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INTERFERON, DOUBLE-STRANDED RNA AND RNA DEGRADATION. FRACTIONATION OF THE ENDONUCLEASE$_{INT}$ SYSTEM INTO TWO MACROMOLECULAR COMPONENTS; ROLE OF A SMALL MOLECULE IN NUCLEASE ACTIVATION

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SUMMARY. We reported earlier that a) the incubation of an extract from interferon-treated Ehrlich ascites tumor cells with double-stranded RNA and ATP results in the activation of an endonuclease and b) after the activation the double-stranded RNA and ATP can be degraded without impairing the activity of the endonuclease. We report now the separation and partial purification of two macromolecular components (DE1$_{INT}$ and DE2$_{INT}$) involved in the process. Upon incubation with double-stranded RNA and ATP component DE1$_{INT}$ generates a heat-stable product of low molecular weight (designated as nuclease activator). On incubation with the nuclease activator a latent nuclease in component DE2$_{INT}$ is activated.

INTRODUCTION

Interferons are glycoproteins which are synthesized in a variety of animal cells upon viral infection or treatment with other interferon inducers. The most efficient artificial inducer is dsRNA, e.g., poly(I)·poly(C). Though dsRNA is accumulated during the replication of some viruses it has not been established if it is a mediator in the induction of interferon by viruses. The interferons are released from the cells in which they are produced, interact with other cells and inhibit in these the replication of various viruses (1)

The treatment of mouse EAT or L929, or human HeLa S3 cells with interferons is manifested in the cell extract in various ways (2-7). Some of these manifestations depend on the addition of dsRNA and ATP to the extract. Thus: a) various single-stranded RNAs are degraded faster in reaction mixtures containing S30$_{INT}$ than in those containing S30$_C$ but only if the reaction mixtures are supplemented with dsRNA and ATP (8,9). These two agents are needed for the activation of an endonuclease activity, but do not have to be present during the cleavage of RNA (3,9). We designated the system in S30$_{INT}$ that is activated by dsRNA and ATP and is responsible for the acceleration of single-stranded RNA degradation as endonuclease$_{INT}$ (8). b) dsRNA also promotes

Abbreviations: DE1a$_{INT}$, DE1b$_{INT}$, DE1$_{INT}$, DE2$_{INT}$ and DE1a$_C$, DE1b$_C$, DE1$_C$, DE2$_C$, different fractions purified by chromatography on DEAE-cellulose of extracts from interferon-treated and from control cells; ds, double-stranded; EAT, Ehrlich ascites tumor; PMSF, phenylmethylsulfonylfluoride; S30$_{INT}$ and S30$_C$, extracts from interferon-treated and from control cells.

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the phosphorylation of a few proteins in $S30_{INT}$ and only much less so in $S30_C$ (4,10,11). A protein kinase activated by dsRNA was partially purified and found to phosphorylate eIF-2, one of the peptide chain initiation factors (Sen et al., submitted for publication). c) dsRNA and ATP added to an extract of interferon-treated L cells promote the formation of a low molecular weight, heat-stable compound. This compound blocks translation in extracts from control or interferon-treated cells (4,5). It is thought to be an oligoadenylate derivative (12).

In this communication we describe the separation and partial purification of two macromolecular components, $DE1_{INT}$ and $DE2_{INT}$, which are involved in the activation and action of $endonuclease_{INT}$. Upon incubation with dsRNA and ATP, $DE1_{INT}$ synthesizes a low molecular weight, heat-stable product (nuclease activator). Incubation of the nuclease activator with $DE2_{INT}$ results in the activation of $endonuclease_{INT}$.

