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Alterations of Transcription and Translation in HeLa Cells Exposed to Amino Acid Analogs

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Amino acid analogs, like other effectors of the stress response, induce in mammalian cells the same gene products that are induced upon heat shock; incorporation of the analog into protein is required for induction. We show here that induction by analogs involves controls operating at the levels of both transcription and translation. The electrophoretic patterns of newly made mRNAs simplify with time such that the putative stress protein mRNAs are the only species transported from the nucleus. Concomitantly, the patterns of protein synthesis simplify such that the stress proteins become nearly exclusive polypeptide products. Although the normal mRNAs are either not used or used with greatly reduced efficiency, they are not degraded and retain translatability when transferred to cell-free systems. Soon after the stress response has been induced, there follows a defect in the initiation of polypeptide chains, as evidenced by examination of polysome profiles. Upon prolonged exposure, polysomes are recovered, and although they give rise to stress proteins almost exclusively, the normal mRNAs are still present in these structures. Thus, in addition to the initiation defect, a lesion in elongation may also be involved. The extreme sensitivity of protein synthesis to the inhibition of RNA synthesis, together with the parallel simplifications in the patterns of newly made mRNAs and polypeptides, may imply that only newly made mRNAs are efficiently translated in analog-treated cells.

As with almost all organisms so far examined, mammalian and avian cells exhibit responses readily comparable to the classical heat shock response of *Drosophila* (1, 7, 9, 23, 33). Because of the diversity of agents capable of inducing these proteins in different organisms, we and others have referred to them as stress proteins (SPs) in the belief that they are induced in response to metabolic stress, however ill defined (1, 6, 33). Similarly, despite the superficial differences among the various inducing situations, it is assumed that each of the various agents elicits a common and highly conserved response, activated by a generalized metabolic stress resulting either directly or indirectly from the actions of the inducer. Such an assumption is supported, in part at least, by the findings reported here, in which it is shown that HeLa cells react to amino acid analogs with a response embodying many of the features recognized in the more widely studied heat shock response of *Drosophila*. Furthermore, we present evidence which may indicate that a novel control mechanism, namely the control of protein synthesis at the level of selective elongation, may also be invoked.

With mammalian cells, exposure to certain amino acid analogs induces a fuller set of SPs than do other agents, including heat shock; particularly efficacious is the proline analog, L-azetidine-2-carboxylic acid (AzC). Using this analog, we have determined the chronology of various aspects of the response in HeLa cells. An initial period of protein synthesis is required for activation of the response, after which a combination of separable controls redirects gene expression to the predominant, and eventually nearly exclusive, production of SPs. These controls affect both protein synthesis and RNA synthesis and appear to proceed independently yet simultaneously. At the level of RNA synthesis, production of the normal spectrum of RNAs is repressed, while a small set of polyadenylated [poly(A)$^+$] RNAs, presumably SP mRNAs, become exclusive products. Protein synthesis upon preexisting mRNAs is shut off with similar kinetics and becomes dependent on continued RNA synthesis. The pattern of protein synthesis parallels RNA synthesis in that their progressive simplifications reduce to nearly exclusive synthesis of the SPs.

Examination of polysome profiles both early in exposure and after prolonged exposure indicates that there is an initial lesion in the initiation of polypeptide chains which with time is at least partially overcome so that polysomes are restored. A second translational control, namely the selective blockage of elongation on normal mRNAs, appears to be established. Further considerations raise the possibility that this defect could be based on the age of the mRNAs rather than the identity of their polypeptide products.

MATERIALS AND METHODS

Cells and general experimental conditions. HeLa cells were passaged the day before experiments to give confluent monolayers of equal age and density. All dishes were rinsed twice with phosphate-buffered saline and then maintained in Dulbecco modified minimal essential medium containing 2% calf serum for the duration of the experiment. When additions were to be made, small volumes of concentrated stock solutions were added directly to the medium (giving no more than a 1% increase in volume), followed by brief swirling to ensure even distributions. All experiments were conducted at 37°C. AzC, actinomycin D (AMD), and cordycepin were all from Calbiochem-Behring, La Jolla, Calif.

For labeling with [^{35}S]methionine, dishes were rinsed three times with prewarmed methionine-free medium and labeled as indicated in the figure legends. AzC was omitted from labeled medium. At the end of the pulse, the cells were washed several times with ice-cold phosphate-buffered saline and then solubilized on the plates with hot sample buffer. Cell lysates were collected and boiled before gel analysis. Linear 7.5 to 15% gradient polyacrylamide-sodium

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dodecyl sulfate gels for polypeptide separations were as described previously (31) and were subjected to fluorography (3). For estimation of protein synthesis rates, autoradiograms were scanned in a densitometer, and the areas under complete lanes were measured.

RNA preparation and cell-free translation. Extraction of total cytoplasmic RNA and translations in a nuclease-treated rabbit reticulocyte lysate were as described previously (31).

Preparation and fractionation of polysomes. Cultures were washed and scraped in ice-cold TGN (146 mM NaCl, 35 mM Tris-hydrochloride [pH 7.4], 11 mM glucose) and pelleted. After rinsing, cells were suspended at ca. 2×10^7 cells per ml in 10 mM Tris-hydrochloride (pH 7.4)–10 mM NaCl–1.5 mM $MgCl_2$ and swollen on ice for 10 min. Cells were lysed by the sequential addition of Nonidet P-40 and sodium deoxycholate to 0.5% each, vortexed, and sedimented first at 2,000 × *g* for 2 min and then at 11,000 × *g* for 10 min. Postmitochondrial supernatants were mixed with 0.25 volumes of 5× KMH (1× KMH is 500 mM KCl, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.4], 5 mM magnesium acetate), and 0.4-ml samples were layered on 12 ml of linear 0.5 to 1.5 M sucrose gradients prepared in KMH. Sedimentation was for 90 min at 40,000 rpm in an SW41 rotor at 4°C. Gradients were collected through a flow cell recording absorbance at 260 nm. Fractions of 0.5 and 1 ml (see Fig. 7 and 6, respectively) were diluted with an equal volume of 2% Sarkosyl containing 20 μg of calf liver tRNA per ml. After sequential phenol-chloroform and chloroform extractions, RNA was alcohol precipitated twice and dried. After being dissolved in suitable volumes of water, equal portions were translated in the reticulocyte lysate at subsaturating mRNA concentrations, and their products were compared by gel electrophoresis. The absorbance profiles were used to estimate relative proportions of ribosomes in polysomes after subtraction of the trace from a blank gradient.

