INVOLVEMENT OF POLY-A IN SELECTIVE GENE EXPRESSION: SUPPRESSION OF ENZYME INDUCTION IN NEURAL RETINA BY INHIBITORS OF POLY-A SYNTHESIS

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SUMMARY

The hormonal induction of glutamine synthetase (GS) in embryonic neural retina involves selective accumulation of stable and active RNA templates for GS synthesis. Cordycepin, (3'-deoxyadenosine) suppresses this induction in close correlation with its inhibition of poly-adenylate (poly-A) synthesis. Similarly ethidium bromide, which also reduces poly-A synthesis, suppresses the induction of GS. Both agents inhibit GS induction by acting at a pre-translational level. The overall results suggest that formation of poly-A is required for the induction of GS; they provide the first indication of a relationship between poly-A synthesis and a specific, inducible, gene-controlled aspect of cell differentiation.

A rapid and many-fold increase in levels of glutamine synthetase (GS)* is characteristic of functional differentiation in the embryonic chick neural retina (1). This enzyme increase can be induced precociously by hydrocortisone (HC) in cultured retinas isolated from early chick embryos, as previously described (2). The induction of GS represents a marked increase in the rate of de novo synthesis and accumulation of enzyme (3); it requires RNA synthesis, with stable templates for GS accumulating during the first few hours of exposure to the steroid inducer (2, 3). In connection with work on mechanisms controlling the provision, stability and expression of the RNA required for GS induction, we have examined whether synthesis of poly-adenylate (poly-A) is involved in this process.

Poly-A sequences, 100-200 nucleotides long, have been detected in heterogeneous nuclear RNA and cytoplasmic messenger RNA of various higher organisms (4-9) and viruses (9, 10-13). While their precise function has not yet been established, it has been suggested that poly-A may be involved in the processing or transport of mRNA from nucleus to cytoplasm (14), or in the stability and activity of templates (6, 11).

As a first step in investigating poly-A in retinal GS induction, we have examined the effects of two reported inhibitors of poly-A synthesis, cordycepin and ethidium bromide (12, 14) on this induction, and on RNA and poly-A synthesis in retina treated with the steroid inducer. Both drugs were found to cause a dose-dependent suppression of GS induction of a relatively greater magnitude than that of RNA synthesis. While in the case of cordycepin, the suppression of GS induction was clearly correlated with a marked inhibition of poly-A synthesis,

*Abbreviations: GS, glutamine synthetase; HC, hydrocortisone; poly-A, polyadenylate.

ethidium bromide reduced poly-A synthesis only at doses higher than those sufficient for partial suppression of GS induction. Thus, ethidium bromide may exert its effect by a more selective interference, than that of cordycepin, with the provision of RNA or poly-A species specific for this induction.

MATERIALS AND METHODS

Neural retina tissue freshly isolated aseptically from 12-day chick embryos was maintained as previously described (3) in flask cultures in culture medium consisting of Tyrode's physiological salt solution with 10% fetal bovine serum and penicillin-streptomycin mixture. GS was induced by adding hydrocortisone (1 µg/ml medium) to freshly prepared cultures (3). Cordycepin (Sigma) or ethidium bromide (Boots Pure Drug Co.) were added 30 minutes before the addition of the steroid inducer. To label RNA, the cultures were pulsed with 17 µCi/ml 2,8-^{3}H-adenosine, or with 10 µCi/ml 5-^{3}H-uridine (New England Nuclear) for 30 minutes, immediately before harvesting at 3 hours. Parallel cultures were harvested at 6 hours for assay of GS specific activity.

For the preparation of RNA and poly-A, radioactively labeled tissue was washed with Tyrode's solution and hypotonic buffer (10mM Tris, pH 7.4, 10mM KCl, 1.5mM $MgCl_2$) and the cells broken in the buffer with a Dounce homogenizer. The 1000xG pellet from the homogenate was treated according to Penman (15) and used as a source of nuclear RNA; in some experiments nuclei were further purified by resuspending the pellet in 2.2M sucrose (in 20mM Tris, pH 7.4, 60mM KCl, 4mM $MgCl_2$) and repelleting at 40,000xG for 1 hour. The nuclei were broken with high-salt buffer and treated with DNAse (RNAse-free; Worthington) (15). The 1000xG supernatant was centrifuged at 15,000xG for 10 minutes, and the final supernatant used for extraction of cytoplasmic RNA.

