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KINETICS AND DISTRIBUTION OF INTERFERON mRNA IN INTERFERON-PRIMED AND UNPRIMED MOUSE L-929 CELLS

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Summary

Mouse L-929 cells that have been primed with 100 U/ml of either crude or electrophoretically pure mouse interferon for two hours before induction with Newcastle disease virus, begin producing interferon about 2 - 3 hours earlier and produce about 10 times more interferon than do unprimed cells. The kinetics of IF mRNA appearance and decay are very similar for primed and unprimed cells, indicating that the differences in interferon production by primed and unprimed cells cannot simply be explained by differences in the rate of transcription, maturation or degradation of IF mRNA. However, significant differences are noted in the distribution of IF mRNA : priming leads to a marked reduction in the amount of IF mRNA in free cytoplasm and, as a result in total cytoplasmic IF mRNA RNA, whereas polysomal IF mRNA content remains essentially unaltered. Only primed cells showed a detectable level of double stranded (ds)-RNA dependent oligo(2'-5')adenylate (2-5A) synthetase activity at the time of RNA extraction. It is possible that the 2-5A system may play a role in the altered distribution of IF mRNA in interferon-primed cells.

INTRODUCTION

Mouse interferon (IF) mRNA can be translated successfully in several cell-free systems including the wheat germ system (1) and the micrococcal nuclease-treated rabbit reticulocyte lysate system (2). We have shown recently that total cytoplasmic RNA from Newcastle disease virus (NDV)-induced L-929 cells can program the synthesis of interferon in Xenopus laevis oocytes. Moreover, if care is taken to purify this RNA partially by sucrose-formamide gradient centrifugation, a linear dose-response relationship is obtained between the amount of RNA injected into the oocyte and the amount of interferon translated (2). Other investigators have obtained similar data with IF mRNA extracted from mouse cells (3,4). A quantitative relationship has also been

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established in the oocyte and wheat germ system programmed with human interferon mRNA (5-7). These observations have facilitated a study of the mechanism of superinduction of human fibroblast interferon production (8,9).

An efficient way to enhance interferon synthesis in human or mouse L cells consists of pretreating the cells with a low dose of interferon for a relatively short time before addition of the interferon inducer. This phenomenon is called "priming" and has been known since Isaacs and Burke (10) (for review see 11,12). Priming has important practical applications since it is commonly used for scaling up the production of interferon both in human diploid cells (13,14) and human leukocyte (15). Furthermore, in normally induced cells early secreted interferon (or in some cell lines spontaneously produced interferon (16)) may prime its own production (17). Since the mechanism of this beneficial action of interferon on its own production has not been elucidated, and since a tool was available for measuring the level of translatable IF mRNA in mouse cells (2), we initiated a study on the effect of interferon priming on the amount, kinetics and distribution of IF mRNA induced in NDV-infected L-929 cells. The results obtained in this investigation do not point to a direct effect of priming at the transcriptional level. They suggest that priming results in a redistribution of intracellular IF mRNA. In interferon-primed cells there was a decrease of the total cytoplasmic IF mRNA content, whereas IF mRNA in the polysomal compartment was unchanged. This may reflect either an increased rate of translation of IF mRNA, or an increased degradation of free cytoplasmic mRNA, or both.

MATERIALS AND METHODS

Cells and viruses were described previously (2).

The procedures for the induction of interferon mRNA and interferon priming have also been described (2). The crude mouse interferon preparation used for interferon priming had a specific activity of $2 \times 10^5 - 1 \times 10^6$ units/mg protein. In some experiments electrophoretically pure mouse interferon was used. This preparation had a specific activity of 1.5×10^9 units/mg protein (17). Where pure interferon was used for priming, unprimed cells were treated with the same dilution (1 : 900) of a 0.1 M citrate buffer as that employed in the final stage of interferon purification

The procedures for cell harvesting and fractionation, IF mRNA extraction and IF mRNA translation in Xenopus laevis oocytes, and interferon assay have been described elsewhere (18). All interferon titers are expressed in NIH mouse reference interferon (G 002-904-511) units.

