Recent Progress in Poxvirus Research

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INTRODUCTION

This review will deal primarily with studies reported within the past 2-year period. Except to place recent works in their proper perspective, no attempt has been made to consider previous advances; for a review of these, the reader should consult Joklik (27).

Recent advances include the development of techniques for the disassembly of poxvirus particles in vitro and for the isolation of the viral deoxyribonucleic acid (DNA) as a single molecule. In addition, a "late" enzyme has been discovered. Recent studies indicate, moreover, that a number of the structural viral proteins are coded for by the "parental" genome. Perhaps the most exciting discovery is the finding that poxviruses contain a DNA-dependent ribonucleic acid (RNA) polymerase. Transcription of the viral genome has been demonstrated in vitro by investigators using intact virus particles and particles of subviral dimensions known as "cores." Recent studies have also forced a complete re-evaluation of the mechanism by which poxvirus particles are disassembled (uncoated) in vivo, and it now appears likely that "derepression" of the host genome is *not* required to initiate infection.

STRUCTURE AND CHEMICAL COMPOSITION

General Features

Before proceeding with recent advances, it will be instructive to consider certain aspects of viral architecture and composition. For convenience, I have illustrated some of these features in Fig. 1.

Three structures are clearly recognizable in sections of the virus particle—the outer coat, the lateral bodies, and the dense central structure known as the "core" or nucleoid (9). It is this last structure, the core, which contains the poxvirus DNA. The addition of trypsin to cores prepared in vitro (15) causes them to rupture, releasing the DNA. Once the DNA has been released, it is sensitive to deoxyribonuclease. The suggestion that a special enzyme, the "uncoating protein" (25), is required to release the DNA during the course of infection will be dealt with in the section on uncoating.

Recent studies indicate that the core consists at least partially of components which are coded for by the parental genome (20, 21). In addition, the recently discovered DNA-dependent RNA polymerase of poxviruses (35) is associated with this structure. At the moment, it is impossible to tell whether the enzyme resides within the core, or whether it is a component of the core structure itself. Chemical composition data have been obtained for vaccinia virus (60) and for fowlpox virus (49). Vaccinia is about 89% protein and 5 to 6% DNA; the rest is lipid. Presumably, the lipid components (phospholipid, cholesterol, and neutral fat) are constituents of the membrane structures (*see* Fig. 1). Copper, biotin, and riboflavine were also detected in these preparations (60). However, at this time it is not known whether these components are constituents or contaminants, as no metabolic role can be demonstrated.

Vaccinia virus, rabbitpox, cowpox, and ectromelia have the same DNA content and base composition. The guanine plus cytosine (GC) content is about 36%, and the base ratio [adenosine plus thymine $(AT)/GC$] is approximately 1.7. The T_m of cowpox DNA is 87 C, and its density in CsCl is 1.695.

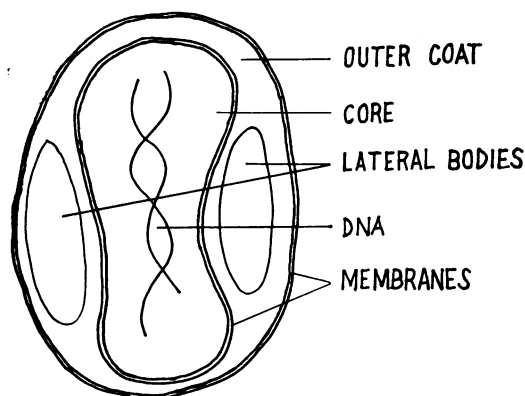It is only recently that the DNA of poxviruses

FIG. 1. *Features of poxvirus architecture.*

had been isolated in an apparently intact form. Recent studies (22, 55) indicate that the DNA is a linear duplex, 87 to 100 μ in length. This would put the molecular weight in the range of 160 $\times$ 10^6 to 200 $\times$ 10^6 daltons, in excellent agreement with previous estimates based on chemical composition (23, 49).

Provided that the entire genome is transcribed, sufficient DNA is present to code for several hundred proteins. Only a few of these are known. Seventeen to twenty have been implicated in virus structure (*see below*), and five (thymidine kinase, DNA polymerase, and three deoxyribonucleases), which are presumably virus-coded, have been detected by enzyme assay (7, 33, 38, 39, 40, 42).