METHODS

Purification of components of the $endonuclease_{INT}$ system

The low speed (30,000 g) supernatant fractions were prepared from EAT cells that were treated with 650 to 1300 units (of NIH mouse reference standard) interferon/ml (sp. act. 2 x 10^7 units/mg protein)(13) for 24 to 36 h ($S30_{INT}$) or from control EAT cells ($S30_C$) according to the procedure of Brown et al. (8). $S30_{INT}$ (2340 mg protein; 80 ml) was supplemented to adjust its final composition to that of buffer A (25 mM TrisCl (pH 7.8), 220 mM KCl, 30 mM beta-mercaptoethanol, 5 mM magnesium acetate, 1 mM ATP, 1 mM ethylenediamine tetraacetic acid (EDTA), 30 µM phenylmethylsulfonylfluoride (PMSF), 0.05% (w/v) Triton X-100), and centrifuged at 200,000 x g and 2° for 2 h. The supernatant fraction ($S200_{INT}$) was removed and the pellet suspended in 80 ml of buffer A (which had been supplemented with KCl to a final concentration of 500 mM) by stirring on ice for 3 h. The suspension was centrifuged as above and the supernatant fraction (ribosomal wash; 132 mg protein) was combined with the $S200_{INT}$. 150 ml of the combined fractions was supplemented with 50 ml of a saturated (sat.) solution of $(NH_4)_2SO_4$ (which had been adjusted to pH 8.2 with $NaHCO_3$) and stirred at 0° for 30 min. The precipitate was collected by centrifugation at 16,000 g and 2° for 2 min and discarded. The supernatant fraction was supplemented with 31 ml of sat. $(NH_4)_2SO_4$ and the precipitate was collected as above and suspended in buffer B (10 mM TrisCl (pH 8.3), 30 mM beta-mercaptoethanol, 1 mM EDTA, 30 µm PMSF, 10% (w/v) glycerol) (fraction $AS25\text{-}35_{INT}$). The resulting supernatant fraction was supplemented with 42 ml of sat. $(NH_4)_2SO_4$ and the precipitate was collected as above and suspended in buffer B (fraction $AS35\text{-}45_{INT}$). The AS fractions were dialyzed against two changes of 2 liters each of buffer B for altogether 15 h. After dialysis the $AS35\text{-}45_{INT}$ fraction (158 mg protein) was diluted to 15 ml with buffer B and applied to a 5 ml DE-52 (Whatman) DEAE-cellulose column which had been equilibrated with buffer B. The flowthrough fraction (19 ml) was designated as $DE1a_{INT}$. The column was eluted first with buffer B containing 50 mM KCl yielding $DE1b_{INT}$ (17 ml; total protein in fractions $DE1a_{INT}$ and $DE1b_{INT}$ 18.4 mg) and subsequently with buffer B containing 200 mM KCl yielding fraction $DE2_{INT}$ (15 ml; 28.8 mg protein). All fractions were stored in small aliquots in liquid nitrogen. 10 ml of $S30_C$ was fractionated as above yielding 4.6 ml of $DE1a_C$ + $DE1b_C$ and 3.2 ml $DE2_C$.

RESULTS

The acceleration of RNA cleavage by dsRNA and ATP in extracts of interferon-treated cells requires at least two macromolecular components: A convenient assay for detecting endonuclease activity was based on the use of labeled bacteriophage R17 RNA as a substrate and gel electrophoresis and radioautography to resolve the cleavage products. The data obtained with this assay verify that dsRNA and ATP accelerate the cleavage of the labeled R17 RNA in $S30_{INT}$, but much less so in $S30_C$ (Fig. 1A).

Using this assay we established that the endonuclease$_{INT}$ activity in $S30_{INT}$ is divided between the high speed supernatant fraction and the high salt wash fraction of ribosomes. These fractions were combined and further fractionated by differential precipitation. All of the activity detected was in the precipitate formed between 35 and 45% saturation with $(NH_4)_2SO_4$. This material was divided into three fractions ($DE1a_{INT}$, $DE1b_{INT}$ and $DE2_{INT}$) by ion exchange chromatography on DEAE-cellulose. None of the three fractions had endonuclease$_{INT}$ activity in itself, however mixtures of $DE1a_{INT}$ with $DE2_{INT}$ or $DE1b_{INT}$ with $DE2_{INT}$ had the activity (Fig. 1B). For subsequent studies the $DE1a_{INT}$ and $DE1b_{INT}$ fractions were combined to yield fraction $DE1_{INT}$.

