Journal of Virology, Nov. 1979, p. 442–448
0022-538X/79/11-0442/07$02.00/0

Vol. 32, No. 2

Macromolecular Synthesis in Cells Infected by Frog Virus 3

XII. Viral Regulatory Proteins in Transcriptional and Post-Transcriptional Controls

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Received for publication 2 March 1979

Using fluorophenylalanine (FPA) to interfere with functional viral protein synthesis, we have investigated the complex transcriptional and post-transcriptional controls that operate in cells infected with frog virus 3. Our previous data, obtained by polyacrylamide gel electrophoresis of viral RNAs and proteins, showed that the addition of FPA at the beginning of infection completely prevented the synthesis of late viral RNAs and late viral proteins and blocked the normal progressive decline in the rates of synthesis of two quantitatively different classes (class I and class II) of early proteins. These results indicated that the initiation of late RNA and late protein syntheses, as well as the post-transcriptional regulation of early protein synthesis, was under the control of virus-specific proteins (D. B. Willis, R. Goorha, M. Miles, and A. Granoff, J. Virol. **24**:326–342, 1977). In this communication, we show that the viral protein required to "turn on" the synthesis of late RNAs and late (class III) proteins was made within 1 to 1.5 h postinfection (p.i.); when we added FPA after this time, we observed the synthesis of all of the late macromolecules. The data also suggest that another viral protein, separate from the "turn-on" protein, controlled the abundance of late RNAs. In addition, at least two separate proteins were involved in the post-transcriptional regulation of two classes of early proteins. When FPA addition was delayed until 2 h p.i., the rate of synthesis of class I proteins (which normally peaked at 2 h p.i.) was reduced by 6 h p.i. just as in a normal infection, but the rate of synthesis of class II proteins (which normally reached a maximum at 4 h p.i. before declining) was reduced only when we waited until 3 or 4 h p.i. to add FPA. These experiments corroborate and extend previous evidence for the existence of numerous viral regulatory proteins in the control of frog virus 3 gene expression at the transcriptional and post-transcriptional levels.

We previously classified the virus-specific proteins in frog virus 3 (FV3)-infected cells as early, intermediate, or late, based upon the time during the growth cycle when their rates of synthesis were maximum (13). Thus, early proteins were synthesized maximally at 2 h postinfection (p.i.), intermediate proteins at 4 h p.i., and late proteins at 6 h p.i. or later. The appearance of late proteins was solely a time-dependent phenomenon and did not require prior synthesis of viral DNA (3). On the other hand, viral RNAs could only be called early (i.e., synthesized at 1 h p.i.) or late (i.e., synthesized at 2 h p.i. and thereafter), because once the synthesis of any viral RNA species had begun, its rate was maintained at the same or at a higher level (13).

The amino acid analog fluorophenylalanine (FPA) aided us in the temporal classification of viral macromolecules. This drug, when incorporated into proteins in an essential site, renders such proteins nonfunctional without inhibiting protein synthesis in general (11). The addition of FPA at the beginning of infection had pleiotropic effects (13). Only the early RNAs were synthesized, even at 6 to 8 h p.i. Both early and intermediate proteins were made, but their rates of synthesis did not decline as in cells infected in the absence of the drug. Since both early and intermediate proteins were synthesized in the presence of FPA, we have now elected to classify all of these proteins as early, to correspond with the early RNAs. However, as these two groups of proteins displayed obvious differences in both the time of their own peak rate of synthesis (13) and the time of synthesis of their regulatory proteins (see Results), we have subdivided them into class I proteins or class II proteins. The difference between early and late proteins is a qualitative one; only early proteins are present at 1 h p.i. or when cells are treated with FPA at 0 h (13). The difference between class I and class II early proteins is a quantitative one. Both can

be detected under the above conditions; however, class I proteins reach a maximum rate of synthesis at 2 h p.i. and decline thereafter, whereas class II proteins display a peak rate of synthesis at 4 h p.i. before their rate of synthesis goes down. In keeping with this terminology, which is based on the rate of synthesis of a protein rather than its presence or absence, we have termed the late proteins, which have a peak rate of synthesis at 6 h p.i., as class III proteins.