Disassembly of the Virus In Vitro

A simple chemical procedure which can be utilized to dissect the virus in vitro has been described by Easterbrook (15). The method is as follows. The virus particles are treated first with a nonionic detergent, NP_{40}, and then with 2-mercaptoethanol. This treatment alone results in rupture and release of the outer coat. Lateral bodies, which are still attached to the cores at this point, can be removed enzymatically by the addition of a small quantity of trypsin. This results in a preparation which consists almost entirely of viral cores. Sonic treatment or excess trypsin will cause the cores to rupture, yielding "ghosts" and naked viral DNA.

Treatment of virus particles with NP_{40} alone or 2-mercaptoethanol alone produces the following result. With 2-mercaptoethanol, the outer coat appears to loosen or swell. Easterbrook suggested that this is due to the presence of disulfide bonds which are involved in maintaining the integrity of the outer coat. NP_{40} was thought to attack the lipoprotein of the membrane layer,

resulting in increased permeability to phosphotungstic acid.

The beauty of the procedure is that it can be applied to milligram quantities of purified virus, each step appears to be reasonably quantitative, and the intermediate structures can be purified by sedimentation in sucrose or tartrate gradients. I suspect that the procedure will eventually have widespread application, especially in the determination of virus composition. The method has already been put to use by Fenner and Sambrook (17) and by Holowczak and Joklik (20, 21).

Structural Proteins of Poxviruses

Adequate studies on the structural proteins have not been undertaken, and, as a result, information is limited. The primary difficulty is that the virus particles are extremely insoluble in all but the most drastic reagents (60). The attempts to solubilize the proteins of the virus by mechanical disintegration and extraction with alkaline buffers have been discussed (27). Although the percentage of the total viral protein solubilized by these techniques is small (as little as 20%), as many as eight antigens have been recognized.

The ultimate description of the number of components present in the outer coat, the lateral bodies, and the core, may well depend upon the development of special techniques such as those described by Easterbrook (15). To acquire such knowledge would indeed be a major achievement. Nevertheless, it should be kept in mind that the ultimate objective of the work is the description of the chemical and physical properties of the constituents. The technical problems involved in elucidating the manner in which each component is associated with the lipid, nucleic acid, and other protein components of the virus are severe, and at the moment it is safe to say the solutions are not in sight.

One procedure is available which results in complete solubilization of the virus particle—treatment with sodium dodecyl sulfate (SDS), urea, and 2-mercaptoethanol. However, in terms of the objectives outlined above, the procedure is clearly of limited value. These reagents not only destroy the secondary and tertiary structure of the proteins but they also produce a complex mixture of polypeptides which is difficult to resolve.

The recent studies by Holowczak and Joklik (20, 21) demonstrate the difficulties. These investigators analyzed the polypeptides of the virus by acrylamide gel electrophoresis. In spite of the length of the gel columns employed and the reproducibility of the electropherograms, the number of polypeptides, though clearly in excess of 17,

could not be ascertained. The location within the virus of certain polypeptide components was determined by application of the Easterbrook (15) technique.

Treatment of vaccinia virus with NP_{40} alone resulted in the release of a single (multiple polypeptide) component (VSP-6). Holowczak and Joklik suggested that this component, which accounted for 18.7% of the total virus protein, is situated at the surface of the virus particle. Treatment of the virus particle with NP_{40} followed by 2,2'-dithiodiethanol liberated all of the polypeptides except those associated with the viral core. The polypeptides of the core, which accounted for 36.7% of the total viral protein, consisted of two components believed to be single polypeptides (VSP-1 and VSP-2) and a third component (VSP-4) which was a mixture of polypeptides. No information was provided on the constituents of the lateral bodies.

An approach which circumvents the problems of solubilization is that of examining the proteins present after infection—i.e., the soluble antigens. At the present time, they represent the best source of virus coded proteins which are suitable for purification and characterization. As many as 17 components have been observed in rabbit skin infected with vaccinia (64), 17 components also have been observed in chick chorioallantoic membranes infected with vaccinia (51), and up to 20 components have been observed in HeLa cells infected with rabbit pox (4). It has *not* been established yet whether all of the components capable of reacting with hyperimmune serum are structural viral proteins. Nevertheless, it is clear that many are identical to the antigens which can be extracted from virus particles (8, 64).

Two of these antigens have been isolated and purified—an LS-antigen which has a molecular weight of approximately 240,000 (61) and an antigen (molecular weight 100,000 to 200,000) which will combine with virus-neutralizing antibody (5). (The LS antigen has two immunogenic sites: one which is labile and is inactivated at 60 C, and a second which is stable even at higher temperatures.) Physical and chemical methods have also been applied recently to the soluble viral antigens of KB cells (8). Three antigens have molecular weights of 200,000 or greater, and the remaining antigens are in the 50,000 to 100,000 range. Of the high molecular weight components, at least one stimulated the synthesis of virus-neutralizing antibody.