RESULTS

Kinetics of interferon production by primed and unprimed NDV-induced L-929 cells

Our initial experiments were aimed at delineating the optimal conditions for priming of NDV-induced L-929 cells. Priming of the cells with a moderate

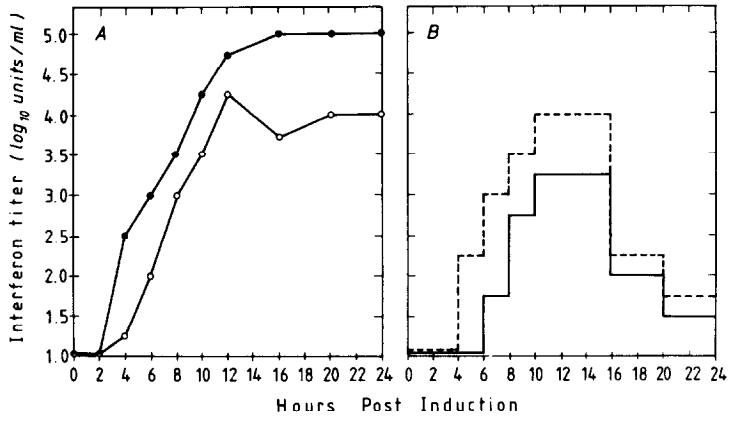

Figure 1. Kinetics of interferon production by primed and unprimed NDV-induced mouse L-929 cells. A) Cumulative interferon production. B) Interval interferon production. For cumulative interferon production (A), the cell cultures were primed (●) or not primed (○) with crude mouse interferon (100 units/ml) for 2 h, induced with NDV, and further incubated with fresh maintenance medium for the indicated periods at which time the media were harvested and assayed for interferon. For interval interferon production (B), the cell cultures were primed (---) or not primed (——) with crude mouse interferon (100 units/ml), induced with NDV, and further incubated with fresh maintenance medium until the next indicated time, when the medium was harvested for interferon assay and replaced with fresh medium until the next interval, at which time this process was repeated.

dose of interferon (i.e. 100 units/ml) for 2 h before induction with NDV, both increased the total amount of interferon produced and accelerated interferon production (or release) (19,20). The priming phenomenon has been described in mouse L cells induced with NDV (21) and polyinosinic acid.polycytidylic acid $\left((I)_n.(C)_n\right)$ (22). Our data on the cumulative production of interferon (Fig. 1A) showed that, in accord with Abreu et al. (21), priming accelerated interferon release in the medium by about 2 - 3 hours, and total interferon yields from primed cells exceeded those from unprimed cells by about 10-fold. In contrast with Abreu et al. (21), however, interferon production did not cease earlier in primed than in unprimed cells : as indicated by interval interferon measurements (Fig. 1B), interferon production peaked and terminated at about the same time for both primed and unprimed cell cultures.

Using a preparation of electrophoretically pure mouse interferon, De Maeyer-Guignard et al. (17) recently showed that interferon itself is responsible for the phenomenon of priming. This was best demonstrated with $(I)_n.(C)_n$ as the inducer, when high doses of interferon still displayed priming activity in C-243 mouse cells. With NDV as the inducer, a small priming effect was observed with the lowest dose of interferon only. We checked whether in our experimental conditions (100 interferon units/ml for 2 h before induction of L-929 cells with NDV) priming could also be obtained with electrophoretically

pure interferon (1.5×10^9 units/mg/protein), and found indeed similar kinetics of both cumulative and interval interferon production as described for crude interferon in Fig. 1A and B (data not shown). Thus, priming is an intrinsic property of interferon itself and cannot be attributed to impurities present in the crude interferon preparation. This was ascertained previously for $(I)_n.(C)_n$-induced C-243 cells (17) and is confirmed herein for NDV-induced L-929 cells.

Kinetics of interferon mRNA appearance in primed and unprimed NDV-induced L-929 cells

Following the quantitative IF mRNA assay developed in our previous studies (2,18), we have measured the kinetics of IF mRNA synthesis in both primed and unprimed NDV-induced L-929 cells. For sucrose-gradient purified total cytoplasmic RNA, maximal IF mRNA production was observed at 12 h after induction with NDV, both in primed and unprimed cells (Fig. 2A). However, the amount of IF mRNA extracted from primed cells at 12 h after NDV infection was considerably (10-fold) lower than that obtained from unprimed cells. On the contrary, there was no reduction or, occasionally, a slight increase in the amount of polysomal IF mRNA extracted from primed cells (Fig. 2B). The kinetics of appearance of polysomal IF mRNA in primed cells was quite similar to the kinetics of polysomal IF mRNA in unprimed cells. In both primed and unprimed cells the polysomal IF mRNA activity dropped to half of its maximum value after about 2 hours. Of course, these kinetics reflect a balance be-