RESULTS

Protein synthesis requirement for induction of SPs by AzC. Exposure to AzC resulted in increased synthesis of the SPs and decreased synthesis of the normal spectrum of HeLa cell proteins by 4 h (Fig. 1, lanes H and J). We therefore used specific inhibitors of either protein synthesis or RNA synthesis at intervals during a 4-h exposure to AzC to elucidate some features of the mechanism of induction at a molecular level.

Cycloheximide (CX) is a specific and rapidly acting inhibitor of polypeptide chain elongation; those mRNAs active in protein synthesis at the time of addition are essentially frozen on polysomes for as long as an appropriately high CX concentration is maintained. It is effectively reversible by washing cells with medium lacking the inhibitor, so that the proteins labeled during a short subsequent pulse (5 to 10 min) largely represent products of the mRNA population engaged in protein synthesis at the time CX was added.

When protein synthesis was blocked either before or at the same time that AzC was added, no induction of SP synthesis was detected upon reversal after 4 h of exposure (Fig. 1, lanes B to D). When CX inhibition was imposed at progressively later times, increasing amounts of SPs were synthesized upon reversal (Fig. 1, lanes E to G). Indeed, the levels of SP synthesis observed in lane G (4 h of exposure to AzC, with CX inhibition during the last hour) were comparable to if not higher than those detected in lane J, in which protein synthesis had not been inhibited by CX. Induced levels of synthesis of these proteins were observed when protein synthesis was blocked between 1 and 2 h of exposure to AzC (Fig. 1, lanes E and F), but an earlier addition prevented their induction (Fig. 1, lanes B to D). The decreased synthesis of the normal HeLa polypeptides observed in lanes B to E (compared with lane A) might have resulted in part from incomplete removal of CX, although a comparable decrease was observed in cells exposed to AzC but not subjected to CX (compare lanes A and H with lane J).

We conclude that 1 to 2 h of protein synthesis in the presence of the analog is necessary for the enhanced synthe-

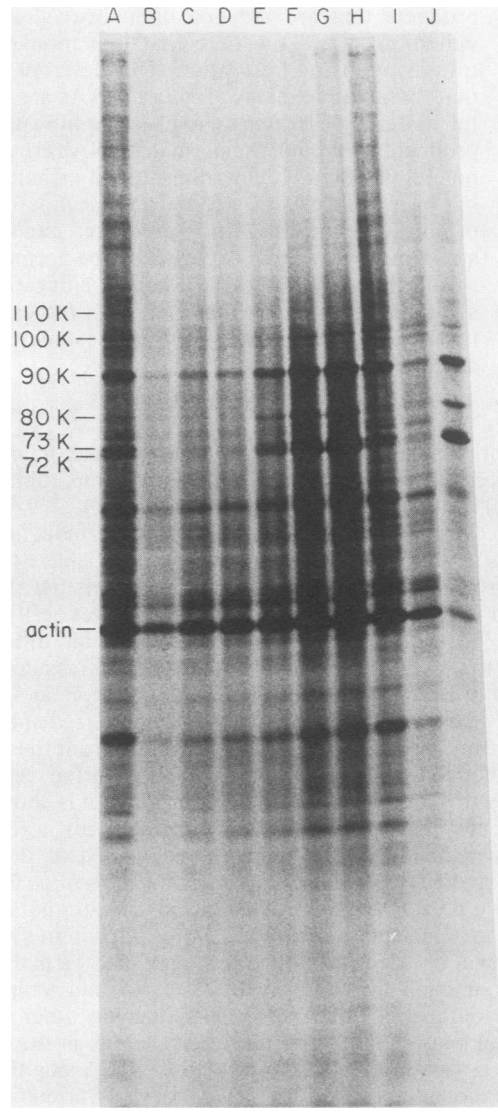

FIG. 1. Induction of SPs by AzC requires protein synthesis. HeLa cells were exposed to 5 mM AzC for 4 h (lane J), and CX was added to a concentration of 200 μg/ml at hourly intervals between 2 h before and 3 h after the addition of AzC (lanes B to G). Control cultures included untreated HeLa cells (lanes A and H) and cells treated with CX alone for 6 h (lane I). At the end of the analog treatment, cultures were rapidly rinsed to remove CX and then labeled for 5 min with 200 μCi of [^{35}S]methionine per ml and solubilized directly in 2× gel sample buffer. Equal portions of the lysates were compared by gel electrophoresis and fluorography. The positions of the HeLa SPs and that of actin are indicated at the left-hand side of the autoradiogram. (Positions designated on subsequent figures are the same as those for Fig. 1.)

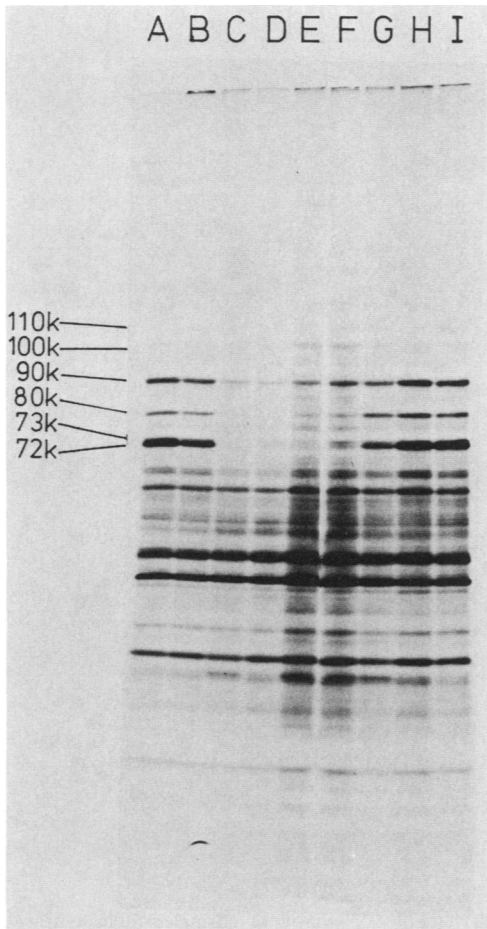

FIG. 2. Protein synthesis is required for increased levels of the SP mRNAs. HeLa monolayers were exposed to 5 mM AzC, with or without 200 μg of CX per ml, for various periods of time as indicated below, and cytoplasmic RNA was extracted. These RNAs were translated at subsaturating concentrations, and 5×10^5 acid-precipitable cpm from each reaction were analyzed by gel electrophoresis. Treatments were as follows: lane A, 4 h of AzC; lane B, 3 h of AzC; lane C, 2 h of AzC; lane D, 1 h of AzC; lane E, no treatment; lane F, 4 h of AzC and 4 h of CX; lane G, 4 h of AzC and 3 h of CX; lane H, 4 h of AzC and 2 h of CX; and lane I, 4 h of AzC and 1 h of CX.