Isolation of the DNA as a Single Molecule

Methods have been described recently by Gafford and Randall (19) and by Sarov and

Becker (55) for isolating intact molecules of poxvirus DNA. In spite of these accomplishments, the reasons for previous failures are not at all clear. It has generally appeared to make little difference whether phenol and SDS were used separately or together, or in conjunction with proteolytic enzymes, or with previously defatted virus. The result in most cases was the same—extensive fragmentation of the DNA (27). Nor is it known whether the major damage to the DNA results during its release from the core or during subsequent manipulations. The latter possibility is a very real one, however, as the DNA is extremely large (larger, in fact, than that of any other known virus).

The DNA of fowlpox virus has been released successfully with SDS by Gafford and Randall (19). These investigators concluded that phenol results in extensive fragmentation, even to purified DNA, and that the extent of the damage is proportional to the exposure to phenol. The possible involvement of protein linkers was investigated, and was ruled out since trypsin had no effect. Purification of the DNA was accomplished by extraction in chloroform-*n*-butyl alcohol followed by methylated albumin kieselguhr (MAK) column chromatography.

In addition to the problems encountered with phenol, the authors discuss the problems involved in converting sedimentation coefficients to molecular weights—a problem in formula selection. The molecular weight of fowlpox DNA by sedimentation techniques was judged to be 200×10^6 to 240×10^6 daltons. The contour length of fowlpox DNA has also been determined (22). Forty-one molecules of DNA were measured; the average length was 100 μ, equivalent to 192×10^6 daltons.

A second procedure for releasing poxvirus DNA using sodium deoxycholate (DOC) and Pronase has been described recently by Sarov and Becker (55). The special virtue of this technique is that it protects the DNA by releasing it in the presence of sucrose. The authors imply that once the DNA has been released in this fashion it can be deproteinized with phenol; however, no data on this point were actually given. The contour lengths of four molecules of vaccinia DNA have been measured; the average length, 87 μ, is equivalent to a molecular weight of approximately 167×10^6 daltons.

In the past 2 years, electron microscopy has been the technique most frequently employed to determine molecular weight. The studies of McCrea and Lipman (41) indicate that the one-step osmotic shock method is to be avoided because it results in extensive fragmentation. Even

when released from cores (16), only 1 of 91 molecules appeared unbroken by this technique. The suggestion that this surviving molecule was at one time circular is, in my opinion, completely unwarranted. No good evidence has yet been obtained to show that animal viruses other than those of the papova group contain circular molecules.

A technique has also been reported for increasing the density difference (normally about 0.004 g/cc in CsCl) between viral DNA and host-cell DNA, based on the selective ability of viral DNA to renature at 60 C. The new value for the density difference was 0.017 g/cc (32).

UNCOATING OF THE VIRAL NUCLEIC ACID

The intracellular uncoating of poxviruses is a two-stage process—the first uncoating step removes the outer coat and the lateral bodies, and the second uncoating step liberates the DNA from the core, rendering it sensitive to deoxyribonuclease (11, 24, 25). Until recently, it was thought that both uncoating steps were required in order for the viral DNA to assume its genetic function. As it turns out, this concept is wrong. Recent experiments show that the poxvirus genome can be transcribed even though it resides within the viral core and is completely resistant to deoxyribonuclease (34, 44, 66). This is apparently due to the fact that poxviruses contain a DNA-dependent RNA polymerase (35, 45). The net result of recent studies is that they discredit, to a large extent, a theory proposed recently to account for the molecular basis of the uncoating phenomenon (25). Before discussing the nature of this proposal, it will be instructive to examine the uncoating process itself. Only the essentials of the process need concern us here.

Knowledge of the uncoating process derives from studies with the electron microscope (11) and from studies employing radioactive isotopes (24, 25). Briefly, the following concept has emerged. Poxviruses are taken into their hosts by a process akin to phagocytosis and eventually reside within phagocytic vesicles within the cell (11, 12). Once within these vesicles, first-stage uncoating commences, resulting in the degradation of the viral coat and, presumably, the lateral bodies. Dales (11) believes that the walls of the phagocytic vesicles collapse simultaneously with the rupture of the viral coat and has suggested that a concerted mechanism is involved. Whether the process is primarily an enzymatic one, or mechanical, or some combination of these factors, is not known. Whatever the mechanism, it accounts for the release of nearly 100% of the viral phospholipid and as much as 50 to 60% of the viral protein (20, 24). These events, which constitute the first stage of disassembly, result in the appearance of viral cores within the cytoplasm. At this stage, the genome is insensitive to deoxyribonuclease.

