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# RNA methylation in vaccinia virus-infected chick embryo fibroblasts treated with homologous interferon

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## ABSTRACT

Interferon-pretreatment of vaccinia-infected chick embryo fibroblasts resulted in a greater than 50% decrease in ribose methylation of the penultimate "cap" nucleotide in virus-specific mRNA. However, in contrast to results obtained with cell-free systems, in intact infected cells there was (a) no detectable reduction in methylation of the 5'-ultimate m$^7$G of viral mRNA; (b) a virus specificity of the interferon-induced change in mRNA "cap"-methylation seems unlikely and (c) analysis of the ribosomal and transfer RNA fractions isolated from interferon-treated and control cells revealed identical patterns of methylated nucleotides. Thus, the interferon-induced change in methylation is specific for mRNA "caps".

## INTRODUCTION

The discovery of methylated nucleotides in eukaryotic mRNA initiated widespread studies on the biological significance of these structures (reviewed in 1, 2). Experimental evidence has been accumulating which indicates a role of the methylated 5'-terminal structures in mRNA translation and stability. Much less evidence has been obtained for possible biological functions of methylated nucleotides in ribosomal and transfer RNA, although attempts have been made to associate these methylations with control of growth (3-6) or malignant transformation (7).

In the interferon-treated chick cell, replication of poxvirus is inhibited, presumably at the level of mRNA translation (8-10). We have been investigating a possible association between this interferon-induced translation block and the degree of RNA methylation in the infected cell. From studies using cell-free protein synthesizing systems, an important role of the methylated "cap" for translation of *in vitro* transcribed vaccinia core RNA is evident (11, 12). The methylated "cap"

 

structures of vaccinia mRNA synthesized in infected HeLa cells have been described (13). Vaccinia mRNA isolated from infected chick cells is also "capped". Upon interferon pretreatment of the cells the methyl group content of the mRNA 5'-terminus is reduced compared to the control from untreated vaccinia-infected cells (14). In order to investigate the functional significance of this interferon-induced change, we have extended the analysis of the methylated nucleotides to transfer and ribosomal RNA isolated from interferon-treated and control chick embryo fibroblasts. The results of our studies using intact vaccinia-infected cells are discussed with respect to the physiological significance of impaired reo-mRNA methylating activities reported from interferon-treated murine cells (25, 26).

## MATERIALS AND METHODS

### Cells, virus, mode of interferon pretreatment and infection

Confluent monolayers of primary chick embryo fibroblasts were infected with vaccinia WR at a multiplicity of 1000 elementary bodies per cell. The absorption period was one hour. Prior to infection, cells were incubated with 200 units/ml of purified chick interferon (specific activity 0.5 - 1.5 x $10^6$ units/ml) for 16 hrs (10). One interferon unit of our laboratory corresponds to 0.6 units of A 62/4 Medical Research Council chick embryo reference standard. Vaccinia virus was grown in mouse L-cells and purified as previously described (15).

### Labelling and isolation of RNA

Cells were labelled from 0-4 hrs after infection with 20 µCi/ml of L(methyl-$^3$H) methionine (Amersham-Buchler, specific activity 14 Ci/mM) in methionine-free medium. Cells were harvested and lysed and RNA was extracted by the cold phenol method as previously described (10). On average, 30-35 $A_{260}$ units of post-mitochondrial radioactive RNA were isolated from 10 Roux culture bottles.

### Oligo-dT chromatography

The polyadenylated fraction was separated from total RNA by chromatography on oligo-dT cellulose columns essentially as described by Aviv and Leder (16). Before a second cycle of

chromatography, the samples were heated for 5 min at 62°C in elution buffer containing 1 mM EDTA. This proved to be necessary in order to remove traces of RNA which may have non-specifically aggregated with the poly(A)-containing material.