The data presented in this paper show that at least one protein regulated transcription by switching on the synthesis of late RNAs. The evidence also suggests that another protein controlled the abundance of certain species of viral RNAs. In addition, we demonstrate that two or more proteins were also involved in the post-transcriptional regulation of FV3 gene expression, because the inhibition of class I and class II protein synthesis late in infection involved regulatory proteins synthesized at disparate times in the infectious cycle.

MATERIALS AND METHODS

Cells and virus. Fathead minnow cells were grown as monolayers at 33°C in Eagle minimal essential medium containing 10% fetal calf serum (MEM-10). A clonal isolate of FV3 was grown and assayed in fathead minnow cells as described previously (8).

Isotopic labeling of RNA and proteins in infected cells. To inhibit host RNA (14) and protein (4) syntheses, we exposed cells to heat-inactivated (Δ)FV3 (56°C, 15 min) in amounts equivalent to 20 PFU/cell for 60 min at room temperature (23°C). The cells were then washed, overlaid with MEM-10, and incubated at 30°C for 2 h. The medium was removed; active virus was inoculated at 20 PFU/cell and adsorbed at 23°C for 60 min. The cells were washed, overlaid with MEM without serum, and incubated at 30°C. This time was considered 0 h for the growth cycle. The cells taken for analysis at 6 or 8 h p.i. did not receive ΔFV3 because by that time host RNA and protein syntheses could be inhibited by active virus alone (4, 14). In experiments with temperature-sensitive mutants, heated mutant virus was used instead of wild-type virus to prevent nongenetic reactivation of the heated viral genome (6).

At various intervals after infection, the medium was replaced with methionine-free MEM containing 25 μCi of [^{35}S]methionine per ml (to label proteins) or 50 μCi of [^{3}H]uridine per ml (to label RNA). After a 30-min labeling period, cytoplasmic extracts were prepared for electrophoresis. FPA (100 μg/ml) was added in phenylalanine-free MEM. At indicated times, medium containing FPA replaced the regular MEM in infected cell dishes.

Polyacrylamide slab gel electrophoresis of RNA and proteins. The procedures for electrophoretic separation of RNA and proteins, as well as for autoradiography of dried gels, have been described (13). For RNA, slab gels consisting of 3.5% acrylamide in 20 mM phosphate-buffered formamide were cast in a Hoefer slab gel apparatus (30 cm by 15 cm by 1.5 mm). Protein samples were layered on wells of a 10-cm, 5 to 15% acrylamide–0.1% sodium dodecyl sulfate gradient slab gel with a 3% stacking gel. Molecular weights of each of the RNA and protein bands were calculated by comparing the distance migrated with R_f of several standards (13).

Computer analysis of data. The developed films were scanned with an Ortec 4310 densitometer interfaced with a PDP 8/1 minicomputer (Digital Electronics Corp.). Other details of computer analysis were previously described (13).

Reagents. [^{35}S]methionine (specific activity, 600 Ci/mmol) was from New England Nuclear Corp.; [^{3}H]uridine (specific activity, 20 Ci/mmol) was purchased from Schwarz/Mann. Formamide, obtained from BDH Laboratories, was deionized with 5% Amberlite MB-1 until the conductivity measured less than 3 mmho. FPA was bought from Sigma Chemical Co.

