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Biogenesis of poxviruses: Role for the DNA-dependent RNA polymerase II of the host during expression of late functions

(effects of α-amanitin on development/host polymerase II mutant in replication/role of polymerase II in late transcription)

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ABSTRACT **The participation of host RNA polymerase II in the vaccinia life cycle was examined by comparing efficiency of multiplication after treating the Ama$^+$ sensitive and Ama 102 drug resistant lines with α-amanitin. In the latter, resistance is due to a mutation in RNA polymerase II. The toxin profoundly reduces synthesis of virus-specified polypeptides and morphopoeisis in Ama$^+$ but not in Ama 102 rat myoblasts without appreciably altering vaccinia DNA replication in either cell type. This implicates RNA polymerase II in the expression of late virus functions. Circumstantial evidence from a model system indicates that γ irradiation of the host prior to infection might disrupt transcription into functional mRNA from the nucleus. Irradiation does not, however, alter the capability of the host to support vaccinia multiplication fully. Therefore, ongoing host nuclear transcription may not be required by this virus. The above results are consistent with the ability of cytoplasts to produce small quantities of mature progeny. Our studies lead us to hypothesize that RNA polymerase II or a subunit of the host enzyme may participate directly in late transcription of the vaccinia genome.**

The highly active toxin α-amanitin, derived from the toadstool *Amanita phalloides*, has been recognized as a specific inhibitor of DNA-dependent RNA polymerase II, hereafter referred to as polymerase II, of animal cells (1). By virtue of its inhibitory specificity, α-amanitin was used to demonstrate that certain DNA and RNA agents, such as papovaviruses, adenoviruses, and influenza viruses, having an obligatory developmental stage in the host nucleus, most probably require polymerase II activity for replication (2–4). By contrast, the poxviruses, which develop in the cytoplasm, were reported to be insensitive to this toxin (as cited in ref. 5) and to contain in the virion core an α-amanitin-insensitive DNA-dependent RNA polymerase (5). However, some requirement for the host nucleus is implied in the replication of vaccinia because virus development in cytoplasts is incomplete (6, 7). The availability of the rat myoblast L_6 cell line, which our initial experiments showed can support the growth of vaccinia and from which a mutant was derived having a polymerase II resistant to α-amanitin (8), prompted us to examine the possible role of host transcriptional function(s) in the life cycle of poxviruses.

MATERIALS AND METHODS

Cells and Viruses. Monolayers of L_2 mouse fibroblasts were used for virus propagation and assays of plaque-forming units (PFU) in nutrient medium and under culture conditions described (9). The viruses used were the hemagglutinin-inducing parental IHD-J or the syncytogenic IHD-W variant of vaccinia (10) and the Indiana strain of vesicular stomatitis virus (VSV). For inoculation, 10 PFU/cell usually were added as reported elsewhere (11).

To investigate the role of host-derived functions in development of vaccinia we used (*i*) a clone L6H9, designated Ama$^+$, and an α-amanitin-resistant mutant Ama 102 derived from this clone of a rat myoblast line (8), both kindly provided by M. E. Pearson (University of Toronto), and (*ii*) temperature-sensitive mutant 422E derived from hamster BHK21 fibroblasts, which is conditional-lethal for 28S ribosomal RNA formation and assembly of the 60S ribosomal subunit (12), provided by H. E. Meiss (New York University Medical School).

Synthesis and Labeling. Cytoplasmic DNA synthesis in IHD-W vaccinia-infected Ama$^+$ and Ama 102 cells was measured by continuous labeling, at 37°C for 4 hr after inoculation, in the presence of 1 μCi of [*methyl*-^{3}H]thymidine per ml (New England Nuclear) as described (13). Briefly, labeled cells were allowed to swell in hypotonic saline/buffer solution, then were disrupted in a Dounce homogenizer. The radioactivity of trichloroacetic acid-precipitable material was measured in a scintillation counter. Procedures used by this laboratory for labeling and characterizing nascent polypeptides have been described (14, 15). Briefly, at 9.5 hr after inoculation, monolayers were exposed for 60 min to 20 μCi of [^{35}S]methionine per ml (New England Nuclear) added to methionine-free nutrient medium. Samples for preparing cytoplasmic extracts were taken either at the end of the pulse or after 8 hr of incubation in chase medium. Aliquots were used for determination of trichloroacetic acid-precipitable radioactivity and for polyacrylamide gel electrophoresis.