DNA-RNA hybridization

Radioactive RNA was hybridized to filters containing a large excess of immobilized vaccinia DNA as described previously (10). Hybridization conditions were as described by Gillespie and Spiegelman except that the RNase treatment was replaced by excessive washing (13). For further analysis of virus-specific polyadenylated RNA, the radioactive material was recovered from the hybrids by heating each filter for 10 min at 82°C in 2 ml of an aqueous solution containing 0.1% SDS and 2 mM EDTA. After repeated alcohol precipitation, the RNA was dissolved in water and lyophilized.

SDS-sucrose gradient analysis

For analytical and preparative separation, RNA was centrifuged for 22 hrs at 65000 x g and 20°C in aqueous 15-30% sucrose gradients containaing 0.02% SDS.

Enzyme digestions

RNA at a concentration of 45 µg/30 µl was digested with 2 units of RNase $T_2$ (Sankyo) in 5 mM potassium acetate pH 4.7 for 4 - 5 hrs at 37°C. For subsequent digestion with bacterial alkaline phosphatase (Boehringer), solutions were adjusted to pH 8.5 with 2 M triethylammoniumbicarbonate and reincubated for 4 - 5 hrs at 37°C in 100 µl using 0.035 units of enzyme/$A_{260}$ unit of RNA.

Two-dimensional thinlayer chromatography of methylated nucleotides

A detailed description of the chromatography system used here has been reported elsewhere (12). Briefly, mRNA digests obtained after RNase $T_2$ and bacterial alkaline phosphatase treatment were applied to 20 x 20 cm cellulose thinlayer plates (Merck) in an aqueous solution containing 9 unlabelled "cap" markers as well as $m^6A$ (P-L Biochemicals). The first dimension was developed in solvent A: isobutyric acid - concentrated ammonia - water, pH 3.7 (66:1:33: v:v:v) for 8 hrs, the second

dimension in solvent B: isopropanol - 1 N hydrochloric acid - ammonium chloride (25:25:1; v:v:w) for 6 - 7 hrs. Optical density markers were located by 254 nm UV light and ($^3$H-methyl) labelled nucleotides were detected by fluorography as described by Randerath et al. (17). For quantitation, the radioactive material was recovered from each spot and the scintillator was removed with ether. The dried material was eluted with water and the eluates were assayed by liquid scintillation counting. For chromatography of the methylated nucleotides obtained after RNase $T_2$ digestion of ribosomal or transfer RNA, solvent A was used in the first dimension, and a solvent containing tert. butanol - conc.hydrochloric acid - water (70:15:15; v:v:v) in the second dimension. The second dimension was developed for 18-20 hrs. Fluorography, detection of radioactive nucleotides and quantitation were as described above.

## RESULTS

### Characterization of the methylated nucleotides in polyadenylated RNA

As shown in Fig. 1, total polyadenylated cytoplasmic RNA double-labelled with ($^3$H-methyl) methionine and ($^{14}$C-) uridine which was isolated from either interferon-treated or control cells revealed very similar sedimentation profiles on SDS-sucrose gradients. The virus-specific fraction of polyadenylated RNA was isolated by hybridization with vaccinia DNA. The methylated nucleotides of this fraction were analyzed by enzyme digestion and two-dimensional thinlayer chromatography as described in Materials and Methods.

There is a reproducible shift in the proportion of complete versus incomplete "cap I" structures induced by interferon. The quantitation of the ($^3$H-methyl) labelled material recovered from the chromatograms is shown in Table I. Of the methylated "cap" structures of vaccinia-specific polyadenylated RNA isolated from control cells, over 80% are of the complete type "I" ($m^7$GpppGmpN, $m^7$GpppAmpN) and less than 20% of the incomplete counterpart ($m^7$GpppG, $m^7$GpppA). In the virus-specific RNA isolated from interferon-treated cells, however, the proportion of incomplete "cap I" structures is increased to approximately 50%, and the proportion of complete "cap I" struc-