RESULTS

Virus-induced protein regulates the rate of viral RNA synthesis. We previously showed that the addition of FPA to FV3-infected cells at the beginning of infection locked the pattern of RNA synthesis into the early phase (13). Figure 1A illustrates the typical 10 early infected cell RNA (ICR) species (ICR 597, 577, 506, 489, 463, 425, 358/345, 322, and 169) synthesized at 1 to 1.5 h p.i. The same species were seen at 6 h p.i. when infected cells were treated with FPA at 0 h (Fig. 1C). The larger RNA species (>597,000 molecular weight) appeared early, but were often poorly resolved, and have not been analyzed further. In Fig. 1B, we can clearly identify the late RNA species—ICR 560, 534, 390, 290, 240, 235, and all RNAs smaller than ICR 169. The increased rate of synthesis of ICR 463 at 6 h p.i. may represent the synthesis of a late RNA superimposed over that of an early RNA of the same molecular weight or an increase in the rate of synthesis of that particular early RNA at late times. The absence of late RNAs in cells treated with FPA indicated that a virus-induced protein was required for late transcription.

To determine when the protein required for late transcription was synthesized, we added FPA at various times after infection and labeled the RNA with [^{3}H]uridine from 6 to 6.5 h p.i. In Fig. 2A, FPA was added at 0 h, and only early RNAs were being synthesized 6 h later (see also Fig. 1). If the addition of FPA was delayed by 1 or 1.5 h (Fig. 2B and C), the late RNAs were synthesized (although at barely detectable levels) at 6 h p.i. All of the late RNA bands were visible in Fig. 2B and C on the original fluorogram, but only the band representing ICR 534, the major late RNA, was sufficiently intense to

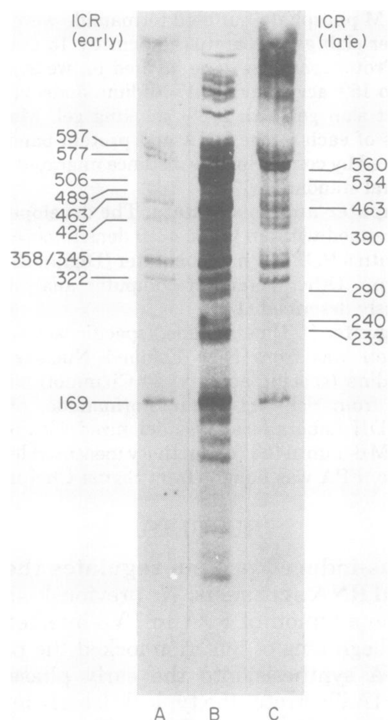

FIG. 1. *FPA inhibits synthesis of late viral RNAs. Cells were treated with ΔFV3 for 4 h before infection with 20 PFU of active FV3 per cell. RNA, labeled for 30 min with [^{3}H]uridine (50 μCi/ml) at indicated times, was extracted from the cytoplasm of infected cells and subjected to electrophoresis as described (13). ΔFV3 treatment was not necessary for 6-h samples, because by that time active virus alone had sufficiently reduced cellular RNA synthesis. (A) 4 h ΔFV3, 1 h active FV3; (B) 6 h active FV3; (C) 6 h active FV3, with 100 μg of FPA per ml added at 0 h. ICR, Infected cell RNA, molecular weight × 10^{-3}.*

be seen after photographic reproduction. The synthesis of late ICRs, albeit at a very low rate, when FPA addition was delayed for 1 to 1.5 h implies that an early protein, synthesized within the 1.5 h, was responsible for turning on late transcription. However, to achieve the maximum rate of late RNA synthesis, addition of FPA had to be delayed for at least 3 or 4 h (Fig. 2E and F). Thus, there may be two different proteins involved in the regulation of late transcription: one responsible for qualitatively turning on the transcription of specific late RNA species, and a second for insuring a high rate of transcription of late messages. Alternatively, only a single protein may be required, with low amounts sufficient to turn on late transcription but continuous synthesis required to achieve the maximum rates of transcription.