The second stage of uncoating proceeds more slowly than the first. Generally, there is a lag of 30 min to 1 hr before the release of the viral DNA begins. The exact timing depends upon the multiplicity of infection: the higher the multiplicity, the shorter the lag. Judging from its susceptibility to deoxyribonuclease, all of the DNA which can be released from cores will be released by 3 to 4 hr. This may be as little as 10% or as much as 60 to 70% of the total input DNA (24).

For second-stage uncoating to occur, there is an absolute requirement for both RNA and protein synthesis. This point is very secure, and it has been demonstrated many times by the use of inhibitors which result in the accumulation of cores (10, 25, 34). The result suggests that information encoded in the base sequence of DNA is required for liberation of the viral DNA from the core.

Several years ago, a scheme was proposed by Joklik (25) to account for these results. He predicted that first-stage uncoating proceeded spontaneously but that second-stage uncoating required information encoded within the host genome. [It was presumed by Joklik (25) on a priori grounds that a viral genome which was insensitive to deoxyribonuclease, and, therefore, presumably still surrounded by protein, was not yet ready to assume its genetic function. In view of the requirement for protein synthesis, this necessitated the involvement of the host genome in the uncoating mechanism.] Joklik believes that the host genome codes for the synthesis of an "uncoating protein," a proteolytic enzyme which is essential in facilitating the release of the viral nucleic acid from the core. Two pieces of information—the lag required before second-stage uncoating commenced and the effect of inhibitors in preventing the release of the viral DNA—suggested to him that the protein was not present in uninfected cells. To complete the theory, it was proposed that a "viral inducer" protein released during the first stage of uncoating "derepressed" the host genome, thus allowing synthesis of the uncoating protein.

Although the theory requires several assumptions, the crucial one is that the viral genome, still within the core and inaccessible to deoxyribonuclease, is an unlikely template for messenger RNA (mRNA). Recent studies (see the following section) indicate that this assumption is unwarranted. Viral cores, blocked in their uncoating by inhibitors, are extremely active in

the synthesis of viral mRNA. As a result, it must be assumed that the DNA of the virus particle is capable of directing its own release and replication.

In his efforts to obtain evidence for the viral inducer concept, Joklik (26) described the properties of a viral protein component which was essential for infectivity. He concluded that the viral inducer protein was sensitive to denaturation by heat, urea, and ultraviolet irradiation. In each case, the virus particles were rendered noninfectious by these treatments, and they failed to undergo second-stage uncoating. Ultraviolet irradiation was presumed to damage the histidine residues of the inducer protein (26).

Recent studies in this laboratory suggest that the inducer protein postulated by Joklik (25, 26) and the DNA-dependent RNA polymerase described by Kates and McAuslan (35) are one and the same. As might be expected, the activity of this enzyme is readily destroyed by heat and urea, but not by ultraviolet irradiation (Woodson, *unpublished data*). The recent findings of Kim and Sharp (37, 57) also support this contention. These investigators concluded that virus particles rendered noninfectious by ultraviolet irradiation and by nitrogen mustard show multiplicity reactivation, whereas those inactivated by heat and urea do not.

The requirement for protein synthesis is indeed real. Inhibitors such as cycloheximide and streptovitacin inhibit second-stage uncoating completely (10, 25, 34). Although no good evidence has been obtained to show that a specific protein is required, this has generally been assumed to be the case. As indicated previously by Joklik (27), attempts to demonstrate the activity of the postulated protein failed. Abel (1) claimed, however, that she could detect uncoating enzymes in extracts of infected chick embryo fibroblasts. Furthermore, she and Trautner (2) reported that infectious DNA, prepared by treating virus particles with these enzymes, could initiate a round of virus replication in *Bacillus subtilis*. Claims of a similar nature have been made recently by Babbar et al. (6). Furthermore, infectious DNA has been isolated from fowlpox (50), and infectious particles (of subviral dimensions) have been obtained from cultures infected with myxoma and fibroma viruses (63). At the present time, these findings are not well understood, and a conclusion must await further studies.