RNA was extracted from both fractions with phenol-chloroform (1:1, v:v) for quantitative recovery of poly-A containing RNA (16). RNA was precipitated from the final aqueous phase overnight at -20°C with 2.0 volumes of ethanol and 0.1 volume of 2M NaCl. The precipitates were washed with ethanol and ether, dried under vaccuum, and dissolved in buffer (0.1M NaCl, 10mM Tris, pH 7.4, 1mM $MgCl_2$). Aliquots of each sample were taken for measurements of absorbance at 260 mµ, and for determination of total radioactivity, following precipitation with trichloroacetic acid (17). To measure radioactivity in poly-A, RNA was digested with T_1 and pancreatic RNAses according to Darnell (14), and the RNAse-resistant fraction (poly-A) was bound to Millipore filters in the presence of high-salt buffer (6).

RESULTS

Table I shows that low concentrations of cordycepin suppressed 41-67% of GS induction and 40-87% of poly-A synthesis. In contrast, synthesis of nuclear RNA was reduced only by 17-32%; the relatively greater reduction of labeled RNA in

TABLE 1

EFFECTS OF LOW DOSES OF CORDYCEPIN ON GS INDUCTION AND ON RNA AND POLY-A SYNTHESIS

			^{3}H-Adenosine dpm X 10^{-3}							
			Nucleus				Cytoplasm			
Additions to medium	GS Sp.A.[1]	% inhibition[2]	RNA	% inhibition	poly-A	% inhibition	RNA	% inhibition	poly-A	% inhibition
HC (control)	1.61	0	168	0	2.25	0	17.7	0	1.71	0
HC+cordycepin (10µg/ml)	1.08	41	140	17	1.35	40	10.2	42	0.78	54
HC+cordycepin (20µg/ml)	0.75	67	115	32	0.30	87	7.2	59	0.49	71
uninduced (no HC)	0.32									

[1]GS specific activity, expressed as µmoles γ-glutamyl hydroxamate formed per mg protein per hour (3).

[2]Inhibition of induction was determined by subtracting the uninduced specific activity (0.32) from the experimental values and taking the specific activity of the control as 100%.

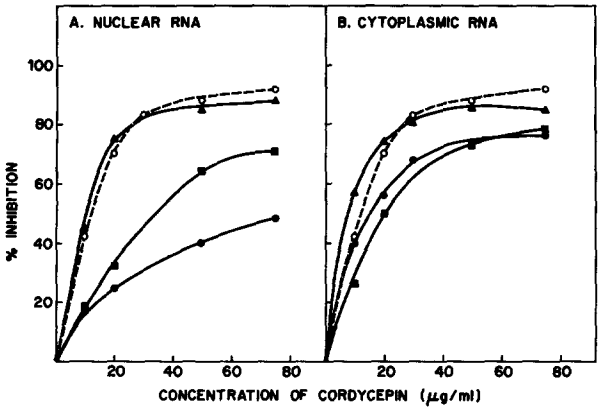

Figure 1. Inhibition by cordycepin of GS induction, and of labeling of RNA and poly-A in nucleus (A) and cytoplasm (B). The dose-response curve for GS induction (% inhibition calculated as in Table 1) is provided in both Figures 1-A and 1-B to facilitate comparison with corresponding inhibitions of RNA and poly-A synthesis. O- - -O, induced GS specific activity; ● ——— ●, ^{3}H-adenosine labeled RNA; ■ ——— ■, ^{3}H-uridine labeled RNA; ▲ ——— ▲, poly-A.

the cytoplasm (40-60%) probably reflects reduced transport of rapidly labeling RNA from nucleus to cytoplasm under conditions of depressed poly-A synthesis (4, 6). Results of a number of experiments using these and higher doses of cordycepin are summarized in Fig.1. At all doses of cordycepin tested there was a good correspondence between the extent of suppression of GS induction and inhibition of poly-A synthesis. In addition, at each concentration of cordycepin, the reduction of ^{3}H-adenosine radioactivity in either nuclear or cytoplasmic RNA was less than that in poly-A; with all doses of cordycepin, labeling of cytoplasmic RNA was affected more than that of nuclear RNA.

While the dose-response profiles for cytoplasmic RNA labeled with either ^{3}H-adenosine or ^{3}H-uridine were very similar, in the case of nuclear RNA incorporation of ^{3}H-uridine was inhibited more than that of ^{3}H-adenosine (Fig.1A); this was particularly evident at higher doses of cordycepin (50-75 µg/ml), which inhibited the incorporation of ^{3}H-adenosine by about 40%, compared to a 60-65% inhibition of ^{3}H-uridine incorporation. A possible reason for this apparent discrepancy may be the differential action of cordycepin on nuclear RNA polymerases: while cordycepin has been reported to prevent synthesis of precursor ribosomal RNA (18) synthesis of heterogeneous nuclear RNA seems unaffected (14, 19). Thus, the differential responses obtained here indicate that the heterogeneous nuclear RNA of the retina may be relatively richer in adenosine than ribosomal RNA precursors, a possibility which is being investigated.