Support for the existence of two different proteins was found by examining the kinetics of RNA synthesis in cells infected with a temperature-sensitive mutant of FV3, ts 2436. Figure 3 illustrates the rates of RNA synthesis at various times after infection by this mutant at the nonpermissive temperature (30°C). This mutant synthesizes viral DNA (10), but even at 8 h p.i. (Fig. 3D) the late viral RNAs (notably ICRs 560, 534, 240, and 235) were synthesized at a much lower rate at 30°C than at 25°C (Fig. 3E). In cells infected with this mutant, late transcription was turned on, and abundant viral DNA template was available, yet the rate of late transcription remained low. This finding indicates that a second protein, independent of both the "turn-on" protein for late RNA synthesis and of DNA

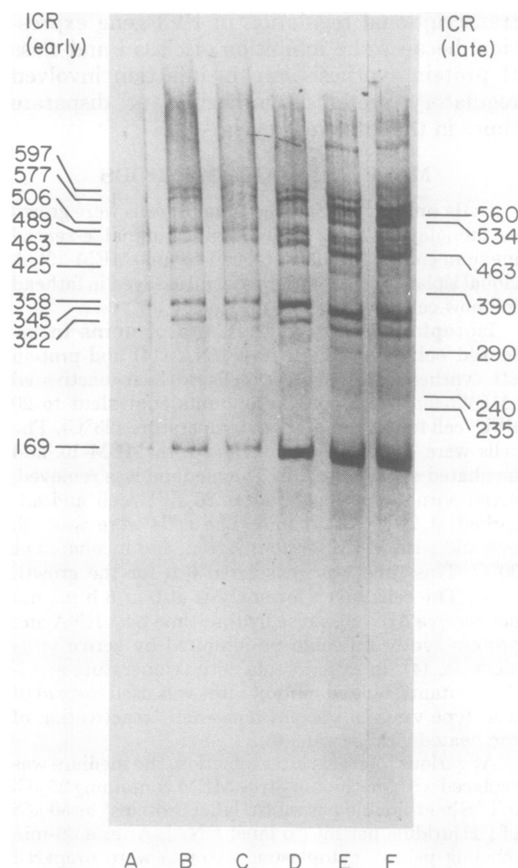

FIG. 2. *Two virus-specific proteins regulate late transcription. All cultures were labeled at 6 h p.i. for 30 min with [^{3}H]uridine (50 μCi/ml). Medium containing FPA (100 μg/ml) replaced the regular medium at the indicated times. Other details are as described in Fig. 1. Time of addition of FPA: (A) 0 h, (B) 1 h, (C) 1.5 h, (D) 2 h, (E) 3 h, (F) 4 h. ICR, Infected cell RNA, molecular weight × 10^{-3}.*

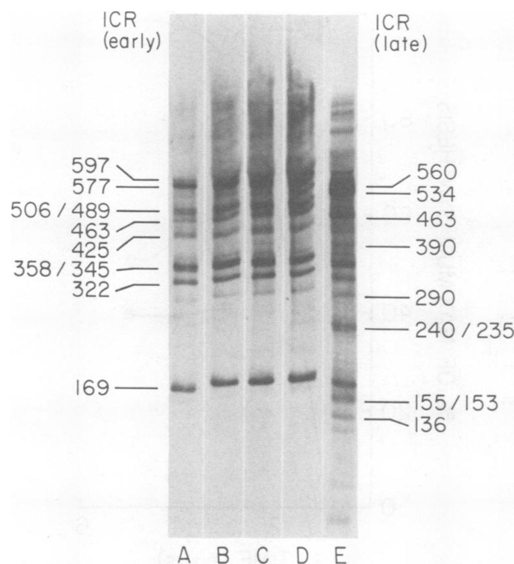

FIG. 3. *ts 2436 is defective in regulating the rate of late transcription. Cultures were exposed to Δts 2436 for 4 h before infection with active ts 2436 (see legend to Fig. 1 for details). Infected cells were incubated at 30 or 25°C and labeled at the indicated times with 50 μCi of [^{3}H]uridine per ml. (A) 2 to 2.5 h, 30°C; (B) 4 to 4.5 h, 30°C; (C) 6 to 6.5 h, 30°C; (D) 8 to 8.5 h, 30°C; (E) 6 to 6.5 h, 25°C. ICR, Infected cell RNA, molecular weight × 10^{-3}.*

synthesis, controlled the rate of late transcription.