TRANSCRIPTION OF THE VIRAL GENOME

Before discussing the problems of transcription, it will be profitable to outline the events
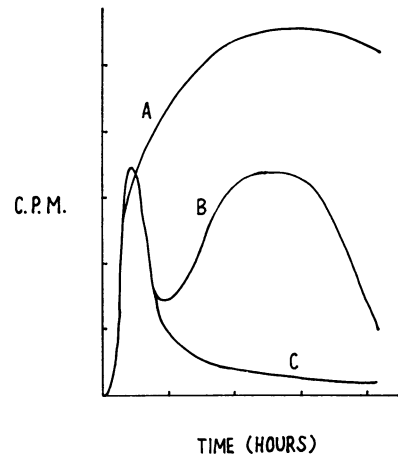

TIME (HOURS)

FIG. 2. *Pattern of vaccinia virus specific mRNA synthesis in HeLa cells (A) in the presence of cycloheximide or with ultraviolet-inactivated infecting virus, (B) no additions, and (C) in the presence of FUDR (66).*

which are associated with the replicative cycle. The exact timing of these events depends largely upon the system employed and upon the multiplicity of infection. At high input multiplicities (several hundred particles per cell), transcription of the parental genome begins immediately upon infection; uncoating of the viral nucleic acid and the "takeover" of host cell functions begin by 30 min to 1 hr, induced enzymes and viral antigens appear between 1 and 2 hr; DNA replication begins at 1 to 1.5 hr and is essentially complete by 4 to 5 hr; and progeny particles, which may number 10^4 per cell at 24 hr, begin to appear as early as 4 to 5 hr after infection. These events take place exclusively within the cytoplasm (27).

The problems of transcription can be handled in a variety of ways. The simplest is to discuss them in relation to my own studies, shown in Fig. 2. This figure describes the relative rate at which transcription of the vaccinia genome proceeds in HeLa cells under three different conditions. In all cases, the pattern of viral mRNA synthesis was determined by administering 10-min pulses of ^{14}C-uridine as described elsewhere (66). Curve C represents transcription of the parental genome only, since viral DNA synthesis has been prevented with fluorodeoxyuridine (FUDR). Curve B shows the pattern of RNA synthesis under normal conditions—i.e., transcription of both parental and progeny genomes. Curve A also represents transcription of the parental genome, but under conditions such that second-stage uncoating (release of the viral genome from the core) does not occur. It is this last result which has prompted the re-evaluation

of the uncoating theory proposed by Joklik (25), since it shows that the virus, though not yet fully uncoated, is capable of synthesizing mRNA. This result is obtained with ultraviolet-treated virus and, provided that protein synthesis is inhibited at zero-time, with normal virus. Evidence has been obtained that the RNA transcribed under the conditions of curve A is viral mRNA. This material associates rapidly with polyribosomes, and, when extracted from these structures, it had the base composition of legitimate viral mRNA (66).

Additional proof that transcription of the viral genome can occur, though not yet fully uncoated, comes from the experiments of other workers. Although Munyon and Kit (44) were the first investigators to come to this conclusion, they made no attempt to identify the RNA transcribed in the presence of inhibitors as being viral. Convincing data have been supplied by Kates and McAuslan (34). These investigators showed that the RNA transcribed under the conditions of curve A will hybridize with poxvirus DNA, but not with host-cell DNA. In fact, on the basis of competition hybridization experiments, these workers concluded that the majority of the genes which can be transcribed from the parental genome can also be transcribed from cores. Additional experiments indicated that certain genes (those controlling the synthesis of DNA polymerase, for example) could be transcribed only after the release of the viral genome from the core.

The factors which regulate transcription of the viral genome are not at all clear. Nevertheless, the following points can be made in reference to Fig. 2. First, in order to study the transcription of parental genomes, one must include either FUDR or cytosine arabinoside in the medium; otherwise, this event is completely obscured by the transcription of progeny genomes. In the presence of FUDR, the initial rate of viral mRNA synthesis is directly proportional to the input multiplicity, over a fairly wide range (66). We ascribe this behavior to the fact that each particle brings into the cell its own DNA-dependent RNA polymerase. Second, transcription of the parental genome under conditions of high multiplicity and maximal synchronization (as in curve C) is tightly controlled and occurs in a burst-like fashion. Finally, under a variety of conditions, transcription of the parental genome occurs in an "uncontrolled" or extended fashion, as in curve A. Kinetics of this nature have been observed with inhibitors of protein synthesis and with ultraviolet-treated virus (34, 66), in studies performed in vitro with virus particles and cores (35, 45), and in studies on interferon (31), in which case presumably no viral mRNA is translated.

These results suggest that, when uncoating does *not* occur, transcription is uncontrolled. An explanation for this behavior will be offered in the following section. When uncoating does occur, transcription of the parental genome appears to subside. The possibility that this behavior results from the dissociation of the RNA polymerase-DNA complex is being investigated.