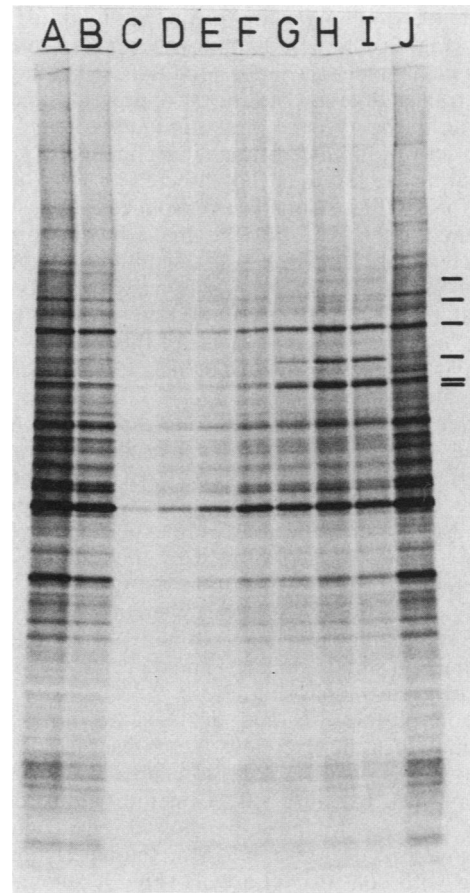

FIG. 3. Two effects of RNA synthesis inhibition during exposure to AzC: induction is prevented and protein synthesis on preexisting mRNA is inhibited. HeLa cells were treated with 5 mM AzC and 5 μg of AMD per ml singly or in combination for various lengths of time. After a 10-min pulse with [^{35}S]methionine (200 μCi/ml), equal portions were run in a polyacrylamide gel and detected by fluorography. Samples were as follows: lanes A and J, normal untreated cells; lane B, 6 h of AMD; lane C, 6 h of AMD and 4 h of AzC; lane D, 5 h of AMD and 4 h of AzC; lane E, 4 h of AMD and 4 h of AzC; lane F, 4 h of AzC and 3 h of AMD; lane G, 4 h of AzC and 2 h of AMD; lane H, 4 h of AzC and 1 h of AMD; and lane I, 4 h of AzC.

sis of the SPs. This experiment can assay only the mRNA actually engaged in protein synthesis rather than the total mRNA content of the cells. However, it could be imagined that mRNA levels for these induced proteins had accumulated in the cytoplasm to the same elevated levels in all cases, but were not detected by pulse-labeling since they could not enter polysomes under conditions of protein synthesis inhibition. Cytoplasmic RNA was therefore prepared from cells subjected to AzC treatment and various periods of CX inhibition and was translated in the reticulocyte lysate at approximately equal, subsaturating concentrations so that the relative level of a particular cell-free product should reflect the level of its mRNA. Figure 2 illustrates the cell-free translation products of cytoplasmic RNA as a measure of mRNAs encoding both normal proteins and the induced proteins over a 4-h time course in the absence of CX and after 4 h of exposure to AzC with increasingly longer periods of protein synthesis inhibition.

In the absence of CX, increased levels of translatable cytoplasmic mRNA encoding the SPs became detectable between 2 and 3 h of exposure to AzC and continued to increase up to 4 h, the latest point in this experiment (Fig. 2, lanes E to A). On the other hand, inhibition of protein synthesis at progressively later times during a 4-h exposure to AzC resulted in increasing levels of the SP mRNAs (Fig. 2, lanes F to I). When CX was added at the same time as AzC (Fig. 2, lane F), accumulation of the SP mRNAs was not apparent. A 1-h delay (Fig. 2, lane G) allowed substantial accumulation of SP mRNAs by a total of 4 h of exposure to AzC, and by 2 h of exposure, the induction of SP mRNAs appeared to be fully established and insensitive to inhibition of protein synthesis (compare lanes H and I). Thus, it appears that protein synthesis during the first 1 to 2 h of exposure to AzC is required for activation of the stress response and that the SP mRNAs accumulate in the cytoplasm of stressed cells between 2 and 3 h after the addition of AzC (compare lanes B and C). In comparing the levels of cell-free products directed by RNA extracted after a 2-h exposure to AzC (Fig. 2, lane C) with those directed by RNA

extracted after a 4-h exposure to AzC but inhibited with CX after 2 h (Fig. 2, lane H), significantly greater levels of the SPs were made under the latter condition, indicating that the levels of translatable mRNA for these proteins continued to accumulate in the absence of continuous protein synthesis. Although less pronounced, a similar conclusion is reached by comparing the levels of SPs directed by RNA from cells exposed for 3 h (Fig. 2, lane B) and from cells exposed for 4 h but inhibited with CX during the final 1 h (Fig. 2, lane I).

By combining the results of CX inhibition obtained from pulse-labeling and cell-free translation experiments, we conclude that 1 to 2 h of incorporation of AzC into protein is sufficient to induce elevated levels of the mRNAs encoding the SPs and that there is little or no delay in their appearance on polysomes.

Inhibition of RNA synthesis during exposure to AzC: prevention of induction of SPs. Comparable experiments in which RNA synthesis, rather than protein synthesis, was inhibited with AMD uncovered two additional features of the reponse. As before, HeLa cells were exposed to AzC for 4 h, and RNA synthesis was inhibited by the addition of AMD either before or after the addition of AzC. The level and nature of polypeptides synthesized were assayed by labeling with [^{35}S]methionine during a 10-min pulse, followed by sodium dodecyl sulfate-polyacrylamide gel analysis of equal portions of each sample (Fig. 3).

Induction of the SPs and decreased synthesis of the background HeLa polypeptides were observed after 4 h of exposure (Fig. 3, lane I). Six h of exposure to AMD in the absence of AzC had little effect on the pattern of protein synthesis detected by pulse-labeling (Fig. 3, lane B) but resulted in a 30% decrease in [^{35}S]methionine incorporation compared with control HeLa cells (Fig. 3, lanes A and J). When AMD was added 2 h before AzC, pulse-labeling after a total of 4 h of exposure to AzC revealed drastic inhibition of the synthesis of both SPs and the normal HeLa cell polypeptide complement (overall rate of protein synthesis, ~20% of control) (compare lanes B and C). This indicated that (i) as expected (7, 9), the increased synthesis of SPs in the presence of AzC required active transcription, and (ii) more surprisingly, in the presence of AMD, AzC interfered with the synthesis of normal cell proteins to a greater extent than it did in cells with otherwise unimpaired transcription (Fig. 1 and 3).