Two viral proteins are involved in post-transcriptional control. Late in the course of a typical FV3 infection, the rates of synthesis of both class I and class II early proteins go down (13). This decreased rate of synthesis occurs even in the face of high rates of early RNA synthesis, indicating that this process is under post-transcriptional control. In addition to blocking the appearance of late viral RNAs, the presence of FPA from the beginning of infection prevented this decline in the rates of synthesis of both classes of early proteins late in infection; this observation suggested that virus-specified proteins were involved in post-transcriptional as well as transcriptional control. We therefore investigated (i) the number of viral proteins involved in post-transcriptional control and (ii) the time during the replication cycle of FV3 at which these regulatory proteins appeared by adding FPA at different times after infection and examining the RNA and protein synthesis patterns late in infection, i.e., at 6 h p.i. We reasoned that, if a regulatory protein were synthesized before we added FPA, then class I and class II protein synthesis would decrease as usual. However, if the regulatory protein were normally synthesized after the time we added FPA, then this protein might not function and the synthesis of class I or class II proteins would continue at a high rate.

Figure 4 shows the changes in the pattern of infected cell protein (ICP) synthesis in FV3-infected cells that occurred when we added FPA at different times after infection. In each sample the cells were labeled with [^{35}S]methionine between 6 and 6.5 h p.i. We previously showed that no late proteins were made when FPA was present from the beginning of infection, presumably because of the lack of late viral mRNA (13). When we added FPA at 1 or 1.5 h p.i. (Fig. 4A and B), we saw that late (class III) proteins (ICP 62, 55, 19) were synthesized at 6 h p.i., although at a markedly lower rate when compared with control cells that had not been treated with FPA (12), or with cells that did not receive FPA until 4 h p.i. (Fig. 4F). However, in contrast to normal infection, the rate of synthesis of class I (e.g., ICP 68, 65, 47/45, 35) and class II (e.g., ICP 18, 50, 37) proteins did not decrease by 6.5 h p.i. when FPA was added at 1 to 1.5 h p.i. When FPA was added at 2 h p.i. (Fig. 4C), the rate of

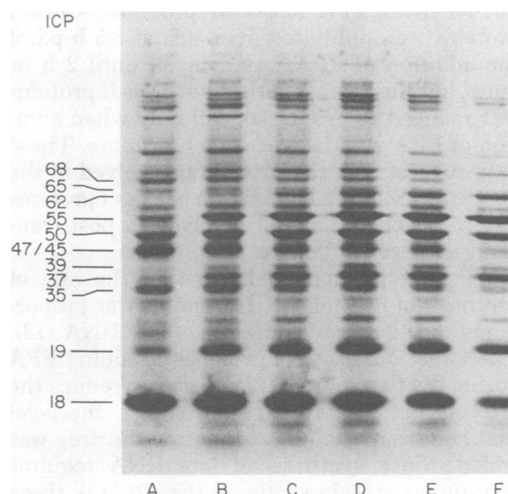

FIG. 4. *Two proteins are involved in post-transcriptional regulation of FV3 protein synthesis. All cultures were labeled with 25 μCi of [^{35}S]methionine per ml at 6 h p.i. for 30 min. Medium containing FPA (100 μg/ml) replaced the regular medium at the indicated times. Cytoplasmic extracts from cells were prepared and electrophoresed as described in the text. From 10 to 20 μg of protein was placed in each well. The film was exposed for 48 h. ΔFV3 was not used for the 6-h sample because active virus alone inhibited cellular protein synthesis at that time. (A) FPA added at 1 h p.i.; (B) FPA added at 1.5 h p.i.; (C) FPA added at 2 h p.i.; (E) FPA added at 3 h p.i.; (F) FPA added at 4 h p.i. ICP, Infected cell protein, molecular weight × 10^{-3}.*

synthesis of class I proteins was reduced. However, class II protein synthesis was not reduced, which suggested that the viral regulatory protein(s) involved in the inhibition of class I (but not class II) protein synthesis was produced between 1 and 2 h p.i. Once synthesized, this protein remained active in the infected cells up to 6.5 h p.i. The rate of synthesis of the class II proteins decreased only when the addition of FPA was delayed by 3 h or more (Fig. 4E and F), giving a pattern of viral protein synthesis similar to the one observed in normal infection (13).

