking, and carbamoylation of cellular proteins and nucleic acids. Given that these highly reactive compounds have pleiotropic effects, we had originally expected that multiple cellular targets would be

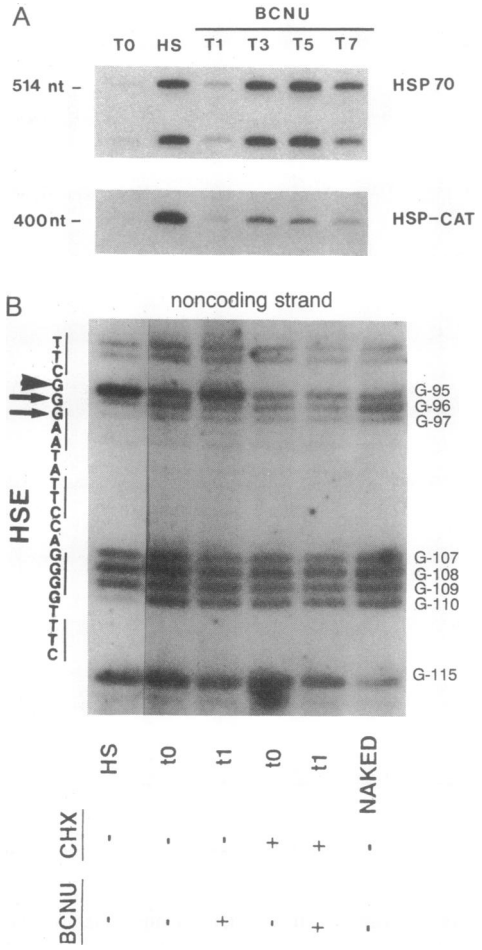

FIG. 4. Nitrosourea-responsive elements within the HSP70 promoter. (*A*) S1 nuclease protection analysis of endogenous HSP70 and HSP-CAT mRNA after heat shock or BCNU treatment. HeLa cells were transiently transfected with the promoter vector LSNWT, subjected to heat shock at 42°C for 1 hr (HS), or treated with 600 μM BCNU and cytoplasmic RNA isolated over the course of 7 hr (T7) from the BCNU-treated cells. The sizes of the correctly initiated transcripts for HSP70 and HSP-CAT are 514 and 400 nucleotides (nt), respectively. (*B*) Genomic footprint of the HSP70 promoter following BCNU treatment: sequence of the HSE corresponding to inverted arrays of NGAAN and its relative position on the footprint. Shown is the noncoding strand methylation pattern in HeLa genomic DNA isolated from cells subjected to heat shock at 42°C for 30 min (HS), control cells (t0), cells treated with 300 μM BCNU at 1 hr (t1), cycloheximide (CHX)-pretreated cells, and cycloheximide-pretreated, BCNU-treated cells. Naked, protein-free genomic DNA methylated *in vitro* is also shown (NAKED). The arrowhead at position −95 corresponds to dimethyl sulfate hypersensitivity and the arrows at positions −96 and −97 correspond to dimethyl sulfate protection observed following heat shock.

