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*REGULATION OF VIRUS-INDUCED DEOXYRIBONUCLEASES**

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Increases in at least three deoxyribonuclease activities occur in HeLa cells after infection with poxvirus. These nucleases can be conveniently described by their substrate preference and pH of maximal activity as (1) an alkaline DNase (double-strand DNA),[1] (2) an acid DNase (single-strand DNA),[2] and (3) a DNase acting maximally at pH 7.8 on single-strand DNA.[3] We shall refer to the latter as neutral DNase. The first two DNases have been tentatively characterized as exonucleases.[4] Since the increase in their activities postinfection can be inhibited by puromycin, it was suggested that the increases are probably due to virus-induced synthesis of the enzymes rather than simple activation.[1, 2] In contrast to the stability of the messenger for virus-induced thymidine kinase synthesis,[5] the messengers for the synthesis of the first two DNases in question are comparatively unstable.[2] We have examined the possibility that the regulation of the synthesis of the DNases might differ in character from that described for induced thymidine kinase synthesis which appears to involve regulation of messenger translation.[5] The regulation of the induced alkaline and acid DNases have been compared with that of thymidine kinase. We present evidence that different control mechanisms exist for different poxvirus-induced enzymes.

Materials and Methods.—Virus strains: The virus strains used were the Utrecht strain of rabbitpox, the Brighton strain of cowpox, a red-pock isolate of the Connaught Laboratories' strain of poxvirus, and the WR strain of rabbitpox. Their preparation, titration, and biological properties have been described.[6, 7] Virus was purified by centrifugation through sucrose.[17]

Cultivation and infection of cells: The cultivation of HeLa S_3 cells in suspension and the procedure used to infect them with virus have been described.[8]

Preparation of cell extracts and assay of enzymes: Cells were harvested by centrifugation at various times after infection, washed once in 0.1 *M* Tris-HCl buffer pH 7.8, and then allowed to swell in hypotonic medium before disruption in a Dounce homogenizer.[9] After removal of nuclei by centrifugation at 600 × *g* for 10 min, the cytoplasmic fraction was used either directly or further centrifuged (10,000 *g* for 30 min) before assay of enzymes. The assays for deoxyribonucleases and thymidine kinase were those described in detail elsewhere.[1, 2, 8] The substrate for DNase assays was tritiated DNA prepared by Marmur's method[18] from *E. coli* T^- grown to a limit in tris-glucose medium containing 1 mc thymine-H^3 per liter. Single-strand DNA for the acid DNase assay was prepared by heating DNA-H^3 (70 μg per ml in 0.01 *M* Tris-HCl pH 7.2) at 100°C for 10 min prior to rapid chilling to 0°.

Polysome preparation: Ribosomes were extracted from normal or infected cells and fractionated on 5–20% sucrose gradients.[10] Pulse-labeling studies using uridine-C^{14} were conducted by using methods described by Becker and Joklik.[11] Under the conditions of the experiment in which cells were subjected to uridine-C^{14} pulses of no longer than 15 min, there is no significant

amount of isotope-labeled host messenger ribonucleic acid in the cell cytoplasmic fraction.[10, 11]

UV irradiation of virus: Purified virus was irradiated with UV light exactly as described by McAuslan.[8]

Inhibitors: Aminopterin, 5-fluorodeoxyuridine, 5-bromodeoxyuridine, and uridine were products of the Calbiochem Co. In experiments in which these compounds were used, the serum normally used in the growth medium was replaced by dialyzed serum.

Results.—Effect of BUdR and FUdR on the regulation of virus-induced thymidine kinase: It has been shown that inhibition of the onset of poxvirus DNA synthesis prevents or delays the termination of poxvirus-induced thymidine kinase synthesis.[8] This result was recently confirmed by Jungwirth and Joklik.[3] Although the experiments have established that DNA synthesis is necessary for the termination of kinase synthesis, it was still of interest to see if it is the amount or the quality of viral DNA synthesized that is important. The question of amount of DNA can be approached by adding FUdR at various times after infection. The question of quality of the DNA can be approached by addition of BUdR to infected cultures. Whereas FUdR causes very rapid cessation of poxvirus DNA synthesis[12] (confirmed by direct experiment with our system), BUdR can be incorporated into poxvirus DNA in place of thymidine with high efficiency.[13] Cells were infected with RP at an input multiplicity of 10 PFU per cell, and at various times after infection, FUdR (10^{-5} *M* FUdR plus 10^{-4} *M* Ur) or BUdR (10^{-4} *M* BudR plus 10^{-3} *M* Ur) was added to infected cultures. At various times cells were harvested and extracts prepared for assay of enzymes. The results (Fig. 1) indicate that both BUdR and FUdR are effective in preventing the onset of kinase repression provided that they are added before 2–$2^1/_2$ hr after infection—that is to say, before the time that poxvirus DNA synthesis commences.[14]