The addition of AMD at progressively later times (from 2 h before to 3 h after AzC [Fig. 3, lanes C to H]) reduced the severity of the inhibition. When AMD was added 2 or 3 h after AzC (Fig. 3, lanes G and H), the level of SP synthesis approached the levels seen after 4 h of exposure to AzC in the absence of RNA synthesis inhibition. By this analysis, the level of translatable SP mRNAs would increase some time between 1 and 2 h of exposure, coinciding with the timing deduced from the protein synthesis inhibition experiments shown in Fig. 1 and 2. Together, the data would suggest that the increased levels of SPs result from de novo RNA synthesis rather than from the conversion of preexisting mRNA from an untranslatable form to a translatable form.

In the presence of AzC for 4 h, the rate of synthesis of the normal spectrum of HeLa cell polypeptides increased with progressively later additions of AMD but did not reach the levels observed after 6 h of AMD inhibition in the absence of AzC (compare lanes I and B of Fig. 3). This inhibition of normal cellular protein synthesis was particularly evident when AMD was given either before or together with AzC (Fig. 3, lanes C to E). To time this shutoff relative to the addition of the analog, AzC was added together with AMD for increasing periods, and the levels and nature of polypeptides synthesized during a 10-min pulse with [^{35}S]methionine were measured as above. This experiment assayed the effect of increasing exposure to AzC upon the activity of preexisting mRNA within treated cells (Fig. 4). A time-dependent decrease in the level of functioning preexisting mRNA was observed, which in the absence of new mRNA synthesis was manifest as the shutoff of protein synthesis. The shutoff first became apparent 1 h after AzC plus AMD, and residual protein synthesis had an estimated half-life of 90 min. Further experiments (data not shown) established that AzC has to be incorporated into protein for this effect on the translation of preexisting mRNAs; its mere presence is insufficient.

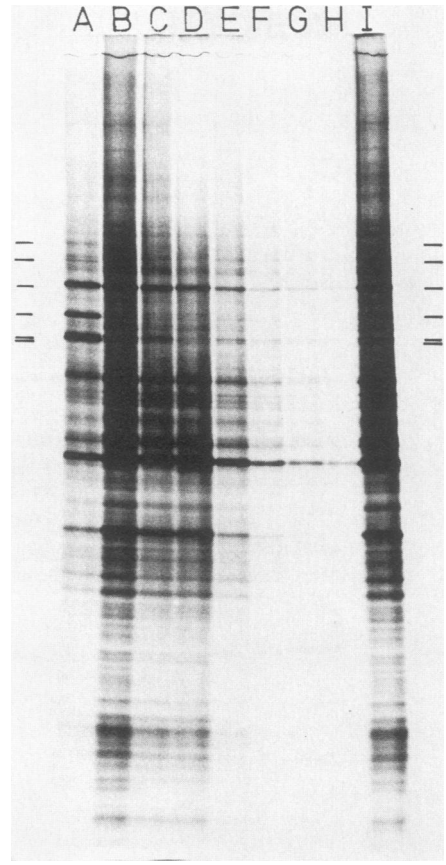

FIG. 4. Kinetics of shutoff of normal protein synthesis. HeLa monolayers were treated simultaneously with 5 mM AzC and 5 μg of AMD per ml for 1 to 6 h, and protein synthesis patterns were visualized by pulse-labeling, followed by gel electrophoresis. Lane A, 6 h of AzC; lanes B and I, no treatment; and lanes C to H, AzC and AMD together for 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h, respectively.

Because of the possibility that these observations resulted from an unexpected side effect of AMD itself (12) rather than from simple inhibition of RNA synthesis, we repeated the experiments above but substituted cordycepin for AMD. The results obtained with cordycepin were identical to those obtained with AMD (data not shown). Therefore, we conclude that incorporation of AzC into protein resulted in decreased synthesis of the normal complement of cell proteins, even in the absence of increased synthesis of the SPs

themselves (when synthesis of their mRNAs was blocked). This must be a direct effect on protein synthesis because the mRNA remains translatable in a cell-free system (see next section, Fig. 5).

Translational shutoff that does not result from degradation of preexisting mRNA. One possible explanation for the shutoff of synthesis of the normal complement of proteins would be that its mRNAs were being degraded in cells treated with AzC and AMD. To test this possibility by direct means, we repeated the experiments shown in Fig. 3 and 4, but rather than pulse-labeling polypeptides, total cytoplasmic RNA was prepared, and the integrity of preexisting mRNA was assessed by cell-free translation (Fig. 5).

Clearly, there was little or no decrease in the translational activity of preexisting mRNA in cells treated for 4 h with AzC alone (Fig. 5, lane 3I). In contrast to the results obtained in vivo, in which there was time-dependent obliteration of the activity of preexisting mRNA in cells treated with AzC and AMD together (Fig. 4, lanes C to H), there was no decrease in the translatability of the normal mRNA complement as assayed in vitro (Fig. 5, lanes 4C to 4H). Likewise, the decreased synthesis of normal cell proteins observed in cells treated with AzC together with progressively earlier additions of AMD (Fig. 3, lanes H to C) was not reflected in the diminished activity of their mRNAs in this cell-free system (Fig. 5, lanes 3H to 3C). On the other hand, in all cases in which increased synthesis of SPs was observed in vivo, increased levels of their mRNAs were detected by cell-free translation.

Thus, analysis of the translatability of RNA from cells in which synthesis of normal proteins had been inhibited by AzC alone or by AzC plus AMD showed that the mRNA remained fully functional when assayed in a heterologous system. This establishes that the selective shutoff of protein synthesis is exerted at the level of mRNA translation, rather than by affecting its stability or intrinsic translatability.

Translational control during AzC treatment. (i) Early in exposure. The induction of SP synthesis and the decline in synthesis of the normal HeLa polypeptide complement both commenced after ca. 2 h of exposure. The reduction in normal RNA synthesis also followed similar kinetics (see Fig. 8). Since the normal mRNAs remained translatable in vitro (Fig. 5), we sought to determine whether altered polysomal locations of normal mRNAs could account for the reduced synthesis of normal proteins and for the almost complete inhibition of protein synthesis seen in cells treated simultaneously with AzC and AMD (Fig. 3 and 4). Postmitochondrial supernatants were prepared by a high-salt, double-detergent procedure and sedimented in sucrose gradients as described above. The polysome absorbance profile was recorded, and RNA was extracted from individual fractions for assay by cell-free translation in reticulocyte lysate (Fig. 6).

The polysome profile generated from control HeLa cells is shown in Fig. 6A: 57% (average of two experiments) of the total absorbance in subunits and polysomes sedimented as a peak of polysomes centered about fraction 8 (corresponding to 10 to 12 ribosomes per message), with 80S ribosomes constituting a small fraction of the total absorbance. Since the lysates and gradients were prepared in high salt, any "runoff" ribosomes were dissociated into subunits, and their relative prominence probably stemmed from this and the fact that the cells used in these experiments were routinely near confluence and maintained in 2% serum.