Inhibitors. Synthesis of RNA was suppressed by adding to the nutrient medium 4 μg of actinomycin D per ml (Sigma); protein was inhibited with 10 μg of streptovitacin A per ml (gift from Upjohn) as described (11). In the experiments involving Ama$^+$ and Ama 102, rat myoblast cultures were treated with 2 μg of α-amanitin per ml (Sigma) according to ref. 8.

Electron Microscopy. The methods for collecting and preparing cell samples for thin sectioning and examination by transmission electron microscopy were the same as those described in previous studies (11).

RESULTS

Vaccinia Virus Replication Examined by Means of Host Cell Mutants and α-Amanitin. To investigate the role of host transcription into mRNA involving polymerase II (1), we used α-amanitin, which specifically inhibits polymerase II of animal cells when used at appropriately low concentrations (1). The host used was a myoblast cell line in which the wild-type cell is Ama$^+$ and the mutant, containing α-amanitin-resistant polymerase II, is designated Ama 102 (8). Because the permeability and time required to inhibit polymerase II activity varies depending on the cell (16), it was necessary to ascertain the

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Abbreviations: polymerase II, DNA-dependent RNA polymerase II; PFU, plaque-forming units; VSV, vesicular stomatitis virus.

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duration of treatment with α-amanitin to effect an inhibition of vaccinia replication. The data in Table 1 show that treatment of Ama$^+$ cells for 5 or 10 hr abolished the capacity of the host to support production of infectious progeny, while shorter treatment resulted in fractional yields of PFU. Because the inhibitor was also kept in the culture medium throughout the growth cycle, it may be concluded that suppression of replication was elicited gradually and progressively. This is consistent with the slow inhibition of polymerase II activity by α-amanitin demonstrated biochemically (17) and implies that this host enzyme or a subcomponent of it is necessary for formation of infectious vaccinia virus. In sharp contrast to the above findings, treatment of Ama 102 cells with the drug had no inhibitory effect on the yield of vaccinia PFU (Table 1), also indicating the involvement of polymerase II in the vaccinia virus cycle.

As a control of the specificity of α-amanitin effects on vaccinia, replication of VSV was tested under parallel circumstances. The data shown in Table 1 revealed that the drug did not suppress VSV production in either Ama$^+$ or Ama 102 cells. Yields in Ama 102 were usually almost an order of magnitude greater than in Ama$^+$, an observation which deserves further attention.

The effects of α-amanitin on synthesis of virus-specified products, including the synthesis of DNA and polypeptides in the cytoplasm, were monitored by isotopic labeling, polyacrylamide gel electrophoresis, and enumeration of progeny particles by quantitative electron microscopy.

Analysis of cytoplasmic extracts from untreated and drug-treated cells labeled with [^{3}H]thymidine showed that, in Ama$^+$, in the absence of α-amanitin about 3300 cpm per 10^6 cells were incorporated and, in cells pretreated for 10 hr and maintained in the presence of the drug during infection, 2600 cpm per 10^6 cells were converted into an acid-precipitable product. In Ama 102, cytoplasmic extracts from both untreated and treated cells contained about 23,000 cpm of [^{3}H]thymidine per 10^6 cells incorporated into a macromolecular product. These data revealed that α-amanitin either does not inhibit or inhibits only partially vaccinia-specified DNA synthesis. As with VSV replication, the much greater rate of vaccinia-related DNA synthesis in Ama 102 cells remains unexplained.

The influence of α-amanitin on the spectrum of vaccinia-specified polypeptides synthesized in Ama$^+$ and Ama 102 cells was ascertained by pulse-chase experiments and polyacrylamide gel electrophoresis. According to the usage adopted in this laboratory, individual polypeptides in gels are identified by their molecular weight so that, for example, p94 is a polypeptide of M_r 94,000. The results illustrated in Fig. 1 show that when Ama$^+$ was the host, the quantity of [^{35}S]methionine-labeled polypeptide formed in the presence of the toxin was

Table 1. Virus replication in wild-type Ama$^+$ and resistant Ama 102 rat myoblasts treated with α-amanitin

Host	Untreated	Hr before inoculation when treatment commenced			
		0	−2	−5	−10
		IHD-W vaccinia virus			
Ama$^+$	1000*	62	39	6	6
Ama 102	700	570	510	630	680
		Vesicular stomatitis virus			
Ama$^+$	860	1,100	ND†	ND	1,200
Ama 102	7700	16,000	ND	ND	18,000

* Virus titers are expressed as 10^4 PFU/10^6 cells.