dsRNA and ATP trigger the synthesis by $DE1_{INT}$ of a heat-stable nuclease activator. The addition of the nuclease activator to $DE2_{INT}$ gives rise to endonuclease$_{INT}$ activity: The experiments in Fig. 2 were prompted by a) the above finding that at least two protein components are involved in endonuclease$_{INT}$ function, b) the recognition that dsRNA and ATP are required only for the activation of endonuclease$_{INT}$ not for its action(s) and c) the discovery that incubation of dsRNA and ATP in $S30_{INT}$ gives rise to a heat-stable small molecule that inhibits protein synthesis (4). Our experiments revealed that upon incubation of $DE1_{INT}$ with dsRNA and ATP a heat-stable product is formed which, if added to $DE2_{INT}$ promotes R17 RNA cleavage (lane 6). We will designate this product as "nuclease activator". $DE1_{INT}$ or $DE2_{INT}$ alone do not cleave RNA even in the presence of dsRNA and ATP (lanes 1 and 4). $DE2_{INT}$ does not substitute for $DE1_{INT}$ in synthesizing the nuclease activator in the absence or presence of dsRNA (lanes 2 and 3). If either dsRNA or ATP is omitted from the reaction mixture with $DE1_{INT}$ the nuclease activator is not formed (lanes 5 and 9). Neither is it formed if the reaction mixture containing $DE1_{INT}$, ATP and dsRNA is heated to 100° prior to incubation (lane 7). Finally $DE1_C$ does not substitute for $DE1_{INT}$ in synthesizing the nuclease activator (lanes 11 to 14).

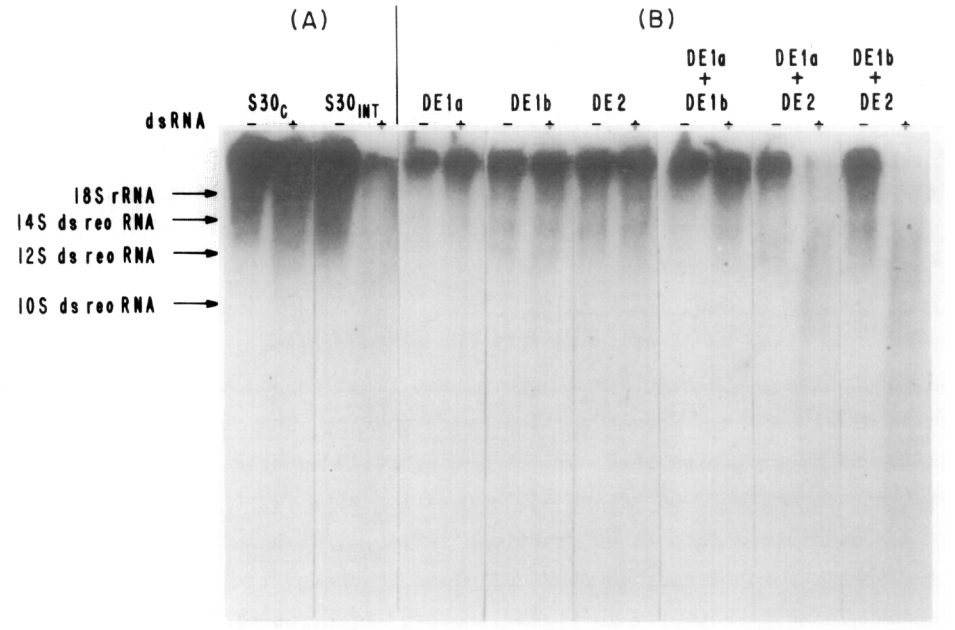

Fig. 1. The promotion of RNA cleavage by dsRNA and ATP involves at least two protein fractions. Assay by gel electrophoresis and radioautography. A) Effect of dsRNA on R17 RNA cleavage in $S30_C$ and $S30_{INT}$. The reaction mixtures (30 µl) contained buffer C (25 mM TrisCl (pH 8.0), 120 mM KCl, 5 mM magnesium acetate, 6 mM beta-mercaptoethanol) as well as 1 mM ATP, 50,000 cpm of [^{32}P]-labeled R17 RNA (sp. act. 1.5-6.0 x 10^5 cpm/µg), 133 µg/ml unlabeled R17 RNA (prepared by a procedure (14) modified from that of J. Steitz) and either 2 µl $S30_C$ or 2 µl $S30_{INT}$ as indicated, and 5 µg/ml poly(I)·poly(C) (Miles Laboratories) if so indicated. They were incubated at 30° for 2 h. Thereafter the reactions were terminated by the addition of 10 µl of a solution containing 50 mM Tris borate (pH 8.6), 2 mM EDTA, 50% (v/v) glycerol, 0.5% (w/v) SDS and 0.002% (w/v) bromophenol blue (serving as an electrophoretic marker). The entire reaction mixtures were analyzed by gel electrophoresis in 3.5% polyacrylamide gels containing 50 mM Tris borate (pH 8.6), 2 mM EDTA and 0.1% SDS (15). Samples were loaded onto 16 cm x 21 cm x 1.5 mm slab gels and were electrophoresed at 200 volts for 3 h and analyzed by radioautography on Kodak XR5 film using a 12 to 14 h exposure time. B) Effect of dsRNA on R17 RNA cleavage in reaction mixtures containing equivalent aliquots of fractions from $S30_{INT}$ obtained by fractional precipitation with $(NH_4)_2SO_4$ and DEAE-cellulose chromatography. (The term equivalent means that the aliquots were obtained from the same volume of cell extract.) The composition of the reaction mixtures was as described in A) except that $S30_C$ and $S30_{INT}$ were omitted and as indicated 3.3 µl $DE1a_{INT}$, or 3.0 µl $DE1b_{INT}$ or 2.6 µl $DE2_{INT}$ or a mixture of two of these fractions was added with or without 5 µg/ml of poly(I)·poly(C) as indicated in the figure. The processing of the reaction mixtures was as described in A). The location of various RNA size markers in the electrophoresis is indicated by arrows.