An overall reduction in the cytoplasmic content of mRNA in cordycepin-treated cells should result in a corresponding decrease in total protein synthesis. Fig.2 shows that, as the concentration of cordycepin in the medium was increased, the rate of overall protein synthesis declined. However, even at a cordycepin dose of 50 µg/ml, inhibition of total protein synthesis (56%) was substantially less than that of GS induction (86%), suggesting a preferential sensitivity of the enzyme induction to cordycepin.

To verify that the RNAse-resistant ^{3}H-adenosine labeled material binding to Millipore filters in high salt buffer was actually poly-A, the material was eluted from the filters, and subjected to polyacrylamide gel electrophoresis. Fig.3 shows that the eluted material localized in the region of 7-10S, as does poly-A of other eucaryotes (4-10). It also shows that treatment of the tissue with 20 µg/ml cordycepin resulted in approximately 70% reduction of the radioactivity in the 7-10S region of the gels, in agreement with the percent value for the inhibition of poly-A synthesis obtained by the Millipore filter binding assay (Fig.1).

Fig.4 summarizes the effects of ethidium bromide on GS induction and on RNA and poly-A synthesis. While GS induction was reduced to 40% of control by 0.2 µg/ml ethidium bromide, no effect of this agent on RNA or poly-A synthesis was detected below the concentration of 0.4 µg/ml. Higher concentrations completely prevented GS induction; they also inhibited synthesis of nuclear poly-A to a greater extent than that of total nuclear RNA.

The inhibition of GS induction by ethidium bromide was not due to a general depression of protein synthesis, as shown in Fig.2. Little or no inhibition of overall protein synthesis was seen in retinas treated with doses of 0.1-0.2 µg/ml ethidiu bromide, which reduced GS induction by 20-40%; higher doses (0.4-1.2 µg/ml) which inhibited 60-100% of induction, suppressed overall protein synthesis by only 15-30%.

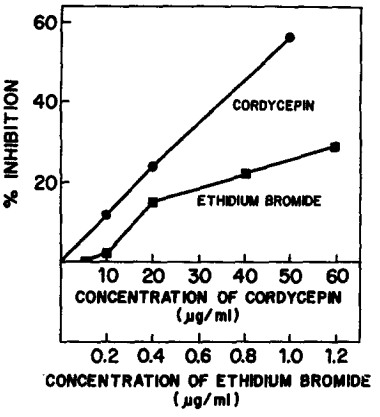

Fig. 2.

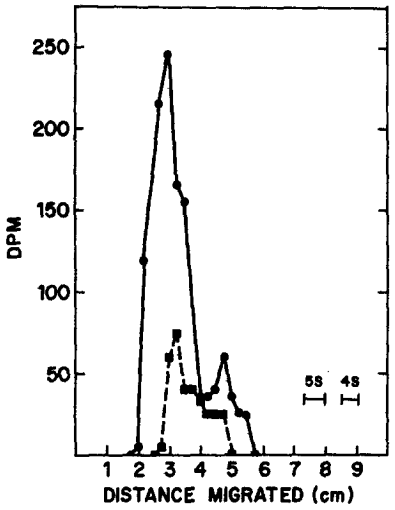

Fig. 3.

Figure 2. Effects of cordycepin and ethidium bromide on overall protein synthesis in induced retina. Retinas were induced with HC for 2 hrs in the presence or absence of the inhibitors, and then pulsed for 30 minutes with 1 µCi/ml ^{14}C-amino acids. Radioactivity incorporated into proteins was measured as previously described (17).

Figure 3. Polyacrylamide gel electrophoresis of nuclear poly-A. ^{3}H-adenosine labeled nuclear RNA samples were digested with DNAse and T_1 and pancreatic RNAses according to Darnell (14), and adsorbed to Millipore filters (6). Poly-A eluted from the filters and reprecipitated using Green's method (13) was electrophoresed on 8% gels (20) for 1.5 hours at 8 mA/gel. Carrier yeast sRNA served as marker. Slicing of gels and determination of radioactivity was according to Dingman (21). ●——●, HC control; ■----■, HC plus 20 µg/ml cordycepin.