† ND, not done. At 3 hr after inoculation the background titer for vaccinia virus-infected Ama$^+$ and Ama 102 cells was ≈6 × 10^4 PFU.

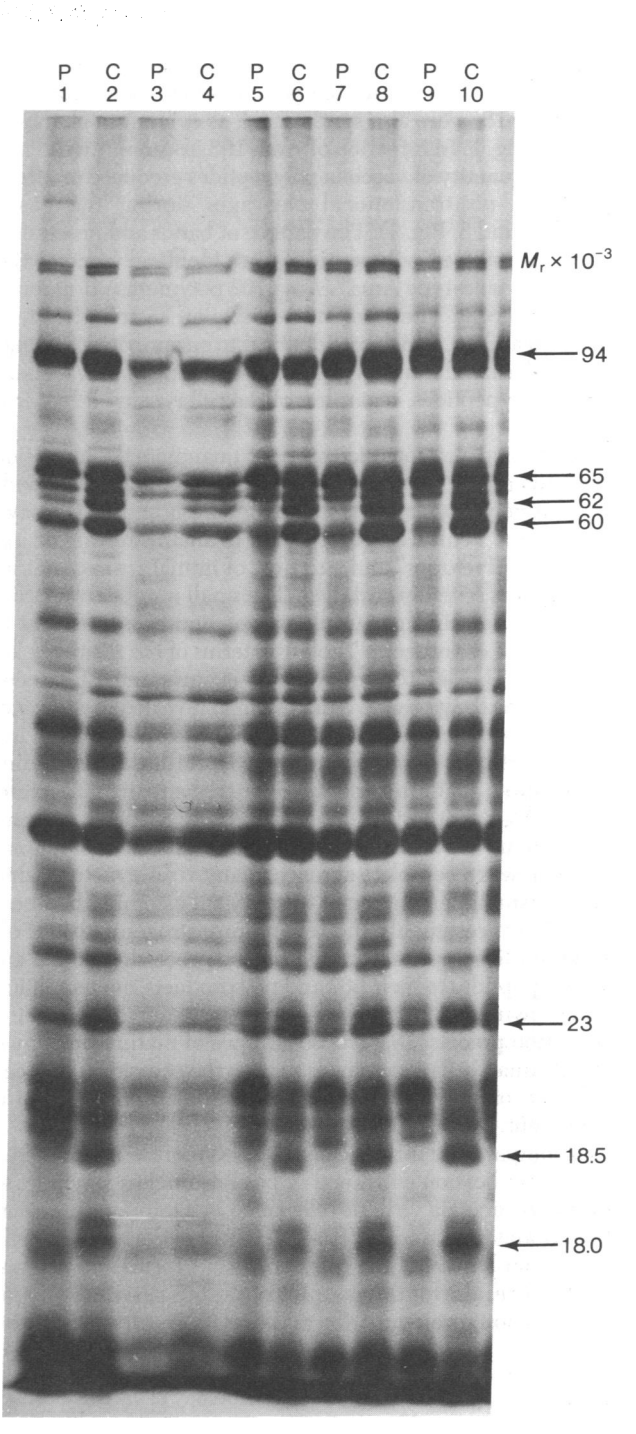

FIG. 1. Fluorogram of polypeptide profiles from untreated and α-amanitin-treated cultures in a slab gel prepared from infected L_2 Ama$^+$ and Ama 102 whole cell extracts according to refs. 14 and 15. After pulse labeling with [^{35}S]methionine and chasing, cells were disrupted by sonication in the presence of a nonionic detergent as described (14, 15), with the exception that the lysate buffer solution contained 1 mM $MgCl_2$. Treatment with 50 μg of pancreatic deoxyribonuclease I per ml (Worthington) for 15 min at 4°C was used to hydrolyze the DNA. Into each channel was placed 10–50 μg of protein and the film was exposed for 3 days. The vertical scale showing $M_r \times 10^{-3}$ was calculated from polypeptides used as M_r standards. Arrows denote the positions of seven polypeptides: p94, p65, p62, p60, p23, p18.5, and p18. P, pulse; C, chase. Channels: (1–4) Ama$^+$ extracts; (5–8) Ama 102 extracts; (9 and 10) L_2 extracts. Channels 3, 4, 7, and 8 show samples from drug-treated cells.