After a 4-h exposure to AzC (Fig. 6C), there were reductions in both the average size (now peaking around fraction 6, five to six ribosomes per message) and the quantity of polysomes. The decrease in polysomal ribosomes (reduced to 43% of total ribosomes) was accompanied by an increase in the number of free subunits and 80S couples. When treatment was with both AzC and AMD (Fig. 6D), there was a much greater loss of polysomes, now reduced to 28% of total ribosomes, and these sedimented mainly as disomes and trisomes. AMD treatment of control HeLa cells resulted in a decreased number of polysomes (42% of ribosomes), although their sedimentation was not noticeably affected. For each of the four cases, the sedimentation of recoverable mRNAs reflected the distribution of polysomes. As expected, the decreased proportion of polysomes was related to the

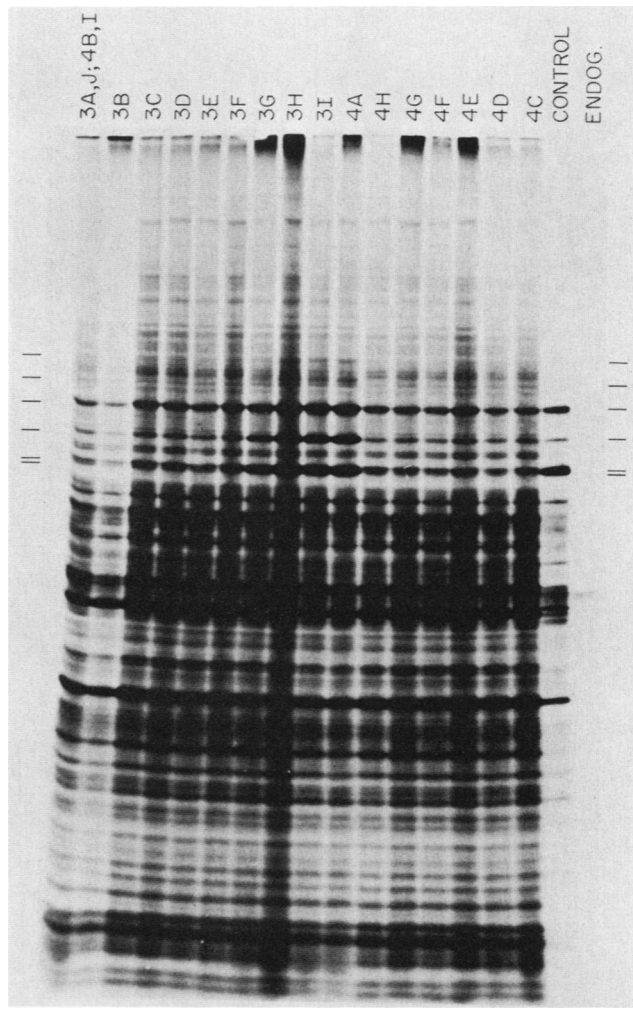

FIG. 5. Degradation of preexisting mRNA does not account for protein synthesis shutoff. The experiments depicted in Fig. 3 and 4 were repeated exactly as described in the legends except that cytoplasmic RNA was extracted and translated in the reticulocyte lysate. The small variations in the incorporation of radioactivity resulted from variations in the yields of cytoplasmic RNA, which ranged from 100 to 163 μg per 10-cm dish (~10^7 cells). Equal portions of the cell-free products were analyzed in a polyacrylamide gel of which a fluorographic exposure is shown. The lanes have been numbered according to the corresponding lanes of Fig. 3 and 4: for example, lane 3H represents the products directed by RNA from cells that had been treated in the same way as cells whose in vivo patterns are shown in Fig. 3, lane H. Control and endog., cell-free products directed by a control preparation of RNA and in the absence of added RNA, respectively.