We next determined that the suppression of GS induction by cordycepin or ethidium bromide was not due to their direct interference with the translation of GS messages. This was tested by taking advantage of the fact that active and stable templates for GS accumulate in the first hours of induction with HC; after 4 hours further transcription can be completely halted with actinomycin D (Act D), but the preformed stable templates continue to make the enzyme for a considerable time (3). Accordingly, if cordycepin or ethidium bromide impaired GS synthesis at the translational level, their addition together with Act D to cultures previously induced for 4 hours should have prevented further enzyme accumulation. The results of this experiment (Fig.5) showed that this was not the case; there was no marked difference in final enzyme levels between control retinas treated only with Act D, and those with added cordycepin or ethidium bromide.

DISCUSSION

Both cordycepin and ethidium bromide suppress the HC-induced increase in

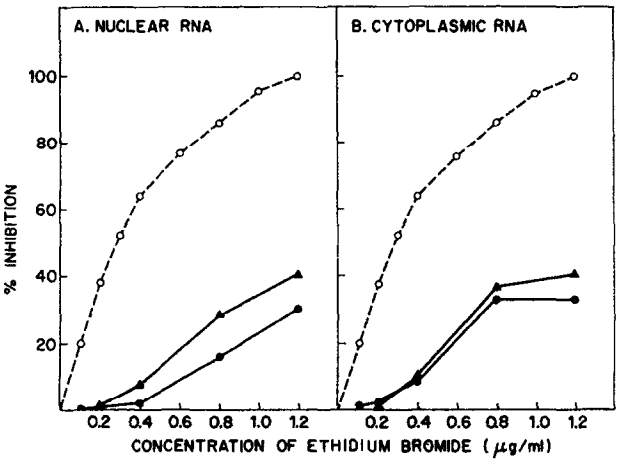

Fig. 4.

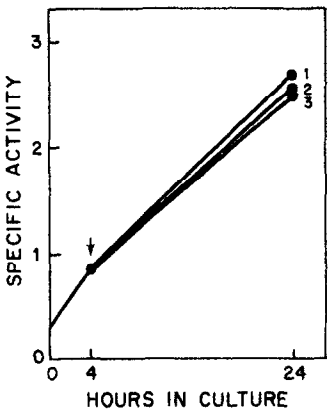

Fig. 5.

Figure 4. Inhibition by ethidium bromide of GS induction, and of labeling of RNA and poly-A in nucleus (A) and cytoplasm (B). The dose-response curve for GS induction (% inhibition calculated as in Table 1) is provided in both Figures 4-A and 4-B to facilitate comparison with corresponding inhibitions of RNA and poly-A synthesis. ○---○, induced GS specific activity; ●——●, ^{3}H-adenosine labeled RNA; ▲——▲, poly-A.

Figure 5. Effects of cordycepin and ethidium bromide on translation of preformed templates for GS. Retinas were cultured for 4 hrs in the presence of HC; further transcription was then stopped (arrow) with 10 μg/ml actinomycin D (1) or with Act D plus 10 or 20 μg/ml cordycepin (2), or 0.2 μg/ml ethidium bromide (3). GS specific activity (expressed as in Table I) was assayed after a total of 24 hrs in culture.

GS levels in the embryonic neural retina. It should be pointed out that since GS induction does not involve or depend on DNA synthesis (22), the latter cannot be implicated in interpretation of our results. Since neither drug appears to directly inhibit translation of GS messages, their suppression of GS induction most likely reflects a reduction in the number of new stable GS templates reaching the cytoplasm. While the possibility has not been entirely excluded that either drug may inhibit the transcription of the RNA essential for GS induction, the present data favor the explanation that they act by inhibiting the synthesis of poly-A and thereby reducing processing, transport or stability of GS messages. In the case of cordycepin, this is strongly suggested by the close correspondence between the extent of inhibition of GS induction and of the labeling of nuclear or cytoplasmic poly-A by different concentrations of this agent. The effects of cordycepin on RNA synthesis described here agree with earlier reports that the drug suppresses labeling of poly-A and of total cytoplasmic RNA more than that of total nuclear RNA (4, 6, 19).

The situation with ethidium bromide requires further analysis. Although at higher concentrations it inhibited poly-A and RNA synthesis, low concentrations showed no measurable effects on either of these while significantly suppressing GS induction. It is possible that the low doses selectively inhibit the synthesis of poly-A or RNA species that are required specifically for GS induction; considering the relatively small amounts of these products, their selective inhibition would not be detectable by the methods employed. Selective inhibition of GS induction at a pre-translational level has been demonstrated before (23).

The results reported here suggest an involvement of poly-A synthesis in the induction of GS and thus point to a possible relationship between poly-A and the expression of a specific, inducible gene-controlled aspect of cell differentiation. Further studies on this problem are in progress.

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