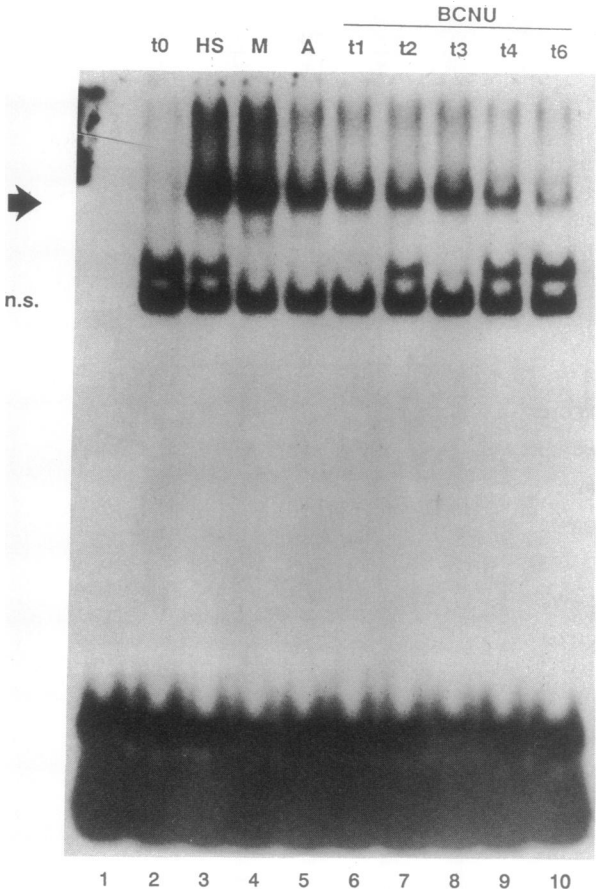

FIG. 5. BCNU activation of HSF in HeLa cells. Whole cell extracts prepared at various times following treatment with 300 μM BCNU were incubated with a ^{32}P-HSE oligonucleotide. The arrow denotes HSF; n.s. denotes nonspecific DNA–protein complexes. Lane 1, no protein; lane 2, control extract; lane 3, heat shock (42°C, 30 min); lane 4, 30 μM $CdSO_4$, 4 hr; lane 5, 5 mM azetidine, 4 hr; lanes 6–10, extracts isolated at 1–6 hr after BCNU treatment.

modified with effects on the expression of numerous genes. The analysis of proteins synthesized following BCNU treatment revealed that the synthesis of only a few proteins is affected—in particular, the major heat shock and stress-induced protein, HSP70. These results together with the transcription data indicate that nitrosoureas appear to have rather selective effects on the expression of HSP70 and, to a lesser extent, HSP90. If the mechanism is through induction of a heat shock-like response, we suggest the HSP90 and HSP70 genes are more sensitive to transcriptional induction. It is unclear why all of the HSE-containing, heat shock-inducible genes are not equally responsive to BCNU to the same extent as HSP70. The HSP90 and HSP70 genes are transcriptionally induced by serum stimulation (21, 22); however, the effects of BCNU cannot be general to all growth regulatory genes, as the transcription of c-*fos*, c-*myc*, and β-actin is not significantly increased.

The experiments using the parental nitrosoureas or the purified isocyanates together with the requirement for protein synthesis reveal that carbamoylation of newly synthesized proteins is required for activation of HSF. Our data do not exclude the involvement of nucleic acid carbamoylation in the induction of this response. However, previous *in vivo* and *in vitro* studies using differentially radiolabeled CCNU have clearly demonstrated negligible isocyanate-derived binding to nucleic acids (23). Unlike the stress response elicited by heat and heavy metals, the activation of HSF by BCNU is dependent upon *de novo* protein synthesis. This observation suggests that the activation of HSF must respond to distinct features and consequences of protein modifications that occur on nascent as opposed to those that occur on preexisting molecules. This hypothesis, however, does not readily explain why transcription of the GRP78 gene, a sensitive and specific indicator of protein alterations within the endoplasmic reticulum, is not induced by BCNU. The lack of GRP78 induction is surprising, since other conditions including heat shock, heavy metals, and amino acid analogues, all of which affect nascent molecules synthesized on both free and bound ribosomes, result in activation of HSP70 and GRP78 genes (24). Therefore, even among the two pools of translational machinery, BCNU appears to have selective effects on the cytoplasmic translational apparatus with little