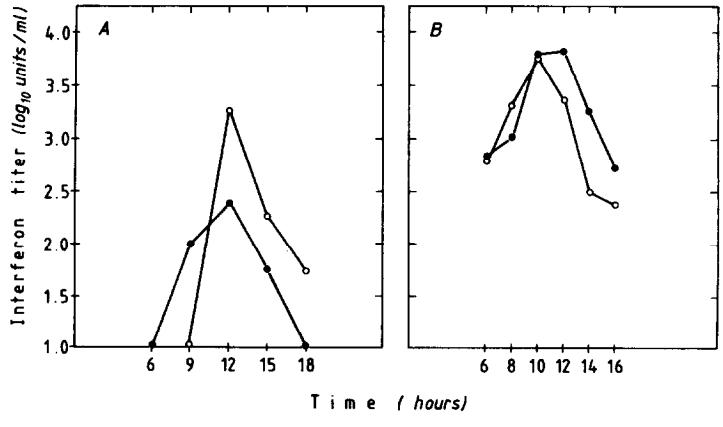

Figure 2. Kinetics of appearance of mouse interferon mRNA in primed and unprimed NDV-induced mouse L-929 cells. A) RNA prepared from total cytoplasmic RNA and purified by sucrose-gradient ultracentrifugation. B) RNA prepared from precipitated polysomes. The cell cultures were primed (●) or not primed (○) with crude mouse interferon (100 units/ml) for 2 h, induced with NDV, and further incubated with fresh maintenance medium for the indicated periods at which time IF mRNA was extracted and processed as described previously (18).

tween the amounts of IF mRNA that are synthesised and degraded and/or exchanged with other cellular compartments. It is, therefore, difficult to draw from these data any definitive conclusions as to the rate of transcription or stability of IF mRNA in primed and unprimed cells.

Others have found that priming may displace peak IF mRNA appearance by about 3 h (21). No such displacement was apparent in our studies (Fig. 2). The discrepancies between our data and those reported previously (21) may at least partially depend on technical differences, such as the method for RNA extraction, as well as biological differences between the cell systems that are evident from the kinetics of interferon production (Fig. 1).

Distribution of interferon mRNA in primed and unprimed NDV-induced L-929 cells

Since no major differences could be detected in the kinetics of IF mRNA appearance in primed and unprimed cells, it was of interest to investigate whether priming would lead to alterations in subcellular IF mRNA distribution. Therefore, IF mRNA activity was measured in different cell fractions of primed and unprimed NDV-induced L-929 cells. Both polysomal RNA and free cytoplasmic RNA (sucrose-gradient purified RNA obtained from the supernatant of the cytoplasmic extract following precipitation of the polysomes), as well as sucrose-gradient purified total cytoplasmic RNA showed a linear interferon response when injected in oocytes (data not shown). Thus, the amounts of IF mRNA could be quantitated for all three RNA fractions. The results of this comparative study are presented in Table 1. It is clear that priming of the cells with 10^2 units/ml interferon resulted in a marked (generally 3- to 6-fold) decrease in the amount of biologically active, sucrose-gradient purified, total cytoplasmic RNA (Table 1, upper part). In contrast, the amount of biologically active mRNA that could be extracted from precipitable polysomes remained essentially unchanged and occasionally increased after the cells had been primed with 10^2 units/ml interferon.

Thus, priming exerted a differential effect on total cytoplasmic (sucrose-gradient purified) IF mRNA and polysomal IF mRNA. It is possible that in primed cells biologically active IF mRNA molecules are shifted to the polysomes (probably membrane-bound polysomes) and do not longer occur as free cytoplasmic RNA molecules. This could also be demonstrated when pure mouse interferon was employed for priming (Table 1, lower part). In this series of experiments, we measured both total polysomal and free cytoplasmic IF mRNA as well as total cytoplasmic IF mRNA. The results suggest that the decrease in IF mRNA activity observed for total cytoplasmic RNA of primed cells results from a reduction of IF mRNA activity in the free (non-polysomal) cytoplasm. The reduction of free cytoplasmic IF mRNA activity in primed cells may arise from a more rapid degra-

Table 1

Quantitation of IF mRNA activity extracted from primed and from unprimed NDV-induced mouse L-929 cells