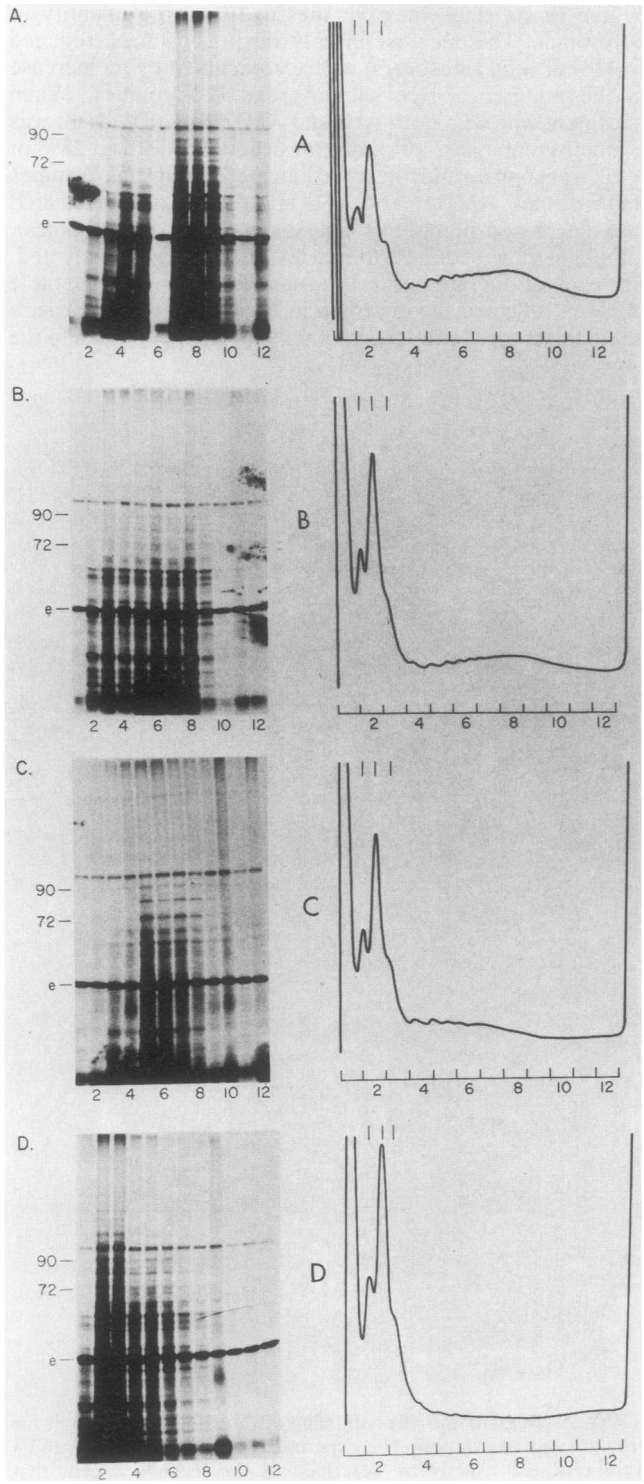

FIG. 6. Polysomal location of mRNAs and the effects of AzC and AMD. Post-mitochondrial supernatants from cultures of HeLa cells exposed for 4 h to 5 mM AzC in the presence or absence of 5 μg of AMD per ml were sedimented in sucrose gradients as described in the text. HeLa cells not exposed to AzC were treated in the same way. The gradients were collected through a flow cell recording absorbance at 260 nm, and RNA was extracted from the fractions. mRNAs were assessed by cell-free translation at subsaturating concentrations in reticulocyte lysate, and the products were displayed by gel electrophoresis. Cell-free products are shown in the left panels, and the optical density profiles of the corresponding gradient are on the right. Sedimentation was from left to right, and the three pointers in the optical density profiles indicate the positions of 40S, 60S, and 80S ribosomes, respectively. (A) Control HeLa; (B) HeLa plus AMD; (C) AzC-treated HeLa; and (D) HeLa cells treated with AzC and AMD. "e" denotes the major endogenous reticulocyte product.

decreased rates of protein synthesis observed by pulse-labeling (Fig. 3 and 4).

A decrease in the number and average size of polysomes with a concomitant increase in the free ribosome population, as observed with AzC treatment, is consistent with a defect at the level of initiation of polypeptide chains. An initiation defect has been indicated in heat shock treatments of a number of cell types (16, 24, 30).

(ii) After prolonged exposure. The preferential synthesis of SPs seen early after the addition of AzC becomes progressively more pronounced as exposure is prolonged (33). Indeed, after 16 h, the SPs are nearly exclusive polypeptide products of cells, and their putative mRNAs are nearly exclusive RNA products (see Fig. 8). As shown in the earlier figures, the mRNAs for the normal complement of polypeptides remain intact (translatable) yet are not used or are used very inefficiently in treated cells. To determine where these normal RNAs were located, polysomes were made from HeLa cells that had been exposed to AzC for 16 h. In the normal cell lysate (Fig. 7C), the bulk mRNA was contained in the main body of the polysome peak (fractions 11 to 18), with enrichment in the larger polysomes for mRNAs encoding larger polypeptides, as expected if ribosome packing density and elongation on different mRNAs were constant. In AzC-treated cells (Fig. 7A), the situation was somewhat different, with some mRNAs sedimenting throughout the gradient on polysomes of all sizes. AzC-treated cells contained polysomes as large as control HeLa cells, but the distribution of absorbance was skewed towards the smaller polysomes. Particularly evident, because of their high abundance, were the mRNAs encoding the 72,000- to 73,000-, 80,000-, and 90,000-molecular-weight SPs. The largest polysomes, in the lower third of the gradient, were relatively enriched in these SP mRNAs, although extended exposure reveals the presence of many of the normal mRNAs. The mRNAs coding for most of the normal polypeptides were found predominantly in the middle third of the gradient, corresponding to polysomes bearing from two to eight or nine ribosomes per message. The proportion of ribosomes in polysomes in AzC-treated cells was 44% compared with 65% for control cells in this experiment. (The mixed lysate [Fig. 7B] was included as an internal control for possible variations in the gradients.)

RNA synthesis in AzC-treated HeLa cells. When exposure to AzC was extended past 8 h, there was a vast decrease in the synthesis of the normal complement of proteins, although their mRNA levels, as measured by cell-free translation, did not decline (Fig. 5) (33). The failure to synthesize the normal proteins must result from discrimination against these mRNAs since they retain activity when removed from treated cells. However, from these data there is no reason to believe that production of the normal cell mRNAs would necessarily be affected by induction of the SPs. To examine RNA synthesis during the stress response, cells were pulse-labeled with [^{3}H]uridine at intervals, and cytoplasmic RNAs were extracted. RNAs were fractionated into poly(A)$^+$ and non-poly(A)$^+$ populations and separated by agarose gel electrophoresis after glyoxal treatment (Fig. 8). The electrophoretic patterns of unfractionated cytoplasmic RNA simpli-