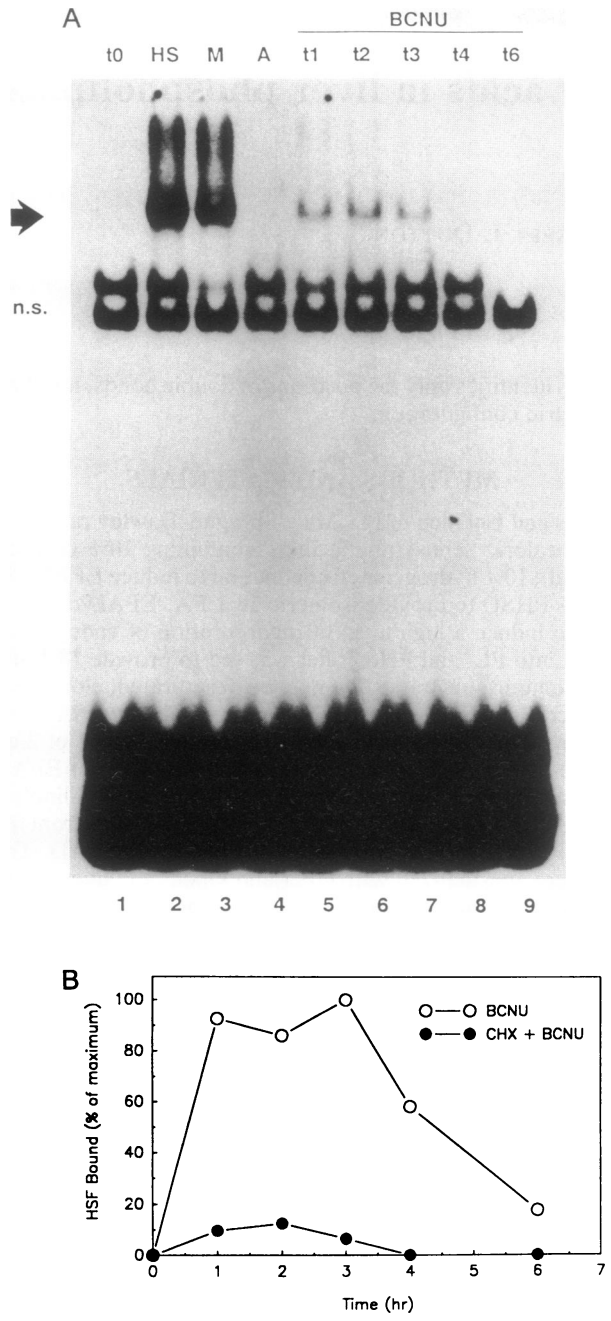

FIG. 6. (*A*) BCNU-induced HSF activation in cycloheximide-pretreated HeLa cells. Cells were treated with 100 μg of cycloheximide per ml prior to heat shock (HS), metal (M), azetidine (A), or BCNU treatment using conditions as described in the legend to Fig. 5. Lane 1, control extract; lane 2, heat shock (HS); lane 3, $CdSO_4$ (M); lane 4, azetidine (A); lanes 5–9, extracts isolated at 1–6 hr after BCNU treatment. (*B*) Quantification of the stress-induced form of HSF in cells exposed to BCNU with or without cycloheximide (CHX) pretreatment. The results were normalized to the highest level of BCNU-induced HSF.

effect on the endoplasmic reticulum-associated cotranslational apparatus.

The observation that nitrosoureas selectively activate a subset of heat shock genes has interesting implications for the role of stress proteins in disease. Stress proteins have been recently shown to have a fundamental role in biological reactions involving protein folding, transport, and assembly (25). Studies on thermotolerence have indicated that the increased or induced synthesis of certain stress proteins has protective value against subsequent exposure to conditions that cause damage (26, 27). Although the concentrations of nitrosoureas that we have used in this study are higher than attained in clinical use, the activation of stress gene expression follows a direct dose–response. We have detected induction of HSP70 synthesis in human peripheral blood monocytes and lymphocytes exposed to 10–20 μM BCNU (R.A.K., unpublished data), a concentration more closely approximating the clinically achievable range (28). Therefore, it will be of interest to ask whether the activation and expression of the 70-kDa and 90-kDa stress genes have protective value against BCNU-induced damage, in a manner analogous to the acquisition of thermotolerence. This also has particular relevance to the ever-growing need for use of combined modalities needed for successful chemotherapy.

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