Transcription of the viral genome has also been studied by Oda and Joklik (46). These investigators examined the behavior of the vaccinia genome in L cells and HeLa cells and found that the two patterns were entirely different. In HeLa cells, the pattern was essentially that which I have shown in Fig. 2; by 2.5 hr, transcription of the parental genome was barely detectable. In L cells, however, the parental genome was transcribed at a much greater velocity, and by 4 hr was still being transcribed at a substantial rate. Competition hybridization experiments were undertaken to determine the nature of the sequences being transcribed in the two cell lines. In HeLa cells, parental genomes were transcribed exclusively from 0 to 2 hr after infection. From this time on, "early" and "late" message sequences were transcribed at nearly equivalent rates. (Late sequences were defined as those which could not be transcribed from parental genomes.) In L cells, parental genomes appeared to be transcribed almost exclusively at all times. In spite of this finding, progeny particles were produced in roughly equivalent numbers in the two cell lines. From these experiments, it may be concluded that there is little or no correlation between the yield of progeny particles and the quantity of late sequences transcribed. Similar observations have been made in regard to protein synthesis (53) and will be discussed later. The experiments also suggest that it is the host which determines the pattern of viral transcription. Additional studies, in a variety of cell lines, will be required to determine whether this is literally true. Finally, there is the possibility that the observed result is due to the efficiency of uncoating. That uncoating can vary, not only among cell lines but among strains of the same cell line, has been shown previously by Joklik (24).

Clearly the problems of transcription are many. The factors which may influence and control transcription are, nevertheless, becoming more apparent. One of these factors is an enzyme involved in the transcription process itself. Evidence that it is a component of the virus particle will be presented next.

POXVIRUS RNA POLYMERASE

Until recently, there was no reason to think that the poxvirus system would behave differently from any other; the input DNA, after its release from the core, would be transcribed by a host-cell enzyme. The first indication that this was not the case came from the experiments (34, 44, 66) which demanded the re-evaluation of the un-coating theory (25). These said, in essence, that the DNA was transcribed prior to its release from the core. Although it is conceivable that a host enzyme could enter the core (though deoxyribonuclease cannot), or that a host enzyme could act upon a few genomes released in some anomalous fashion, the evidence clearly indicates that the enzyme responsible resides within the core, and that it is a component of the virus particle. This was demonstrated by the experiments of Kates and McAuslan (35). These investigators examined the cytoplasmic extracts of uninfected cells and of infected cells containing viral cores for polymerase activity. No activity was found in the cytoplasm of uninfected cells in spite of the addition of rabbitpox DNA (100 μg, either native or denatured) in amounts equivalent to the combined genomes of 3.6×10^{11} virus particles. In contrast, the extracts containing viral cores rapidly catalyzed the incorporation of labeled uridine triphosphate (UTP) into an acid-insoluble product. The activity required the presence of all four ribonucleoside triphosphates, but it was not stimulated by the addition of primer DNA, nor was it inhibited by the addition of deoxyribonuclease. Transcription was inhibited by actinomycin D, and the product was sensitive to ribonuclease.

Further evidence that the enzyme is a component of the virus particle was obtained by showing that highly purified preparations of cores will catalyze RNA synthesis in vitro. The reaction requires only three additional components—Mg^{++}, triphosphates, and buffer. Virus particles are also active in vitro, provided that the outer coat has been loosened or made permeable by exposure to mercaptoethanol or trypsin. Similar observations have now been made in this laboratory (Woodson, *unpublished data*) and by Munyon et al. (45). Although no one has yet been successful in obtaining from poxviruses an enzyme which will respond to exogenous primer, efforts with this goal in mind are presently being made in several laboratories.

The nature of the product which is transcribed in vitro has been investigated to some extent. When the product was examined after a period of limited transcription (30 sec to 2 min), the bulk of it was found to be core-associated and in-sensitive to ribonuclease. However, after extended periods of transcription (8 to 10 min is sufficient), the majority of the material was then ribonuclease-sensitive (Hedgpeth and Woodson, *unpublished data*). Further evidence for the release of the product from the core has been obtained by sedimentation techniques (35, 45). In addition, Kates and McAuslan (35) have shown that a portion of the product is the same size as that which is transcribed in vivo, and that it can be annealed to poxvirus DNA.

A comparison of the properties of the poxvirus system with those of the DNA-dependent RNA polymerase from *Escherichia coli* reveals several interesting features. When the *E. coli* enzyme is primed by native DNA, chains are first initiated, followed by chain growth. At the end of the reaction, nascent RNA chains remain bound to the polymerase which, in turn, remains firmly bound to the DNA (39). The poxvirus system behaves quite differently. First, overall transcription proceeds in a linear fashion for a period of several hours (35, 45). During this time, quantities of RNA equivalent to the weight of the poxvirus genome are transcribed and released (35). The results imply that portions of the poxvirus genome are being transcribed in a repetitive fashion. Experiments which should confirm or deny this result are presently in progress.