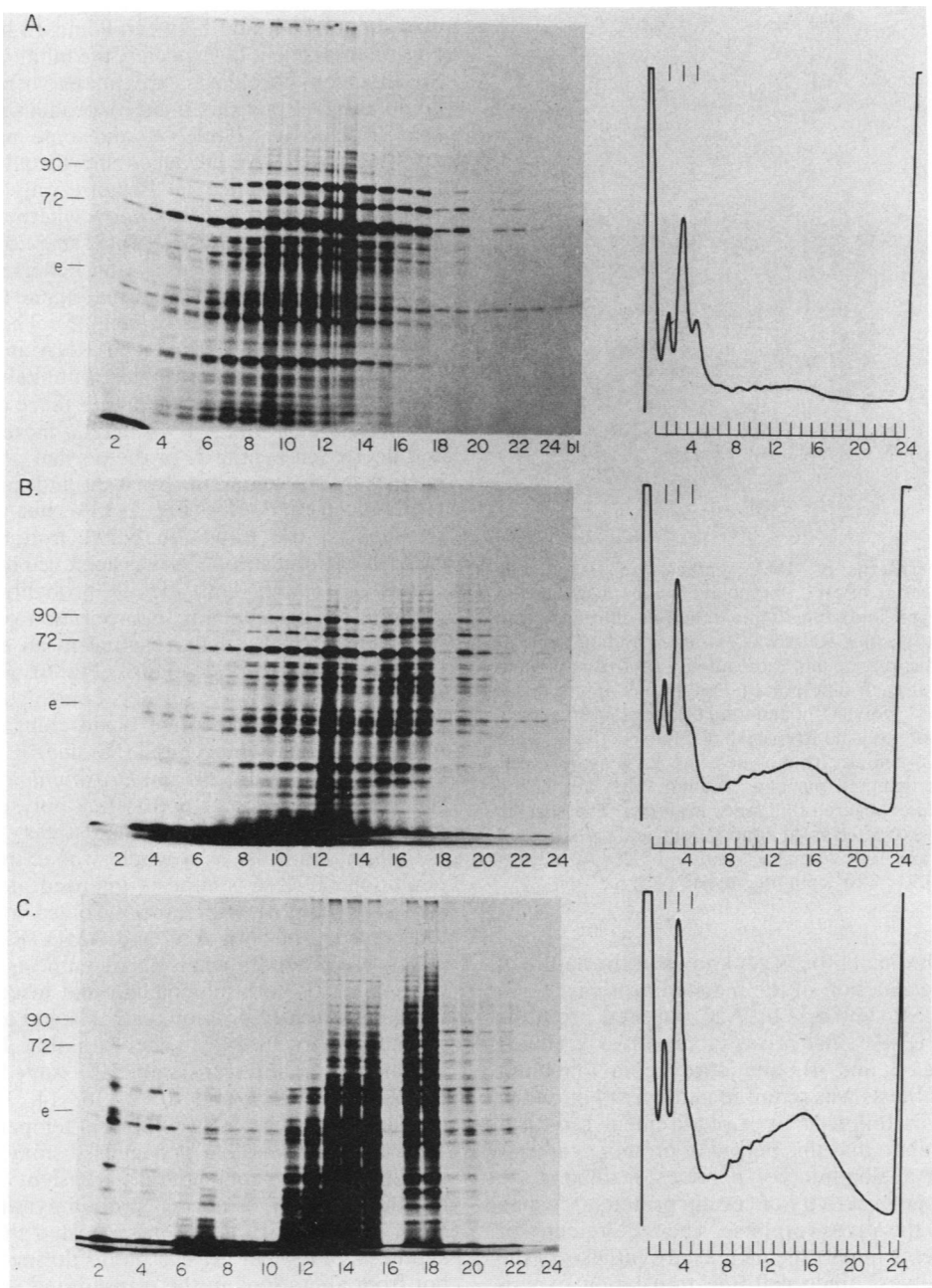

FIG. 7. Polysomal distributions of mRNAs after prolonged exposure to AzC. HeLa cells exposed for 16 h to 5 mM AzC and a control culture were lysed, and post-mitochondrial supernatants were prepared. Analysis of polysomes and mRNA distributions were as described in the legend to Fig. 6. (A) AzC-treated HeLa cells; (B) equal parts of the control and treated cell lysates mixed before analysis; and (C) control HeLa cells; bl, blank. e, Endogenous reticulocyte product.

fied with time, reducing to a situation in which the only species labeled during the pulse were a small number of poly(A)$^+$ RNAs. Thus, the overall decrease in RNA synthesis resulted from the production of an increasingly restricted population of RNA species.

rRNA production declined noticeably after as little as 2 h of exposure, and inhibition was complete between 4 and 8 h. Residual production of 18S rRNA was more protracted than that of 28S rRNA, as previously observed in cases in which protein synthesis has been directly inhibited by the use of drugs (22, 35, 36). A certain amount of low-molecular-weight RNA was also produced in long-term treated cells (not seen in these gels), but this has not been investigated in detail.

DISCUSSION

We have examined certain aspects of the induction of SPs in HeLa cells upon exposure to the proline analog, AzC. Alterations in both RNA and protein synthesis were observed and were found to be generally similar to alterations brought about by other inducers of the SPs (1, 23). This apparent conservation of mechanism as well as size and number of SPs further supports the suggestion that a ubiquitous and conserved response is being activated by the

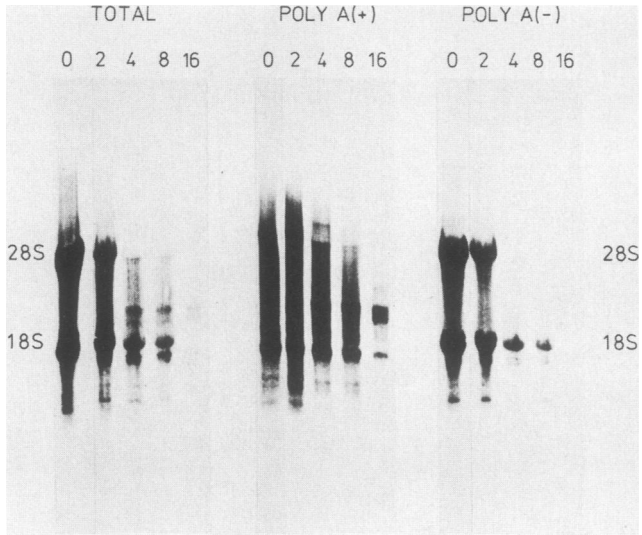

FIG. 8. Changing patterns of RNA synthesis in HeLa cells treated with AzC. A series of HeLa monolayers was pulse-labeled with [^{3}H]uridine (0.5 mCi/ml) for 30-min intervals during a 16-h exposure to AzC. Cytoplasmic RNA was extracted and quantitated with respect to UV absorbance and radioactivity; essentially equal yields were obtained at each time point (~140 μg per 10-cm dish). Poly(A)$^+$, non-poly(A)$^+$ [poly(A)$^-$], and total fractions, corresponding to 15, 7.5, and 5% of the yield from each dish, respectively, were denatured with glyoxal and electrophoresed in a 1.2% agarose gel. ^{3}H-labeled RNA was detected by using sodium salicylate as the scintillant (4); an equal exposure of all lanes is shown. The specific activities of the RNA extracted from control cells and cells treated with AzC for 2, 4, 8, and 16 h were 3.8×10^4 and 3.2×10^4, 2.7×10^4, 1.93×10^4, and 0.91×10^4 cpm/μg, respectively.

various inducers, although little is yet known of the nature of the targets or of the function of the induced proteins.

The induction of SP synthesis by AzC required synthesis of both protein and RNA, since it was blocked by CX (Fig. 1 and 2), AMD (Fig. 3 and 4), and cordycepin (data not shown). Protein synthesis was required in the first hour or so of exposure and was followed by a requirement for RNA synthesis. We speculate that this period of protein synthesis permits entry of the analog into polypeptides, leading to the inactivation or change in activity of certain protein(s), which ultimately activates the stress response. The requirement for RNA synthesis reflects in part the increased synthesis of the SP mRNAs themselves, since cell-free translation experiments (Fig. 2 and 5) demonstrated that increased synthesis of the SPs paralleled the appearance of higher cytoplasmic levels of their mRNAs. These mRNAs are probably the same species detected by direct labeling of cells after exposure to AzC (Fig. 8). There is further reason to suspect that these are the SP mRNAs, since messenger activity for the SPs comigrates with these labeled species (data not shown). The possibility that additional RNA species are synthesized remains open.

Concomitant with SP induction was decreased synthesis of the normal complement of HeLa cell polypeptides, and this also required incorporation of AzC into protein (Fig. 1) (unpublished data). In this and other stress situations in which shutoff of normal protein synthesis is marked (1, 23), it is apparently the consequence of translational as well as transcriptional controls.