Treatment		Interferon titer (log_{10} units/ml)		
		Total cytoplasmic RNA	Polysomal RNA	Free cytoplasmic RNA
Exp. I	unprimed	3.5	2.1	ND
	primed	2.7	2.7	ND
Exp. II	unprimed	3.2	3.2	ND
	primed	2.7	3.0	ND
Exp. III	unprimed	2.7	2.1	2.7
	primed	2.2	2.1	2.0
Exp. IV	unprimed	1.6	2.85	2.1
	primed	< 1.2	2.7	< 1.2

All data represent the titer of mouse IF in an homogenate of 10 oocytes injected with either sucrose-gradient purified total cytoplasmic RNA, free cytoplasmic RNA or polysomal RNA at 100 ng per oocyte, and incubated for 17 h at 19°. All RNA preparations were obtained from primed and unprimed cells 12 h after infection with NDV. All the numbers represent mean values from duplicate assays. For experiments I and II the cells were primed with crude mouse interferon (100 units/ml). For experiments III and IV the cells were primed with electrophoretically pure mouse interferon (100 units/ml). ND : Not done.

dation of IF mRNA in this fraction, and/or the accumulation of a ribonuclease or translational inhibitor.

2-5A synthetase activity in the cytoplasmic extracts prepared from primed and unprimed NDV-induced L-929 cells

The sequential events of interferon priming, followed several hours after NDV infection by the accumulation of viral double-stranded (ds)RNA intermediates, may provide the conditions suitable for the induction and activation of an oligo(2'-5')adenylate (2-5A) synthetase and the synthesis of (2'-5')oligoadenylate molecules. These molecules would then activate an endoribonuclease that degrades mRNA and would themselves be degraded by a phosphodiesterase (for review see 23). This nucleolytic activity may eventually account for the decreased free cytoplasmic IF mRNA content noted in primed NDV-induced cells. In a preliminary attempt to assess the validity of this hypothesis we sought to detect 2-5A synthetase in primed and unprimed NDV-induced L-929 cells at the time they were generally processed for RNA extraction (12 h after

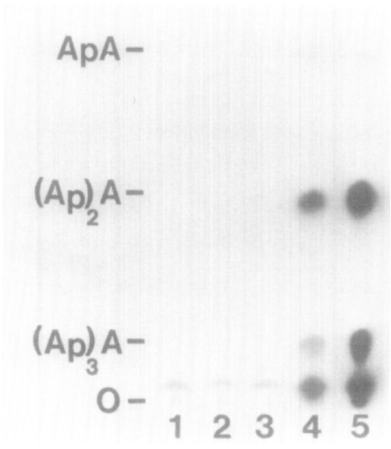

Figure 3. Detection of 2-5A synthetase activity in S10 cytoplasmic fraction prepared from primed and unprimed NDV-induced mouse L-929 cells. The cell cultures were primed or not primed with crude mouse interferon (100 units/ml) for 2 h, induced with NDV, and further incubated with fresh maintenance medium for 12 h, at which time the cells were washed, lysed homogenized and centrifuged at 10,000 x g. The S10 supernatant was assayed in a 12.5 µl reaction containing 25 mM $Mg(OAc)_2$, 100 mM KOAc, 5 mM ATP, 4 mM fructose 1-5 diphosphate, 1 mM dithiothreitol and 75 µCi of lyophylised $(\alpha^{32}P)$-ATP (200 Ci/mmole, The Radiochemical center, Amersham, UK). $(I)_n.(C)_n$, when present, was added at 20 µg/ml. After incubation for 2 h at 30°, 2-5A oligonucleotides were isolated by chromatography on DEAE cellulose columns as described previously (24). The 2-5A containing fractions were precipitated with 6 volumes of acetone, resuspended in water and treated with bacterial alkaline phosphatase before they were separated by thin layer chromatography on polyethyleneimine cellulose in 1 M acetic acid (25).