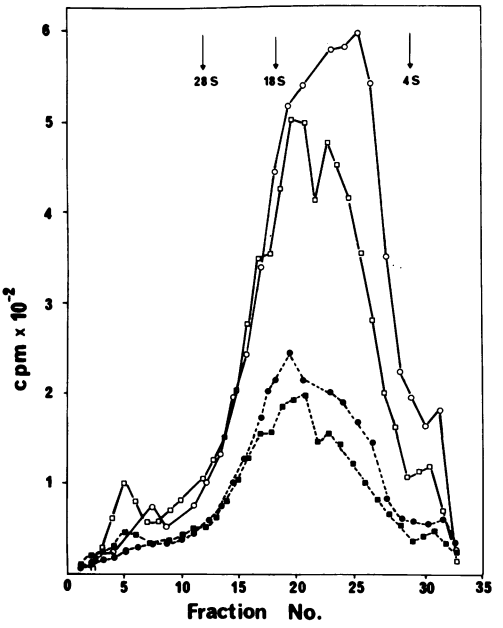


Fig. 1 Sedimentation profile of mRNA from vaccinia-infected interferon-treated and control cells. $RNA_3$ was double-labelled with ($^3$H-methyl) methionine and ($^{14}$C-)uridine 0-4 hrs after infection. Polyadenylated fractions were analyzed on SDS-sucrose gradients. TCA-precipitable radioactive material in each gradient fraction was assayed by liquid scintillation counting. (□—□) $^3$H-methyl-, (■- -■) $^{14}$C-uridine label from interferon-treated cells. (○—○) $^3$H-methyl-, (●- -●) $^{14}$C-uridine label from control cells. Positions of added optical density markers are indicated by arrows.

tures is correspondingly decreased to approximately 50%. The amount of internal $m^6A$ is not influenced by interferon-pretreatment.

A very similar effect of interferon on ribose methylation of the penultimate 5'-nucleotide was detected also in the fraction of polyadenylated RNA that did not hybridize with vaccinia DNA (Table 1b). Based on the assumption that this fraction contains the majority of cell-specific mRNA, a virus-specificity of the interferon-induced change in "cap" methylation is unlikely.

Analysis of the methylated nucleotides in non-polyadenylated RNA

Similar to the situation in other cells, the relative amount of methylation of ribosomal and transfer RNA in the chick cell is roughly ten times that of polyadenylated mRNA (18, 19). Thus, only up to 0.3% of the total ($^3$H-methyl) labelled cytoplasmic RNA can be recovered in the purified polyadenylated fraction (14). Hence, it was quite of interest to investigate, whether the interferon-induced change in methylation was limited to the mRNA fraction. Our initial comparison of the ($^3$H-methyl)

| methylated nucleotide | a (hybridized) interferon | a (hybridized) control | b (not hybridized) interferon | b (not hybridized) control |
|---|---|---|---|---|
| cap I ($m^7$GpppGmpN, $m^7$GpppAmpN) | 33 | 51 | 12 | 26 |
| cap 0 ($m^7$GpppG, $m^7$GpppA) | 32 | 10 | 20 | 6 |
| $m^6$A | 35 | 39 | 62 | 62 |
| unidentified | | | 6 | 6 |

Table I: Effect of interferon-pretreatment on mRNA methylation. ($^3$H-methyl) labelled polyadenylated RNA from infected cells was digested with RNase T2 and bacterial alkaline phosphatase. 5000-10000 cpm of the methylated nucleotides were analyzed by twodimensional thinlayer chromatography and fluorography. The radioactive material from each spot was recovered from the chromatograms and quantitated by liquid scintillation counting (see Materials and Methods). All numbers indicate percent of total cpm recovered from the corresponding chromatogram. Numbers in column a represent mean values from two separate experiments (maximum standard deviation ± 17%).

labelled nucleotides of poly(A)-minus RNA obtained after one cycle of oligo-dT chromatography revealed no significant difference between the samples from interferon-treated (Fig. 2A) and control cells (Fig. 2B). Virtually identical patterns of methylated nucleotides were obtained. No methylated "cap" structures typical for mRNA were detectable in this fraction. The similarity of the patterns of poly(A)-minus RNA isolated from interferon-treated or control cells is even more evident after quantitation of the radioactive material recovered from the chromatograms. This is shown in Table II.