Figure 5 shows the quantitative determination of the molar rates of synthesis of representative class I (ICP 68), class II (ICP 18), and class III (ICP 55) proteins. FPA was added at the indicated times, and the infected cells were labeled for 30 min at 6 h p.i. The relative amount of radioactivity distributed in the individual protein bands, measured by computer planimetry of the densitometric tracing of the autoradiogram (13), was compared either with that of the same protein in cells which received FPA at the beginning of infection (class I and II proteins), or with that of the polypeptides in cells not receiving FPA (class III proteins). Class I proteins were inhibited over 40% at 6.5 h p.i. if the addition of FPA was delayed until 2 h or more, but the rate of synthesis of class II proteins was reduced to 50% of control only when addition of FPA was delayed by 4 h or more. These data suggest that the protein(s) involved in the post-transcriptional regulation of class I proteins differed from the one(s) involved in post-transcriptional control of class II proteins.

We have previously shown that the rate of synthesis of late (class III) proteins was proportional to the rate of synthesis of late RNA (13). The effect on class III proteins of adding FPA during the first 2 h p.i. (Fig. 5) was to reduce the rate of class III protein synthesis to the level that had been attained at the time the drug was added. Since synthesis of late RNA requires continuous protein synthesis (Fig. 2) (13), these data confirm the conclusion that late protein synthesis is regulated at the level of transcription.

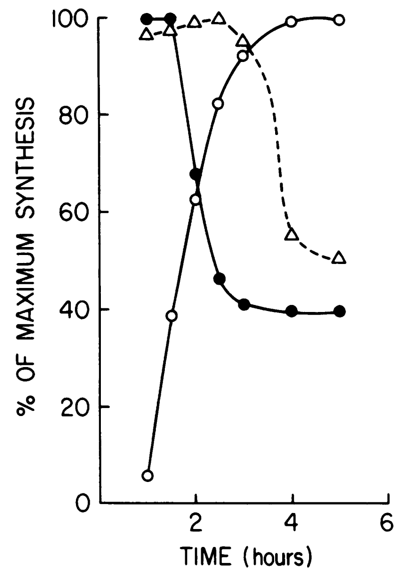

FIG. 5. *Quantitative analysis of the rates of synthesis of selected FV3 proteins. Cultures were labeled for 30 min at 6 h p.i. with 25 μCi of [^{35}S]methionine per ml. The time on the abscissa indicates the hour when FPA was added to the infected cell cultures. The specific activity of each protein was determined by scanning the autoradiogram with an Ortec densitometer interfaced with a PDP 8/1 minicomputer (13) and determining the percentage of density of the area under each peak to that of the total scan. Changes in the rates of class I and class II protein synthesis are expressed as the percent change in the specific activity of these viral proteins in cells treated with FPA at indicated times to those in infected cells which received FPA at the beginning of infection. Changes in the rate of late protein synthesis are expressed as the percent change in the specific activity of ICP 55 in treated cells to that in infected cells which were not treated with FPA. Symbols: ●, relative rate of synthesis of a representative class I protein (ICP 68); △, relative rate of synthesis of a representative class II protein (ICP 18); ○, relative rate of synthesis of a representative late protein (ICP 55).*