EARLY AND LATE FUNCTIONS

The multiplication of certain bacteriophages (phage λ, phage 2C, phage T4, etc.) requires the formation of virus-specific enzymes which appear "early" or "late" in the replicative cycle. The synthesis of these enzymes, and the expression of the genome generally, is under strict control (18, 47, 59). For poxviruses, a similar regulation of gene action has been postulated (30). Induced enzymes (7, 33, 38, 39, 42) appear shortly after infection which are subject to switchoff control (38, 39) when progeny genomes appear. In addition, certain of the structural viral proteins are synthesized early and others are synthesized late (21, 53, 65).

Available evidence suggests that expression of the poxvirus genome is controlled largely at the level of transcription. Thus, a portion of the parental genome is transcribed at the core level, and additional genes are presumably transcribed once the genome is free (34). These sequences of mRNA presumably code for the early enzymes and for the early structural viral proteins. Late sequences of mRNA are transcribed after the appearance of progeny genomes, and presumably these result in the activation of the switchoff

mechanism and the synthesis of the late structural viral proteins (46).

At the present time, there is no indication of the mechanism by which the information is selected. Presumably this would occur in a fashion analogous to that which has been predicted for other systems—i.e., different portions of parental and progeny genomes are available for transcription owing to physical and chemical differences, or enzymes of different specificity recognize one portion of the genome or another (47). In addition, repressor proteins may be involved in regulating the expression of the DNA (48). At the moment, there is no information on whether the poxvirus genome is transcribed symmetrically or asymmetrically (62), or whether early and late message sequences derive from distant segments of the poxvirus genome, as in the case of phage λ (59).

Once a particular mRNA molecule has been transcribed, or while it is in the process of being transcribed, it will associate with ribosomes (29) in such a fashion as to initiate polypeptide synthesis. As a result of this act, or owing to the presence of soluble degradative enzymes within the cell, or both, it will be degraded in some fashion rendering it nonfunctional. The observation that vaccinia mRNA transcribed early in HeLa cells decays slowly and that late message sequences decay more rapidly has led to speculation that protein synthesis is controlled additionally at the level of translation.

In recent studies by Sebring and Salzman (56), it was found that at early times mRNA synthesis proceeded at substantial rates and that the half-life of the RNA was in excess of 2 hr. At late times, the quantity of mRNA which could be extracted from polyribosomes was sharply reduced, and the half-life of this material was about 13 min. In spite of these events, protein synthesis was observed to continue at a substantial rate until late in infection. Similar studies were undertaken by Oda and Joklik (46). These investigators found that, in HeLa cells, less than 20% of early vaccinia mRNA was degraded in a period of 5.5 hr. Late sequences had a half-life of less than 1 hr. The situation in L cells was quite different—vaccinia mRNA transcribed at early and late times had identical half-lives (about 2 to 3 hr).

The fate of the messenger molecules which are subject to switchoff control has also been touched upon to some extent. The molecular basis of this phenomenon is still completely unknown, and at the present time there is insufficient evidence to support strongly either the notion that a specific protein is coded for by the progeny genome (18, 39) or that new transfer RNA (tRNA) molecules

arise which result in misreading of early (stable) mRNA sequences (18). Two studies have been reported recently which bear upon the problem.

The studies by Sebring and Salzman (56) indicate that a sizable fraction of vaccinia virus mRNA synthesized late sediments in the 30 to 74S region. In contrast to the results obtained by Joklik and Becker (29), this RNA was judged to be nonfunctional in that it did not decay in the presence of actinomycin and it could not be chased into the polysome region. Additional studies will be required to determine the nature of the binding of this RNA to other cell constituents, and to determine the sequence composition of the RNA—i.e., whether it is predominantly early or late, or both. Oda and Joklik (46) found that early sequences transcribed at a late time enter the polyribosome region. A similar observation has been reported in the case of bacteriophage T4 (18).

In the remainder of this section, I would like to discuss (i) the synthesis and assembly of the structural viral proteins and (ii) the function of several nonstructural proteins which arise during the course of infection.

(i) Recent studies show that three to five of the structural viral proteins are coded for by the parental genome, and that these proteins are synthesized early. This is a rather unusual situation, and, as far as I know, it has not been observed in any other systems.