We wanted to exclude the possibility, that the major part of complete "cap I" structures of mRNA isolated from interferon-treated cells was lost during the necessarily rigorous purification procedure (see Materials and Methods). Therefore, we analyzed the poly(A)-minus fraction obtained after the second cycle of oligo-dT chromatography. This fraction should contain mostly ribosomal and transfer RNA. SDS-sucrose gradient analysis of this fraction did reveal material with sedimentation behaviour expected for ribosomal and transfer RNA (data not shown). However, analysis of the methylated nucleotides (Fig. 3)

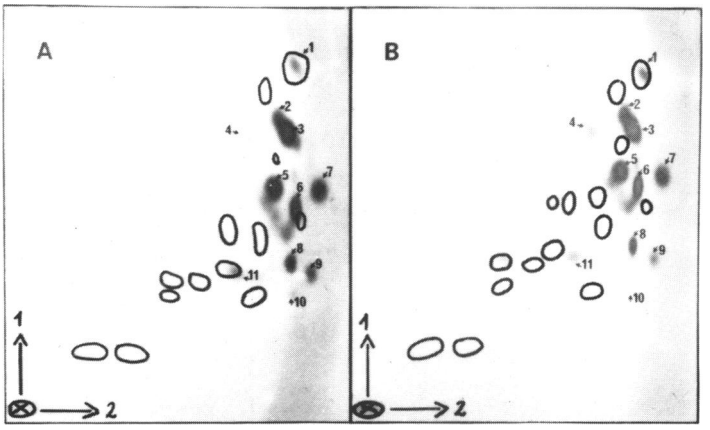


Fig. 2. Methylated nucleotides of poly (A)-minus RNA from vaccinia-infected interferon-treated (A) and control cells (B). Approximately 50 000 cpm of the ($^3$H-methyl) labelled digest obtained after RNase T2 and bacterial alkaline phosphatase treatment were subjected to two-dimensional chromatography in the same system as was used for analysis of the mRNA digests described in Materials and Methods. Positions of optical density markers are outlined solid, positions of ($^3$H-methyl) labelled nucleotides are numbered 1 - 11. X-ray films were exposed for 4 days.

showed that indeed some mRNA "caps" were lost into this fraction during purification. Nevertheless also in this fraction, the same effect on "cap" methylation is evident in the sample from interferon-treated cells exactly as was shown for the "caps" of purified polyadenylated RNA. Thus, the interferon-induced change in "cap" methylation is not a result of a specific loss of complete "cap I" structures during the purification procedures.

Analysis of the methylated nucleotides of separated ribosomal and transfer RNA

It has been demonstrated that the function of ribosomes in extracts from interferon-treated murine cells may be impaired (20, 21). Therefore, we were particularly interested in possible interferon-induced structural alterations of ribosomal RNA isolated from vaccinia-infected chick cells. Of the poly(A)-minus fraction obtained after one cycle of oligo-dT chromatography, more than 90% of the ($^3$H-methyl) label is incorporated into acid-insoluble material sedimenting in the 5S region on SDS-sucrose gradients. Thus, only a very small proportion of the ($^3$H-methyl) label is incorporated into ribosomal RNA sedimen-

| Spot No | Interferon | Control |
|---|---|---|
| 1 | 5.7 | 6.4 |
| 2 | 8.5 | 10.1 |
| 3 | 28.0 | 27.2 |
| 4 | 2.2 | 2.8 |
| 5 | 15.9 | 17.7 |
| 6 | 13.9 | 14.1 |
| 7 | 11.1 | 9.6 |
| 8 | 7.7 | 6.6 |
| 9 | 3.3 | 2.3 |
| 10 | 0.9 | 0.8 |
| 11 | 2.8 | 2.4 |
| (total) | (100.0) | (100.0) |

Table II: Quantitation of ($^3$H-methyl) labelled nucleotides recovered from the chromatograms shown in Fig. 2. All numbers represent percent of total cpm recovered from the corresponding chromatogram.