The nuclease activator is a small molecule: Nuclease activator was generated by incubating $DE1_{INT}$ with dsRNA and ATP, and subsequently $DE1_{INT}$ was inactivated by heating. The reaction mixture was lyophilized, dissolved

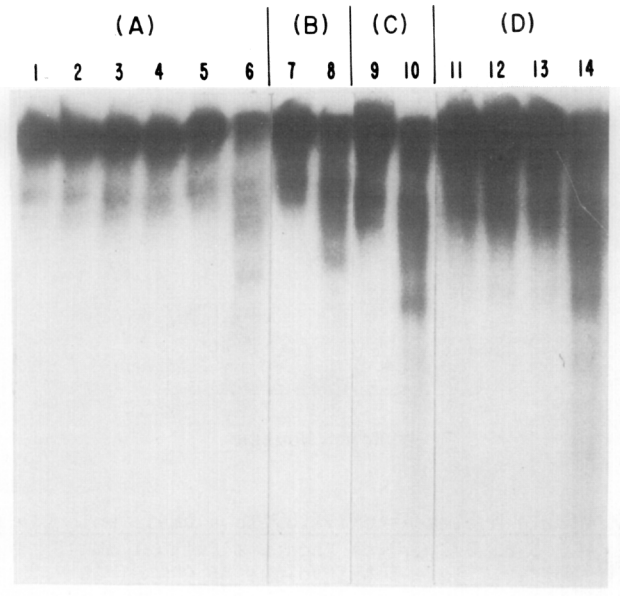

Fig. 2. Incubation of $DE1_{INT}$ (but not of $DE1_C$) with dsRNA and ATP give rise to a heat-stable nuclease activator which if added to $DE2_{INT}$ activates a nuclease cleaving R17 RNA. Assay by gel electrophoresis and radioautography. A) dsRNA promotes the synthesis of a heat-stable nuclease activator by $DE1_{INT}$. Fractions $DE1a_{INT}$ and $DE1b_{INT}$ were combined in a volume ratio of 3.3 to 3.0 and the mixture was designated as $DE1_{INT}$. Either 2.6µl $DE2_{INT}$ (lanes 2, 3) or 3.1 µl $DE1_{INT}$ (lanes 5,6) or no protein fraction (lanes 1, 4) were incubated in a 19 µl reaction mixture containing buffer C, 1 mM ATP as well as 5 µg/ml poly(I)·poly(C) (lanes 3, 6) or no poly(I)·poly(C) (lanes 1, 2, 4, 5) at 30° for 2 h (first incubation). Thereafter the reaction mixtures were kept at 100° for 5 min and at 0° for 20 min. Some of the reaction mixtures were supplemented with 3.1 µl $DE1_{INT}$ (lanes 1 to 3) others with 2.6 µl $DE2_{INT}$ (lanes 4 to 6) and all with 1 mM ATP, 50,000 cpm [^{32}P] R17 RNA and 133 µg/ml of unlabeled R17 RNA in a final volume of 30 µl in buffer C. The reaction mixtures were incubated at 30° for an additional 2 h and were processed for gel electrophoresis as described in Fig. 1A. B) $DE1_{INT}$ is heat labile; the synthesis of a heat-stable nuclease activator by $DE1_{INT}$ in the presence of dsRNA requires incubation. The composition, incubation and processing of the reaction mixtures in lanes 7, 8 was as in the case in lane 6 (in A)) except that the reaction mixture in lane 7 was shifted to 100° immediately after mixing (i.e., the first incubation at 30° for 2 h was omitted). C) ATP is required (in addition to dsRNA) for the synthesis of the nuclease activator by $DE1_{INT}$. The composition, incubation and processing of the reaction mixtures in lanes 9, 10 was as in the case of that in lane 6 (in A)) except that ATP was omitted from the first incubation of the sample in lane 9. D) $DE1_C$ does not substitute for $DE1_{INT}$ in synthesizing the nuclease activator. The composition, incubation and processing of the reaction mixtures in lanes 11 to 14 was as in the case of that in lane 6 (in A)) except that no poly(I)·poly(C) was added to the reaction mixtures in lanes 11 and 13 and that 3.1 µl of $DE1_C$ was added (instead of $DE1_{INT}$) to the reaction mixtures in lanes 11 and 12.