much less than in its absence (channels 3 and 4). However, the inhibitor did not abolish the synthesis of the p94 and p65 precursors nor their processing to the p62 and p60 products. Most or all of the other identifiable polypeptides, including p23, p18.5, and p18, were also synthesized, as evident in channels 3 and 4 of Fig. 1. In infection of Ama 102, α-amanitin did not reduce the quantity of vaccinia polypeptides produced or affect the normal posttranslational cleavages during the chase (channels 7 and 8, Fig. 1). The pattern of bands and processing of vaccinia-induced polypeptides were identical whether the host was the wild-type Ama$^+$, Ama 102 polymerase II mutant, or mouse L_2 cells. The comparative data on L_2 cells shown in channels 9 and 10, which are identical to our previous findings (18), emphasize the uniformity of the spectrum of vaccinia-induced polypeptides, regardless of the cell type used for virus replication.

Results using electron microscopy to quantitate the formation of vaccinia related structures (Table 2) corroborated the findings from polyacrylamide gel electrophoresis and PFU assays. Only in Ama$^+$ cultures treated with α-amanitin was the appearance of "factories" and assembly of immature and mature progeny virus severely curtailed. The small number of mature progeny evident in this sample was obviously insufficient to alter the titer, expressed in Table 1 in terms of the relatively less sensitive PFU. Electron microscopic examination of 210 cell profiles in the drug-treated Ama$^+$ sample revealed the presence of 27 mature virions, all situated in only 3 profiles, and 48 immature particles, observed in only 6 profiles. This finding demonstrated that after treatment with the toxin, when rates of virus DNA synthesis were only partially reduced, vaccinia development was arrested to a varying degree, so that in some cells there was a complete absence of any virus structure while in a very small percentage of others virus assembly had been completed. Presumably the few mature progeny virions observed in this sample accounted for the processing of p94 and p65 polypeptides to their p62 and p60 products, evident in Fig. 1 and shown previously to be an obligatory step in the vaccinia maturation process (18). The occurrence of mature progeny in treated Ama$^+$ cells also implies that, in the occasional host cell, inhibition of polymerase II may have been only partial or nonexistent.

To compare the influence of the polymerase II function in vaccinia replication with another possible nuclear function, we examined vaccinia virus development in a temperature-sensitive mutant 422E derived from BHK21 cells. The 422E cell is conditional-lethal for 28S ribosomal RNA formation and, as a consequence, also for the assembly of the 60S ribosomal subunit. With this host, preincubation at the restrictive temperature of 39°C for 24 hr or longer followed by infection with IHD-W vaccinia, also at 39°C, failed to suppress virus development, as judged by a comparison of the amount of virus formed at 39°C and at the permissive temperature of 33°C. This finding implies that formation of nascent ribosomes prior to or during infection was not required for vaccinia replication.

Effects of γ Irradiation on Vaccinia Replication. Published evidence implicating a role of the host nucleus in vaccinia biogenesis (6, 7) suggested that the continued template activity of the host DNA might be required for completion of the virus life cycle. This idea was tested by exposing monolayers of L_2 cells to intense γ irradiation from a ^{60}Co source 4 hr prior to inoculation. Treatment with 70,000 rads (1 rad = 1.0×10^{-2} J/kg) was lethal for these cells, the majority of which died within 48 hr. Nevertheless, when infected 4 hr after exposure they remained fully competent to produce infectious vaccinia, as evident by the burst size, ≈100 PFU/cell, obtained from both control and irradiated cultures. In these cells mitosis can be abolished by <5000 rads. Our observation suggests that extensive damage to the host DNA by γ irradiation did not impair vaccinia production.