ting at 28S and 18S (Figure 4). A similar distribution of the ($^3$H-methyl) label in the poly(A)-minus fraction has been described for RNA isolated from adenovirus-infected cells (22). Hence, we carried out separate analyses of the methylated transfer and ribosomal RNA obtained after SDS-sucrose gradient

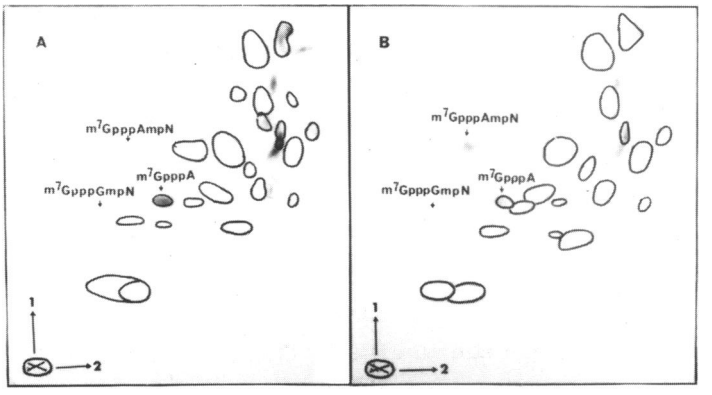


Fig. 3. Methylated nucleotides of mRNA "contaminants" obtained during purification. Poly(A)-minus ($^3$H-methyl) labelled RNA obtained after two cycles of oligo-dT chromatography was subjected to enzyme digestion and two-dimensional chromatography as described for Fig. 2. Approximately 30 000 cpm obtained from interferon-treated (A) and 20 000 cpm obtained from control cells (B) were applied to chromatography. Positions of added optical density markers are outlined solid. X-ray films were exposed for two weeks.

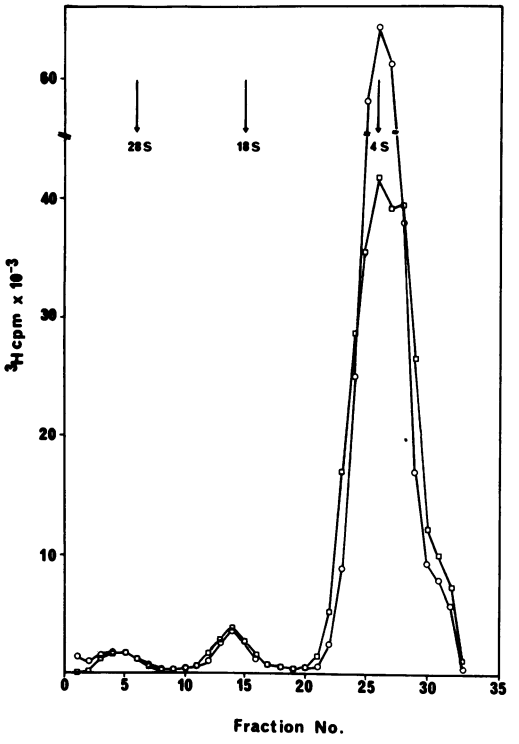


Fig. 4. SDS-sucrose gradient analysis of ($^3$H-methyl) labelled poly(A)-minus RNA from vaccinia-infected interferon-treated (□—□) and control cells (○—○). TCA-precipitable aliquots of each gradient fraction were assayed by liquid scintillation counting. Peak positions of optical density are indicated by arrows.

centrifugation. The gradient fractions corresponding to transfer RNA and ribosomal RNA, respectively, were pooled and subjected to analysis of the methylated nucleotides. The radioactive material was digested with RNase T2 only, and the 3'-monophosphates obtained were separated by two-dimensional chromatography, using the solvent tert.butanol - hydrochloric acid - water in the second dimension (see Materials and Methods). The results of this analysis are shown in Figs. 5 and 6. The patterns of methylated nucleotides in transfer RNA (Fig. 5) as well as in ribosomal RNA (Fig. 6) isolated from interferon-treated or control cells are identical. Moreover, quantitative comparison of the radioactive material recovered from the chromatograms rendered no significant differences in the methylation of transfer RNA (Table IIIa) or ribosomal RNA (Table IIIb) from interferon-treated or control cells. Because of the similarity of the chromatograms obtained from interferon-treated and control cells, the methylated nucleotides were not further analyzed. In the case of transfer RNA, the