in water, supplemented with labeled R17 RNA and ATP (to serve as size markers) and fractionated by gel filtration on a Sephadex G-25 column. The resulting

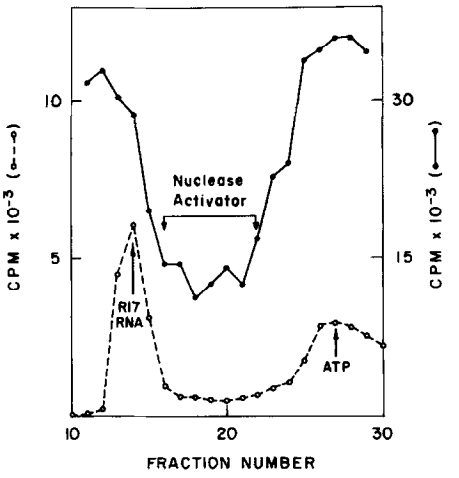

Fig. 3. The heat-stable nuclease activator is a small molecule as determined by gel filtration. 155 µl $DE1_{INT}$ was incubated with 1 mM ATP and 5 µg/ml poly(I)·poly(C) in buffer C in a total volume of 950 µl at 30° for 2h. The reaction mixture was kept at 100° for 5 min, frozen at -20°, lyophilized to dryness and suspended in 150 µl of water. It was supplemented with 200,000 cpm each of [^{32}P]-R17 RNA and γ[^{32}P]ATP (serving as size markers) and was applied to a 0.6 cm x 24 cm Sephadex G-25 (fine) column equilibrated with 10 mM Tris Cl (pH 8.3). (γ[^{32}P]ATP was synthesized by the procedure of Farrell et al., ref. 16.) The column was developed with the equilibrating buffer. 0.3 ml fractions were collected and 20 µl of each was counted in a liquid scintillation system. 10 µl of each fraction was added to a reaction mixture containing 1.3 µl $DE2_{INT}$, 100,000 cpm [^{32}P]RNA and 133 µg/ml of unlabeled R17 RNA in a final volume of 15 µl in buffer C. The reaction mixtures were incubated at 30° for 2 h and were processed for gel electrophoresis as described in Fig. 1. The segment of the gel containing the undigested R17 RNA, the top 1.5 cm of each lane, was cut out and counted in 4 ml of formula 963 scintillation fluid (New England Nuclear). o----o, cpm/20 µl fraction (indicating the location in the effluent of the size markers: labeled R17 RNA and ATP); ●——●, cpm/gel segment indicating the amount of uncleaved R17 RNA in the various reaction mixtures supplemented with different effluent fractions. The reaction mixtures supplemented with fractions 16 to 22 contained the least amount of uncleaved R17 RNA. Thus, these are the fractions containing the most nuclease activator. The contribution to the cpm/gel segment by the [^{32}P]R17 RNA added as size markers is less than 3%, i.e., negligible.

fractions were counted in a scintillation counter to locate the size markers in the effluent (see the legend to Fig. 3). Aliquots of each fraction were added to reaction mixtures containing $DE2_{INT}$ and R17 RNA to test for nuclease activator. The data in Fig. 3 show that the nuclease activator is eluted in the included volume (i.e., after R17 RNA) but before ATP. Thus it is presumably a small molecule (smaller than 5000 daltons but larger than ATP).