ts 3674 is defective in the protein that regulates the synthesis of class II proteins. In a previous study of the altered regulation of viral protein synthesis by temperature-sensitive mutant ts 3674, we similarly concluded that a virus-specific protein was responsible for the decrease in the rate of synthesis of some of the early proteins (4). However, in that study, the resolution of infected cell proteins was poor; a detailed analysis of the rates of synthesis of various proteins was not performed, and the early proteins were not separated into two distinct classes. Therefore, we repeated the analysis of protein synthesis in ts 3674-infected cells (Fig. 6). After 7 h p.i. at nonpermissive temperature (30°C) in ts 3674-infected cells (Fig. 6A), the rate of synthesis of class III (e.g., ICP 62, 55, 19) proteins was high and that of class I (e.g., ICP 68, 65, 47, 45, 35) proteins was low, as in wild-type FV3-infected cells (13). However, there was no reduction in the rate of synthesis of class II proteins (e.g., ICP 50, 39/37, 18). When ts 3674-infected cells were shifted to permissive temperature (25°C) at 7 h p.i. and labeled (7 to 8 h p.i.)

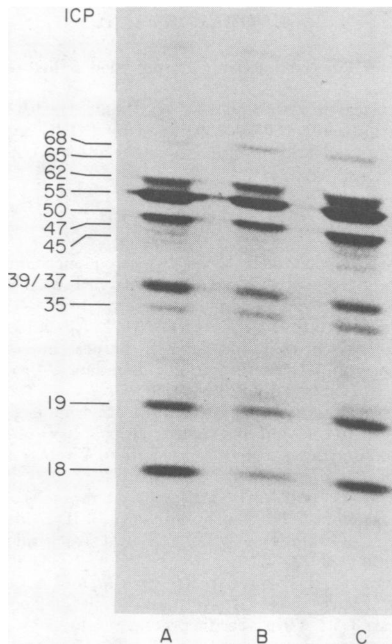

FIG. 6. *Class II protein synthesis is altered in ts 3674-infected cells. Cultures were labeled with 25 μCi of [35S]methionine per ml for 60 min at indicated times. Other details are as described in the legend of Fig. 4. (A) Infected cells incubated at 30°C and labeled from 7 to 8 h p.i. (B) Infected cells incubated at 30°C for 7 h and then shifted to 25°C and labeled immediately for 1 h. Actinomycin D (10 μg/ml) was added at 6.75 h p.i. and was present during the labeling period. (C) Cells incubated at 30°C and labeled from 7 to 8 h p.i. Actinomycin D (10 μg/ml) was added at 6.75 h p.i. and was present during the labeling period.*

with [^{35}S]methionine, there was an immediate reduction in the rate of synthesis of class II proteins, even in the presence of actinomycin D (Fig. 6B). Actinomycin D, added to ts 3674-infected cells kept at 30°C (Fig. 6C), caused no reduction in the rate of class II protein synthesis, showing that the drug alone was not responsible for the sudden decrease in class II protein synthesis.

The half-life of FV3 mRNA's is about 5 h (4), which eliminates the possibility that the reduction in the synthesis of class II proteins upon shift-down to 25°C was due to cessation of synthesis of mRNA for class II proteins. Our interpretation of these results is that, in ts 3674-infected cells, the viral regulatory protein that inhibited the rate of synthesis of class II proteins was defective at nonpermissive temperature; upon shift-down this protein immediately became functional and acted to reduce the synthesis of this class of proteins.