The radioautogram depicts the results obtained for 5 reactions. Reactions 1 and 3 contained 7 µl S10 from unprimed NDV-induced L-929 cells. Reactions 2 and 4 contained 7 µl S10 from interferon primed (100 units/ml, 2 h before infection), NDV-induced L-929 cells. Reaction 5 contained 7 µl of S10 from uninduced L-929 cells which were treated for 20 h with 200 units of interferon. This additional reaction is served here as the reference for the migration of di, tri and tetramers of 2-5A. To reactions 1 and 2 no $(I)_n.(C)_n$ was added. Reactions 3, 4 and 5 contained 20 µg/ml of $(I)_n.(C)_n$.

infection with NDV : see Table 1). As shown in Fig. 3 only primed cells contained a detectable 2-5A synthetase activity. (^{32}P)-ATP labelled products with identical chromatographic mobility as authentic 2-5A oligomers appeared in the primed cell lysates that had been incubated with exogenous dsRNA $((I)_n.(C)_n)$. No such products were detectable when dsRNA was omitted from the incubation mixtures, or when normal unprimed cell lysates were incubated in the presence or absence of exogenous dsRNA. These results imply that, if NDV dsRNA were present in the induced cells, the amounts recovered in the cell lysate were not sufficient to activate the 2-5A synthetase to a level that was detectable under our assay conditions. In studies with antibodies specific for dsRNA, we were unable to detect a significant increase (> 2 fold) in the amount of

dsRNA primed NDV-infected cells compared to that from unprimed NDV-infected cells (B.D. Stollar, M.I. Johnston, J. Content and E. De Clercq, unpublished data).

The other steps in the 2-5A synthetase-endoribonuclease pathway have not yet been analyzed. It would be interesting to know, whether primed NDV-infected cells, as compared to unprimed NDV-infected cells, contain significantly different amounts of intracellular 2-5A (26), 2-5A dependent endonuclease or 2-5A degrading phosphodiesterase. It would also be worth investigating whether the phosphoprotein kinase, the other major interferon-induced enzyme pathway (23), is affected in primed NDV-infected cells.

DISCUSSION

In both primed and unprimed NDV-induced L-929 cell cultures, IF mRNA synthesis peaks at 10 - 12 h after NDV infection. The appearance of IF mRNA is clearly a transient phenomenon, reflecting a delicate balance between the initiation and shut-off of RNA transcription, and RNA degradation. Kinetic data similar to those presented here have been reported previously for IF mRNA from $(I)_n.(C)_n$-induced human diploid fibroblasts (5,6). The most characteristic feature noted upon priming of the cells with interferon was a reduction in the total cytoplasmic IF mRNA fraction, whereas the polysomal IF mRNA fraction remained constant or rather increased. Thus, priming significantly altered the subcellular distribution of IF mRNA. The change in IF mRNA distribution upon interferon priming appears reminiscent of the findings of Abreu and Bancroft (27) who reported that in $(I)_n.(C)_n$-induced human fibroblasts most (85 - 90 %) of the active IF mRNA was shifted from the membrane fraction to the free cytoplasmic fraction when translation was inhibited by cycloheximide.

Abreu *et al.* (21) inferred from their data, which, like ours, were derived from unprimed and primed NDV-induced mouse L cells, that the enhanced interferon production in primed cells resulted from enhanced efficiency of translation of IF mRNA in the primed cells. Our data corroborate the hypothesis that interferon priming is indeed mediated by an effect at the translation level. In addition, we have shown that interferon priming leads to a shift in the subcellular distribution of IF mRNA, thereby diminishing the levels of free cytoplasmic IF mRNA, and that the latter reduction may possibly be linked to the 2-5A synthetase-endoribonuclease pathway (23).

The 2-5A mediated degradation of free cytoplasmic IF mRNA may occur faster in primed than in unprimed cells, since, under our experimental conditions, we could detect 2-5A synthetase activity only in primed cells. Polysomal mRNA might at least partially be protected from this degradation, which could explain why the total polysomal mRNA activities of primed and of unprimed cells

were virtually identical. However, there is no known evidence for the protection of polysomal mRNA against degradation by the 2-5A activated nuclease.

The role of 2-5A synthetase in the altered distribution of IF mRNA in interferon-primed NDV-induced L cells remains speculative at present, since neither the activation of the 2-5A dependent endonuclease nor the presence of 2-5A itself has directly been demonstrated. It is not clear whether the proposed 2-5A mediated degradation of RNA in the free cytoplasmic compartment would be specific for IF mRNA or for any particular class of mRNA, i.e. discriminative (28). Neither is it evident how this degradation could be related to the enhanced production of interferon in primed cells. It is conceivable, however, that degradation of untranslated mRNAs in the free cytoplasmic pool might indirectly increase the rate of initiation of IF mRNA translation, by reducing its competition with other cellular mRNAs.