After 2 h of exposure to AzC, the complexity and overall amount of newly synthesized cytoplasmic RNA was noticeably diminished, and between 8 and 16 h, RNA production was restricted to a few species, the putative SP mRNAs (Fig. 8). In chick fibroblasts, treatment with arsenite leads to comparable alterations in RNA production (8), and in *Drosophila* cells, the SP mRNAs and some mitochondrial RNAs are the only RNAs produced shortly after heat shock (28; reviewed in reference 1). Because only cytoplasmic RNA was studied here, we do not know whether control is exerted at the level of synthesis, processing, or transport to the cytoplasm. However, Findly and Pedersen have shown that transcription of a sample "normal" gene (actin) is depressed in heat-shocked *Drosophila* cells (5). The rapid turnover of newly synthesized normal cell RNA in the cytoplasm is another possibility but is rendered unlikely by the stability of normal cell RNA as a whole under these conditions (Fig. 5).

In HeLa cells treated with AzC, increased SP synthesis and decreased synthesis of the normal polypeptide complement became apparent by 4 h and progressively more pronounced thereafter (Fig. 1) (33), but with heat shock of *Drosophila*, the transition between normal patterns and exclusive synthesis of SPs was achieved rapidly (within 10 to 15 min [1, 13, 21]). This "delay" probably reflects at least in part the requirement for incorporation of the analog. AzC treatment of HeLa cells resulted in an early defect in the initiation of polypeptide chains (Fig. 6), which at later times was accompanied by further controls which apparently permit efficient polypeptide chain elongation on particular mRNAs but not others (Fig. 7). Similarly, with heat shock of both mammalian (16, 24) and *Drosophila* cells (18), there is a rapid disappearance of preexisting polysomes, followed by gradual recovery upon continued stress.

Protein synthesis as a whole was extremely sensitive to inhibition of RNA synthesis imposed at the same time as AzC (Fig. 3 and 4), with few polysomes remaining after 4 h in the presence of both AzC and AMD (Fig. 6). Comparable effects are observed when RNA synthesis is inhibited during heat shock of both mammalian and insect cells; there is a rapid cessation of protein synthesis and a failure to recover polysomes (16, 18, 24). In each situation, however, preexisting mRNA remains translatable as assayed by either cell-free translation (Fig. 5 and 6) (2, 10, 14, 19, 21, 27, 30) or reutilization upon return to normal temperature (14, 17, 30). Yeast and *Escherichia coli* cells display stress responses, which with heat shock are only transient (11, 14, 15, 20, 36), but the recovery of normal protein synthesis requires new RNA synthesis. It has been suggested that the decrease in synthesis of normal yeast proteins during heat shock results not from alterations in the translational apparatus but from the decay of mRNA according to its (normally) short half-life (14).

AMD inhibition of analog-treated mammalian cells, or of yeast, insect, or mammalian cells subjected to heat shock, shows that protein synthesis under stressed conditions is contingent on the synthesis of new RNA. That this is an artifact of AMD seems unlikely, because that same result is obtained with an inhibitor of unrelated mechanism (cordycepin) and with a conditional mutant defective in RNA production (20). Bearing in mind that the preexisting normal RNA remains in translatable form, there are three plausible explanations for this requirement; under these conditions, it could be that (i) only newly synthesized mRNAs can be translated, (ii) synthesis of a noncoding RNA is required for protein synthesis per se (as originally proposed by McCormick and Penman [16]), or (iii) synthesis of a coding RNA specifying some protein other than SPs is required for protein synthesis. None of these hypotheses can be rigorously proven or

excluded at the moment; neither are they necessarily mutually exclusive.

Assuming that the progressive decline in normal protein synthesis with AzC alone (33) is the same phenomenon that is revealed when RNA synthesis is blocked at the same time stress is imposed, our preference would lie with the first of the three possibilities. In support of this, Scott et al. (25) have shown that in contrast to cellular mRNAs, translation of HPS-1 virus mRNAs in heat-shocked *Drosophila* cells is refractory to AMD. Because the virion transcriptase is also AMD insensitive, the virus would be the major (or only) source of new mRNA. Whether the virus mRNAs escape the translational blocks by virtue of some feature of structure or sequence or because they are newly made has yet to be determined. Nevertheless, protein synthesis upon such mRNAs takes place in the absence of the RNA species required by the other explanations (unless supplied by the virus itself).

After long-term AzC exposure, the vast bulk of normal mRNA was found on polysomes roughly half the size of those found in normal cells (Fig. 7). If this were due solely to a defect in initiation (Fig. 6), one would predict protein synthesis at ca. 50% the rate in control cells, assuming that elongation on these mRNAs continued at the normal rate. However, pulse-labeling of cells after 16 h of exposure revealed a greater loss of normal protein synthesis (33), implying selective control of synthesis of normal proteins, presumably at the level of elongation. The persistence of normal mRNAs in polysomes of heat-shocked *Drosophila* cells has led Ballinger and Pardue to propose differential elongation on select classes of mRNAs as a translational control mechanism (perhaps) unique to the stress response (2). If elongation control were the only factor, one would expect an increase in the size and amount of polysomes (29). Since polysomes decrease in size and number (by ca. 40 to 50%) (Fig. 7), we presume that in addition to this elongation defect, the early initiation defect persists late in the response. The possibility that the block to elongation on certain mRNAs is complete seems remote because early in the response, preexisting polysomes are seen to disaggregate and then be rebuilt (16, 18, 24).

When polysomes from AzC-treated HeLa cells were run off in vitro, SPs were the only recognizable products (32), suggesting that polysomes bearing the normal mRNAs were incapable of efficient elongation and that the modification whereby this is effected was stable during preparation. In addition, cell-free systems derived from normal and heat-shocked *Drosophila* cells mimic the in vivo situation and will discriminate for SP mRNAs in exogenous messages (10, 30). This selectivity appears to be a property of ribosomes that is stable to high-salt treatment (26), implying modification of an integral component of ribosomes.

In summary, we suggest that in HeLa cells exposed to AzC, the changing patterns of protein synthesis and its dependence on RNA synthesis would be explained if translation were restricted to utilization of newly made RNA and that the feature through which the RNAs were recognized as being new were lost with time. Although not supported by direct quantitation, the concurrence between patterns of RNA synthesis and protein synthesis, together with inferences from the HPS-1 virus studies, represent (albeit) circumstantial support for this notion. The sequestration of ribosomes into "blocked" polysomes, bearing the older populations of mRNA, need not present a problem, since the bulk of this mRNA is found on smaller polysomes, thereby providing a pool of ribosomes potentially available for translation of the decreasing amounts of new mRNA in the face of a limited supply of ribosomes. Without major adaptation, such a hypothesis could also account for many other stress responses that have been described. It should, however, be emphasized that although disparate treatments all lead to superficially similar responses, it may transpire that the responses to different stimuli may not be directly comparable.

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