The early structural proteins begin to appear at about 1 to 2 hr after infection and, like the induced enzymes, are subject to switch-off control at 4 to 6 hr. They constitute about 15 to 25% of the total protein of the virus particle (20, 21, 53). The studies by Shatkin (58) were probably the first to indicate that structural viral proteins were produced early. He showed that a sizable fraction of the total viral antigen was produced in the presence of FUDR. Five early antigens have now been recognized by Appleyard (3) and by Salzman and Sebring (53), four by Wilcox and Cohen (65), and three polypeptide components have been detected by Holowczak and Joklik (21).

The location of the early components within the virus particle has been examined, and it is clear that some are the structural proteins of cores. This was first suggested by Wilcox and Cohen (65) on the basis of the fact that the low molecular weight antigens of vaccinia virus which were synthesized early did not elicit the formation of virus-neutralizing antibody. The studies by Holowczak and Joklik (21) offer a more direct proof. These investigators have utilized a combination of techniques to investigate the location and time of synthesis of various polypeptide

components. Two of the polypeptides synthesized early are components of cores. A third component synthesized early apparently resides at the surface of the particle.

Studies with the electron microscope suggest that the structural components of cores, which are coded for by the parental genome, can be assembled into structures which have the appearance of cores, but which lack DNA. These structures, which are known as "immature forms," accumulate in the presence of FUDR (14), hydroxyurea (52), and IBT (isatin-β-thio-semicarbazone) (13). Unlike FUDR and hydroxyurea, IBT does not inhibit viral DNA synthesis (13, 67). It inhibits protein synthesis, but only after the appearance of progeny genomes (presumably by interfering with the functioning of "late" mRNA molecules). Apparently the packaging of DNA into the core requires the synthesis of late proteins. This conclusion is supported by the finding that one of the polypeptide components of cores (VSP-4) is synthesized late (21), and by the studies with puromycin (28) which showed that proteins synthesized late were necessary to convert progeny DNA to deoxyribonuclease-resistant subvirion forms.

As I have just indicated, structures (presumably intermediates in the assembly process) can be accumulated by the use of inhibitors. More than likely, structures of a similar nature would be accumulated in cells infected by conditional lethal mutants. Now that such mutants have been isolated (54), it is probably safe to assume that a serious study of poxvirus assembly will soon get underway. Fenner and Sambrook (17) have analyzed certain of these mutants already. Many of the host cell-dependent mutants are capable of synthesizing DNA, all four antigens of the core, and all but a few of the remaining proteins. [In view of the studies suggesting that some of the structural viral proteins are coded for by the parental genome (21, 53, 65), it is clear that DNA synthesis alone is no longer a sufficient criterion for classifying a mutant as either "early" or "late."] It would be interesting indeed to determine whether these components are assembled into cores or lateral bodies or both.

(ii) The relationship between protein synthesis and poxvirus DNA synthesis has been investigated by Kates and McAuslan (36). These investigators have provided evidence that an early protein is required both to initiate and to sustain viral DNA synthesis. The protein, which was itself stable, was required in stoichiometric rather than catalytic amounts and could be distinguished from DNA polymerase and thymidine kinase because of the instability of mRNA

coding for its synthesis. The specific function of the protein is not known.

The synthesis and regulation of virus-induced deoxyribonucleases has also been investigated by these workers. Recent studies by McAuslan and Kates (40) indicate that, of three nucleases which are induced by cowpox in HeLa cells, one acts optimally and almost exclusively on denatured DNA at pH 4.5, a pH at which the enzyme is insoluble. The enzyme has been purified (3,000-fold), and its chemical and physical properties have been assessed. It is synthesized continuously from about 3 to 18 hr postinfection, and during this time undergoes a sharp transition from the soluble to the bound state. No enzyme was synthesized in the presence of FUDR, and the rate of enzyme synthesis in the absence of FUDR was strictly proportional to the quantity of DNA synthesis allowed. In all respects, the enzyme appears to be a "late" function.

CONCLUSION

The major accomplishments of the past 2 years may be summarized as follows. The viral DNA has been isolated in a form which, for the first time, appears to be reasonably intact. In addition, a method has been described for disassembling the virus in vitro. Two enzymes have been discovered, a DNA-dependent RNA polymerase which resides within the virus particle, and a deoxyribonuclease which appears to be a late function. Recent experiments have indicated, moreover, that a number of the structural viral proteins are coded for by the parental genome. Our concept of uncoating has been revised completely, and our understanding of the factors which regulate transcription and the expression of early and late functions has increased substantially. Although many problems remain to be solved, most of these are presently under attack. If the progress of the past 2 years can be taken as a measure of what is yet to come, then prospects for the next 2 years should be excellent indeed.

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