In a different system consisting of primed and unprimed $(I)_n.(C)_n$-induced mouse cells, Fujita et al. (4) observed a correlation between the amount of interferon produced and the level of detectable IF poly(A)-mRNA. Their conclusion was that priming influenced the rate of IF mRNA transcription and/or stability of the mRNA product. In view of our findings, we favor the second hypothesis, that is an effect of priming on translation rather than transcription. Since Fujita et al. (4) used $(I)_n.(C)_n$ instead of NDV as the interferon inducer, our data are not directly comparable to theirs. More work is required to define the biochemical changes that occur in primed cells as a function of the inducer.

Finally, all the data presented here concern biologically active (translatable) mRNA. It would be valuable to reevaluate the present findings once an appropriate probe is available for quantitating IF mRNA by nucleic acid hybridisation.

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References

1. Thang, N.N., Thang, D.C., De Maeyer, E., and Montagnier, L. (1975) Proc. Nat. Acad. Sci. USA 72, 3975-3977.
2. Lebleu, B., Hubert, E., Content, J., De Wit, L., Braude, I.A., and De Clercq, E. (1978) Biochem. Biophys. Res. Commun. 82, 665-673.
3. Raj, N.B.K., Fernie, B.F., and Pitha, P.M. (1979) Eur. J. Biochem. 98, 215-221.
4. Fujita, T., Saito, S., and Kohno, S. (1979) J. Gen. Virol. 45, 301-308.
5. Sehgal, P.B., Dobberstein, B., and Tamm, I. (1977) Proc. Nat. Acad. Sci. USA 74, 3409-3413.

6. Cavalieri, R.L., Havell, E.A., Vilcek, J., and Pestka, S. (1977) Proc. Nat. Acad. Sci. USA 74, 4415-4419.
7. Raj, N.B.K., and Pitha, P.M. (1977) Proc. Nat. Acad. Sci. USA 74, 1483-1487.
8. Sehgal, P.B., Lyles, D.S., and Tamm, I. (1978) Virology 89, 186-198.
9. Sehgal, P.B., and Tamm, I. (1979) Virology 92, 240-244.
10. Isaacs, A., and Burke, D.C. (1958) Nature 182, 1073-1074.
11. Rozee, K.R., and Lee, S.H.S. (1977) Interferons and their actions. W.E. Stewart, Ed. CRC Press, Cleveland, pp 133-143.
12. Torrence, P.F., and De Clercq, E. (1977) Pharmacology and Therapeutics, part A 2, 1-88.
13. Havell, E.A., and Vilcek, J. (1972) Antimicrob. Agents Chemother. 2, 476-484.
14. Billiau, A., Joniau, M., and De Somer, P. (1973) J. Gen. Virol. 19, 1-8.
15. Tovell, D., and Cantell, K. (1971) J. Gen. Virol. 13, 485-489.
16. Tovey, M.G., Begon-Lours, J., Gresser, I., and Morris, A.G. (1977) Nature 267, 455-457.
17. De Maeyer-Guignard, J., Cachard, A., and De Maeyer, E. (1980) Virology 102, 222-225.
18. Content, J., De Wit, L., Johnston, M.I., and De Clercq, E. (1980) in "Methods in Enzymology". S.P. Colowick and N.O. Kaplan, Eds. Volume on Interferons, S. Pestka, Ed. Academic Press, Inc. New York.
19. Friedman, R.M. (1966) J. Immunol. 96, 872-877.
20. Levy, H.B., Buckler, C.E., and Baron, S. (1966) Science 152, 1274-1276.
21. Abreu, S.L., Bancroft, F.C., and Stewart II, W.E. (1979) J. Biol. Chem. 254, 4114-4118.
22. Rosztoczy, I. (1977) Archivum Immun. Therap. Experiment. 25, 631-639.
23. Baglioni, C. (1979) Cell 17, 255-264.
24. Minks, M.A., Benvin, S., Maroney, P.A., and Baglioni, C. (1979) J. Biol. Chem. 254, 5058-5064.
25. Verhaegen, M., Divizia, M., Vandenbussche, P., Kuwata, T., and Content, J. (1980) Proc. Natl. Acad. Sci. USA, in press.
26. Williams, B.R.G., Golgher, R.R., Brown, R.E., Gilbert, C.S. and Kerr, I.M. (1979) Nature 282, 582-586.
27. Abreu, S.L., and Bancroft, F.C. (1978) Biochem. Biophys. Res. Commun. 82, 1300-1305.
28. Nilsen, T.W., and Baglioni, C. (1979) Proc. Natl. Acad. Sci. USA 76, 2600-2604.