DISCUSSION

The results reported here strongly support our previous conclusions (3-5, 13, 14) that viral regulatory proteins control both viral transcription and viral protein synthesis in FV3-infected cells. In the realm of transcriptional control, this study shows that one virus-specific protein turned on the synthesis of specific late viral RNAs, whereas another regulated the rate at which these RNAs were produced. Both qualitative (on-off) and quantitative (abundance) controls of transcription have been described for two other large DNA viruses—herpesvirus (2, 12) and poxvirus (1). In each of the above investigations the data were obtained by hybridization kinetics, because resolution of individual RNA species had not yet been achieved in these systems. The analysis of the hybridization data from herpesvirus- or poxvirus-infected cells during inhibition of protein or DNA synthesis could be interpreted to mean that a virus-specific protein was directly required for either on-off or abundance transcriptional controls. However, the accepted explanation was that the necessary viral protein was involved in DNA rather than RNA synthesis, and that the state of the template (replicating or not) controlled the class and rate of RNA synthesis. Using FPA or a transcriptional defective mutant, as reported here and in a separate communication (D. B. Willis, R. Goorha, and A. Granoff, Virology, in press), we have demonstrated that at least one FV3-specific protein is required for late transcription without regard to DNA synthesis. Using a temperature-sensitive mutant (ts 2436), we have presented evidence that a second viral protein may also be required for the increased rate of transcription of late viral RNA species and that this increased rate of transcription is not coupled to viral DNA replication (Fig. 3). However, evidence for the existence of the second protein is not unequivocal. It is possible that the protein which turns on late transcription also controls the abundance; i.e., more of this protein is required for an increased rate of transcription. In ts 2436-infected cells at nonpermissive temperature this regulatory protein may retain some functional activity; thus, late transcription is turned on, but an increased rate of transcription is not achieved.

Our results also indicate that at least two virus-specific proteins were involved in the post-transcriptional regulation of FV3 protein synthesis. The evidence for the involvement of two proteins in post-transcriptional control of protein synthesis is, however, indirect. Direct proof for the existence of these proteins can only be obtained by devising a suitable in vitro protein-synthesizing system in which inhibition of syn-

thesis of class I and class II proteins by two different viral regulatory proteins can be demonstrated. Post-transcriptional control of viral protein synthesis has been suggested in herpesvirus- (7) and vaccinia virus-infected (9) cells. Viral proteins in herpesvirus infection have been classified, based on the time at which they appear, into three groups—α, β, and γ (6). Honess and Roizman (7) used the amino acid analog canavanine to show that the presence of a functional herpesvirus β polypeptide was required to shut off the synthesis of α polypeptides and that a functional γ polypeptide was required to shut off the synthesis of β polypeptides. This inhibition was probably mediated at the post-transcriptional level.

One major difference between the synthesis of FV3 and herpesvirus proteins seems to be that the presence of an amino acid analog from the beginning of herpesvirus infection resulted in the production of α polypeptides only (7), whereas in FV3 infection both class I and class II proteins were produced in the presence of FPA. However, only a subset of the FV3 RNAs synthesized in the presence of FPA was produced when cycloheximide was used to inhibit protein synthesis (15). These "cycloheximide RNAs" could not be translated in vivo upon removal of the drug, but made authentic viral proteins in a messenger-dependent reticulocyte lysate (Willis and Raghow, manuscript in preparation). If we use the α, β, γ terminology of Honess and Roizman (7) to classify groups of proteins whose synthesis is qualitatively turned on in a temporal manner, then the FV3 proteins identified by translation of the cycloheximide RNAs in vitro would be equivalent to the herpesvirus α proteins. The FV3 α proteins include in their number both class I and class II proteins, which are defined on a quantitative basis. It is possible that, as with herpesvirus, an FV3 α protein switched on the synthesis of the FV3 β proteins, hereby defined as all remaining early proteins not synthesized by cycloheximide RNAs in vitro. However, in the case of FV3, this function of the α proteins was not affected by analog substitution. The FV3 β proteins also include representatives of both class I and class II; all FV3 γ proteins are class III. Translation of FV3 mRNA's in vitro, which has recently been accomplished in our laboratory, will enable us to decide which RNAs are translated into specific proteins, and may even allow us to isolate and assay in vitro the factors responsible for the post-transcriptional control we observe in vivo.

ACKNOWLEDGMENTS

Ramona Tirey and Susan Carr provided skilled technical sssistance.

This research was supported by Public Health Service research project grant CA-07055 and by CORE grant CA-21765 from the National Cancer Institute and by ALSAC.

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