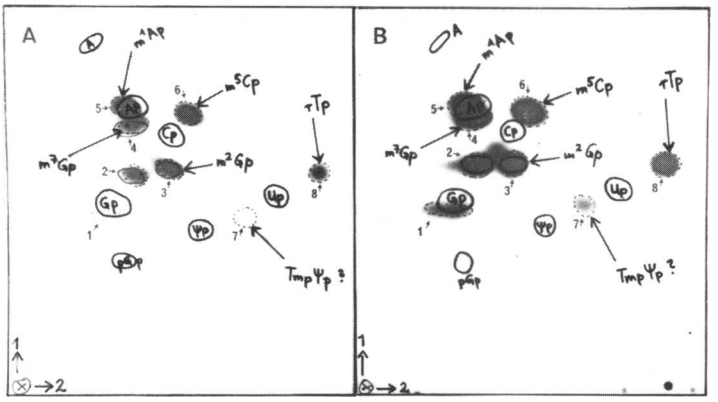


Fig. 5. Two-dimensional thinlayer chromatogram of the ($^3$H-methyl) labelled transfer RNA from vaccinia-infected interferon-treated (A) and control cells (B). Approximately 250 000 cpm (A) and 60 000 cpm (B) of the RNase T2 digest were subjected to chromatography and developed using the same solvent as described in Fig. 3 for the first dimension and a different solvent for the second dimension (see Materials and Methods). Positions of optical density are outlined solid, positions of ($^3$H-methyl) labelled nucleotides are numbered 1 - 8. X-ray films were exposed for 4 days.

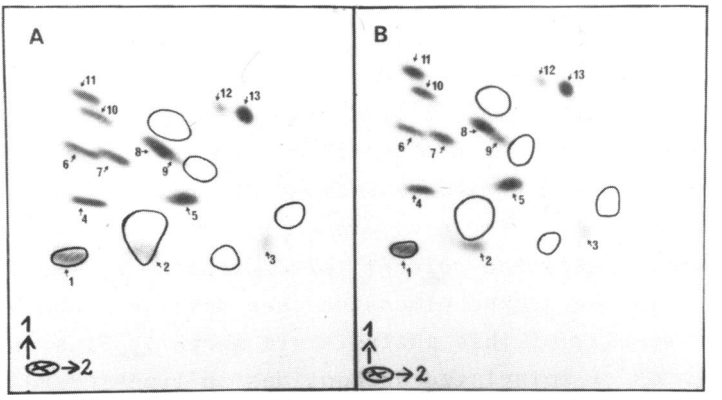


Fig. 6. Two-dimensional thinlayer chromatogram of the ($^3$H-methyl) labelled ribosomal RNA from vaccinia-infected interferon-treated (A) and control cells (B). Approximately 40 000 (A) and 30 000 (B) cpm of the RNase T2 digest were subjected to chromatography as described for Fig. 5. Positions of optical density are outlined solid, positions of ($^3$H-methyl) labelled nucleotides are numbered 1 - 13. X-ray films were exposed for 10 days.

majority of the methylated nucleotides could easily be identified by comparison with similar chromatograms of transfer RNA digests obtained from other eukaryotic cells (23).