To ascertain whether high doses of γ irradiation can cause the abolition of gene expression, we used as a model of transcription and translation infection by IHD-J vaccinia. This model was selected because cytoplasmic virus DNA replication occurs synchronously 1–4 hr after infection and because the IHD-J strain induces production of hemagglutinin as one of the late, late viral functions (19), whereby the requisite transcription and translation commences at approximately 4 hr after infection, when late virion-related polypeptide synthesis is already well underway (11). The results (Table 3) revealed that cells irradiated with 50,000 rads immediately after completion of virus DNA synthesis were able to produce only small quantities of hemagglutinin and infectious particles. Exposure to 5000 rads caused only a partial inhibition of virus-specified synthesis. Application of inhibitors, used as the controls, revealed that actinomycin D if added after DNA synthesis at a concentration sufficient to block transcription rapidly, caused reduction of hemagglutinin and virus formation to approximately the same low level as a dose of 50,000 rads. As anticipated, streptovitacin A, an inhibitor of translation, also inhibited the formation of virus materials. These combined data imply that intense irradiation of the host before infection probably caused severe damage to nuclear DNA and affected transcription into functional mRNA without reducing synthesis of infectious vaccinia virus.

Table 2. Quantitative electron microscopy of vaccinia virus replication in wild-type Ama$^+$ and resistant Ama 102 rat myoblasts treated with α-amanitin

Host cell and treatment	Vaccina virus products			% cell profiles with mature progeny virions
	"Factories"	Immature particles	Mature virions	
Ama$^+$, no drug	41	436	325	35
Ama$^+$ + α-amanitin	8	23	13	1–2
Ama 102, no drug	56	600	332	30
Ama 102 + α-amanitin	78	946	844	56

The data are normalized as counts per 100 profiles of thinly sectioned cells. In the sample of Ama$^+$ with α-amanitin, over 200 profiles were examined.

Table 3. Comparison between effect of γ irradiation and inhibitors of synthesis on vaccinia virus replication and hemagglutinin production

Treatment	% virus produced	HA end-point titer
None	100	1024
5000 rads	26	512
50,000 rads	16	64
Actinomycin D	6	64
Streptovitacin	6	16

L-cell monolayers were inoculated with IHD-J vaccinia and incubated for 4 hr at 37°C. Individual cultures were either irradiated by a ^{60}Co γ source (calibrated to deliver 120 rads/sec) or placed in medium containing actinomycin D or streptovitacin A, then incubated for an additional 20 hr. Hemagglutinin (HA) was assayed as described (10), with the exception that Ca^{2+} and Mg^{2+} were omitted from the phosphate-buffered saline. Virus yields in PFU and HA titers were calculated per 10^6 cells. In untreated cultures ≈300 PFU/cell were formed.

DISCUSSION

Current experiments using as the host the α-amanitin-sensitive Ama^+ and drug-resistant Ama 102 mutant rat myoblasts clearly demonstrate the involvement of a host function related to polymerase II in the life cycle of a poxvirus. The question as to whether ongoing transcription from the nucleus of the host is absolutely required has not been resolved unequivocally. Our observations with host cells extensively γ irradiated before infection favor the idea that transcription from the nucleus into functional mRNA may not be obligatory for completion of the vaccinia cycle of development. This presumption might appear to be paradoxical in relation to data indicating that vaccinia fails to complete its maturation in infected cytoplasts (6). However, upon careful examination of the article detailing the results with cytoplasts (6), it becomes clear that occasionally enucleated cells can produce mature vaccinia progeny. Because polymerase II occurs in and is able to be isolated from the cytoplasm, it could be available to the virus even in the absence or dysfunction of the cell nucleus. Turnover would, of course, deplete the pool of this enzyme with time.

Concerning pulse–chase experiments and polyacrylamide gel electrophoresis of the synthesis of vaccinia-specified polypeptides, once again information derived from infection of cytoplasts (6, 7) might appear to be in conflict with our previous findings which indicated that posttranslational cleavage, including that of p94 and p65 precursors to the p62 and p60 products, is obligatory for completion of virion maturation (18). However, if one keeps in mind the results of Pennington and Follett (6) concerning the variable virus development among cytoplasts sometimes culminating in the formation of mature progeny, then our present findings with Ama^+ cells treated with α-amanitin as the host are quite consistent with observations made on cytoplasts. In both systems the mass of isotopically labeled virion proteins is reduced but processing occurs normally, accounting for the presence of a few mature virions.