DISCUSSION

The following observations indicate that dsRNA and ATP are not needed for

the activation of $DE2_{INT}$ by the nuclease activator: Nuclease activator that has been purified by gel filtration to remove ATP and dsRNA, does activate $DE2_{INT}$. Moreover the addition of ATP and poly(I)·poly(C) to a reaction mixture containing $DE2_{INT}$ and a nonsaturating concentration of purified nuclease activator, does not result in a further increase in nuclease activity (Ratner et al., manuscript in preparation).

The nuclease activator shares the following characteristics with the low molecular weight, heat-stable compound reported by Kerr et al. (12) to be synthesized in $S30_{INT}$ from L929 cells in the presence of dsRNA and ATP: It is inactivated by snake venom diesterase but not by P_1 RNase (Ratner et al., manuscript in preparation).

We purified the agent(s) that is (are) converted into an active endonuclease upon treatment with the nuclease activator at least 50 fold. The partially purified agent(s) is (are) responsible for most of the increase in nuclease activity in $S30_{INT}$ resulting from the addition of the nuclease activator.. It remains to be seen if the same nuclease activator activates one or several inactive nucleases.

Preliminary experiments reveal that $DE2_C$ does contain significant amounts of the agent(s) activated by the nuclease activator but not more than half as much as $DE2_{INT}$. $DE1_C$ seems to be much less active than $DE1_{INT}$ in generating the nuclease activator (Ratner, et al., manuscript in preparation).

In conclusion it should be noted that it is in line with the involvement of a nuclease in mediating (at least some of) the antiviral effects of interferons that the average half life of reovirus mRNAs is about 12 h in control L929 cells but only about 4 h in interferon-treated L929 cells (Desrosiers et al., manuscript in preparation).

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REFERENCES

1. Friedman, R. M. (1977) Bact. Rev. 41, 543-567.
2. Sen, G. C., Shaila, S., Lebleu, B., Brown, G. E., Desrosiers, R. C., and Lengyel, P. (1977) J. Virology 21, 69-83.
3. Ratner, L., Sen, G. C., Brown, G. E., Lebleu, B., Kawakita, M., Cabrer, B., Slattery, E., and Lengyel, P. (1977) Eur. J. Biochem. 79, 565-577.
4. Roberts, W. K., Hovanessian, A., Brown, R. E., Clemens, M. J., Kerr, I. M. (1976) Nature 264, 477-480.
5. Revel, M., Gilboa, E., Kimchi, A., Schmidt, A., Shulman, A., Shulman, L., Yakobson, E., and Zilberstein, A. (1977) Proc. 11th FEBS Meeting, Pergamon Press, Oxford. in press.
6. Samuel, C. E., Farris, D. A., Eppstein, D. A. (1977) Virology 83, 56-71.

7. Shaila, S., Lebleu, B., Brown, G. E., Sen, G. C., and Lengyel, P. (1977) J. Gen. Virol. 37, 535-546.
8. Brown, G. E., Lebleu, B., Kawakita, M., Shaila, S., Sen, G. C., and Lengyel, P. (1976) Biochem. Biophys. Res. Commun. 69, 114-122.
9. Sen, G. E., Lebleu, B., Brown, G. E., Kawakita, M., Slattery, E., and Lengyel, P. (1976) Nature 269, 370-373.
10. Lebleu, B., Sen, G. C., Cabrer, B., and Lengyel, P. (1976) Proc. Natl. Acad. Sci. USA 73, 3107-3111.
11. Zilberstein, A. P., Federman, P., Shulman, L., and Revel, M. (1976) FEBS Lett. 68, 119-124.
12. Kerr, I. M., Brown, R. E., Hovanessian, A. G. (1977) Nature 268, 540-542.
13. Kawakita, M., Cabrer, B., Taira, H., Rebello, M., Slattery, E., Weideli, H., and Lengyel, P. (1978) J. Biol. Chem. in press.
14. Brownlee, G. G. in "Laboratory Techniques in Biochemistry and Molecular Biology", (eds.) T. S. Work and E. Work, North-Holland, Amsterdam, 1972, vol. 3, p. 248.
15. Peacock, A. C. and Dingman, W. (1968) Biochem. 7, 668-674.
16. Farrell, P. J., Balkow, K., Hunt, T., Jackson, R. J., and Trachsel, H. (1977) Cell 11, 187-200.