| a) transfer RNA | | | b) ribosomal RNA | | |
|---|---|---|---|---|---|
| Spot No | Interferon | Control | Spot No | Interferon | Control |
| 1 | 4.3 | 2.8 | 1 | 12.3 | 9.5 |
| 2 | 12.7 | 12.5 | 2 | 8.3 | 8.4 |
| 3 | 16.6 | 16.6 | 3 | 2.9 | 3.2 |
| 4 | 12.1 | 12.5 | 4 | 8.9 | 9.9 |
| 5 | 19.2 | 23.5 | 5 | 11.5 | 12.6 |
| 6 | 24.6 | 24.5 | 6 | 8.6 | 7.6 |
| 7 | 1.7 | 1.4 | 7 | 7.3 | 8.1 |
| 8 | 8.8 | 6.3 | 8 | 13.8 | 10.7 |
| (total) | (100.0) | (100.1) | 9 | 2.3 | 3.9 |
| | | | 10 | 7.3 | 6.4 |
| | | | 11 | 6.8 | 8.0 |
| | | | 12 | 2.5 | 2.6 |
| | | | 13 | 7.4 | 9.1 |
| | | | (total) | (99.9) | (100.0) |

Table III: Quantitation of the ($^3$H-methyl) labelled nucleotides of transfer (a) and ribosomal RNA (b). Numbers represent percent of total cpm recovered from the corresponding chromatogram. Column a refers to Fig. 5, column b refers to Fig. 6.

## DISCUSSION

We have shown that vaccinia virus-specific mRNA isolated from infected chick cells contains a reduced amount of complete "cap I" structures, if the cells had been pretreated with homologous interferon (14). A qualitatively different reduction in ribose methylation leading to a shift from "cap II" to "cap I" structures in interferon-treated reovirus-infected mouse L-cells has been reported in a recent abstract (24). An impairment of reovirus mRNA methylating activity had also been detected in cell-free extracts from Ehrlich Ascites cells pretreated with interferon (25, 26). However, it was concluded from these studies, that in order to ascertain the physiological role of these findings, investigations of the mRNA methylation pattern in interferon-treated infected cells were required.

The results of our studies on RNA methylation in the living chick cell differ not only from the previously mentioned shift from "cap II" to "cap I" structures in interferon-treated reovirus-infected mouse L-cells; they differ also from the results obtained with murine cell-free extracts in several ways: First-

ly, there is no detectable reduction in methylation of the 5'-ultimate 7-methyl-guanosine of vaccinia-specific mRNA isolated from the infected chick cells. Thus, the over 60% decrease in 5'-ultimate 7-methyl-guanosine formation reported from Ehrlich Ascites cell-free extracts might not truly reflect the situation in the living cell (24, 26). Secondly, we have so far been unable to obtain results indicating a virus-specificity of the interferon-induced change in cap methylation in the intact infected cell. Thirdly, methylation of added transfer RNA has also been reported to be significantly decreased in the cell-free system from interferon-treated Ehrlich Ascites cells (25). Our analysis of the methylated nucleotides of ribosomal and transfer RNA proves that the effect of interferon is limited to methylation of the 5'-termini of vaccinia virus-specific and presumably also cellular mRNA. Considering the small amount of mRNA-specific methylation and the great variety of RNA-methylating enzymes present in living cells, the specificity of the interferon-induced change is remarkable. Moreover, all inhibitors of methylation studied so far are of little, if any, specificity for one particular kind of RNA (27).

Possible explanations for the interferon-induced reduction in 5'-penultimate ribose methylation remain of speculative nature. For example, demethylating enzymes as a possible cause have so far not been detected. "Cap" analysis by two-dimensional thinlayer chromatography has failed to detect significantly altered distributions of the bases at penultimate 5'-position of mRNA isolated from interferon-treated or control cells (14). Hence, an explanation for the reduced methylation via interferon-induced changes in base composition of 5'-termini can be ruled out.

Another explanation could be provided by evidence for a specific locus for secondary methylation of mRNA "caps" which would be affected by interferon-pretreatment. However, although a separation of "cap" methylation into early transcriptional (nuclear) and secondary post-transcriptional (cytoplasmic) steps has been elucidated, a specific site of action of any of the enzymes involved has so far not been identified (18, 19, 28-32).

At the present, we still favour the possibility of a specific interferon-induced action on a very specific enzyme. However, this explanation remains hypothetical and will have to be proved by further studies.

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