From the above, the most plausible hypothesis, which takes into consideration all the available information, should assume that early vaccinia functions, expressed initially from the virion core, then after uncoating, and including those required for DNA replication, are catalyzed by a virus DNA-dependent RNA polymerase whereas some or all of the late functions involve host polymerase II. Whether the entire polymerase II is monopolized in this process or perhaps only one of the enzyme subunits is unknown. It should, however, be remembered that both the vaccinia RNA polymerase and polymerase II are multicomponent enzymes (1, 20). Because *in vitro* experiments demonstrate specificity of low concentrations of toxin for polymerase II of Ama^+ cells (16) and experiments on attachment with radioactive α-amanitin by use of a cross-linking agent suggest, but by no means certify, that the toxin acts by specific binding to the p140 subunit of polymerase II (21), it is not inconceivable that this subunit functions in concert with vaccinia RNA polymerase subunits in the late transcription process. Precedents for this notion have been established in the prokaryotes, as documented on the modification of host RNA polymerase by some bacteriophages of *Escherichia coli* and *Bacillus subtilis* (reviewed in ref. 22). The possibility that viral and host polymerase enzyme subunits can be assembled into a hybrid transcriptional enzyme may be testable if, in the future, host mutants become available in which different polymerase II subunits are genetically altered.

After the initial submission of this article a paper appeared dealing with the role of the host cell nucleus in vaccinia replication (23). The data of Hruby *et al.* (23), like our own, document the sensitivity of vaccinia virus replication to α-amanitin. However, our hypothesis and that of the other workers diverge in that Hruby *et al.* implicate direct involvement of the host nucleus in the vaccinia cycle.

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1. Roeder, R. G. (1976) in *RNA Polymerase*, eds. Losik, R. & Chamberlin, M. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 285–329.
2. Amati, P., Blasi, F., Di Porzio, V., Riccio, A. & Traboni, A. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 753–757.
3. Ledinko, N. (1971) *Nature (London)* **233**, 247–248.
4. Lamb, R. A. & Choppin, P. W. (1977) *J. Virol.* **23**, 816–819.
5. Costanzo, F., Fiume, L., LaPlaca, M., Mannini-Palenzona, A., Novello, F. & Stripe, F. (1970) *J. Virol.* **5**, 266–269.
6. Pennington, T. H. & Follett, E. A. C. (1974) *J. Virol.* **13**, 488–493.
7. Hruby, D. E., Guarino, L. A. & Kates, J. (1979) *J. Virol.* **29**, 705–715.
8. Somers, D. G., Pearson, M. L. & Ingles, C. J. (1975) *J. Biol. Chem.* **250**, 4825–4831.
9. Dales, S., Milovanovitch, V., Pogo, B. G. T., Weintraub, S. B., Huima, T., Wilton, S. & McFadden, G. (1978) *Virology* **84**, 403–428.
10. Ichihashi, Y. & Dales, S. (1971) *Virology* **46**, 533–543.
11. Dales, S. & Mosbach, E. H. (1968) *Virology* **35**, 546–583.
12. Toniolo, A., Meiss, H. E. & Basilico, C. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 1273–1277.
13. Pogo, B. G. T. & Dales, S. (1971) *Virology* **43**, 144–151.
14. Stern, W. & Dales, S. (1976) *Virology* **75**, 242–255.
15. Lake, J. R., Silver, M. & Dales, S. (1979) *Virology*, in press.
16. Ingles, C. J., Beatty, B. G., Guialis, A., Pearson, M. L., Creras, M. M., Lobban, P. E., Siminovitch, L., Somers, D. G. & Buchwald, M. (1976) in *RNA Polymerase*, eds. Losik, R. & Chamberlin, M. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 835–853.
17. Somers, D. G., Pearson, M. L. & Ingles, C. J. (1975) *Nature (London)* **253**, 372–374.
18. Stern, W., Pogo, B. G. T. & Dales, S. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2162–2166.
19. Ichihashi, Y., Matsumoto, S. & Dales, S. (1971) *Virology* **46**, 507–532.
20. Nevins, J. R. & Joklik, W. K. (1977) *J. Biol. Chem.* **252**, 6939–6947.
21. Brodaner, O. G. & Wieland, T. (1976) *Biochemistry* **15**, 3480–3484.
22. Losik, R. & Pero, J. (1976) in *RNA Polymerase*, eds. Losik, R. & Chamberlin, M. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 227–246.
23. Hruby, D. E., Lynn, D. L. & Kates, J. R. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1887–1890.