Effect of BUdR and FUdR on the regulation of virus-induced deoxyribonucleases: The increases in poxvirus-induced alkaline and acid deoxyribonucleases in poxvirus-infected HeLa cells are terminated about the same time as is the increase in induced thymidine kinase activity.[1, 2] We have now confirmed these findings using the RP and WR strains of poxvirus in addition to the CP strain. Although the increases in DNase activities can be prevented by puromycin, one cannot be sure that such increases are an indication of enzyme synthesis. Increase in the DNase activities can be blocked by puromycin, but the effect is not reversible;[2] puromycin also blocks viral DNA synthesis which, as we will show, is required to bring about an increase in acid DNase activity.

From the prompt cessation of further increase in induced DNase activity after addition of actinomycin D to infected cells,[2] it is concluded that unlike thymidine kinase synthesis, increase in the activities of the deoxyribonucleases is mediated by an unstable messenger. Therefore, it was of interest to see if the termination of the increase in induced DNase activity was prevented by thymidine analogues. Experiments similar to those described above for thymidine kinase regulation were conducted using HeLa cells infected with CP or RP at an input multiplicity of 10 PFU per cell. Either FUdR or BUdR prevented the termination of CP- or RP-induced alkaline DNase. The analogues BUdR or FUdR must be added prior to about $2^1/_2$ hr postinfection to have an effect on the regulation of DNase (Fig. 2).

If FUdR or BUdR were exerting their effect by stabilizing a messenger for alkaline DNase activity, addition of actinomycin D should not effect the continued

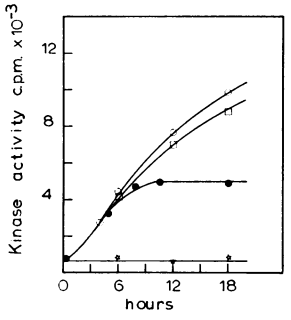

FIG. 1.—Increase in thymidine kinase in RP-infected HeLa cells in the presence or absence of thymidine analogues. Kinase activity in cells infected in the presence of FUdR or BUdR (○); kinase activity in infected cells with FUdR or BUdR added 2 hr postinfection (□); kinase activity in infected cells when FUdR or BUdR either omitted or added 3 hr postinfection (●); kinase activity in uninfected cells (☆).

synthesis of alkaline DNase in the presence of FUdR. As shown in Figure 2, actinomycin D at a concentration of 2 μg per ml caused a rapid cessation of alkaline DNA synthesis in FUdR-treated cells. Although actinomycin D is a potent inhibitor of the action of DNases,[15] the concentration of actinomycin in the enzyme extract was too small to cause any measurable inhibition in the assay for alkaline DNase.

In striking contrast to the effect on alkaline DNase regulation, addition of FUdR to HeLa cells at the start of infection completely prevented the normal increase of CP-induced acid DNase activity. The results were repeated using aminopterin (aminopterin, 100 μg per ml, plus adenosine, 40 μg per ml; plus glycine, 40 μg per ml, cf. Simon[16]), indicating that the FUdR effect is not due to incorporation of fluorouracil into messenger RNA (Fig. 3).

If BUdR was used in place of FUdR, then an increase in acid DNase activity did take place, but the rate of increase was somewhat lower than it was in cells not treated with BUdR.

It was also of interest to see if a mechanism for terminating the increase in acid DNase activity could be established if viral DNA synthesis was prevented. Cells

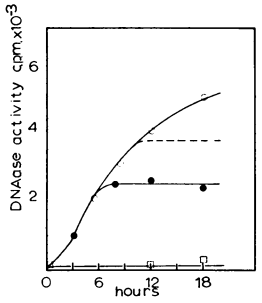

FIG. 2.—Increase in alkaline DNase in CP-infected HeLa cells in the presence or absence of thymidine analogues. No analogue added (●); FUdR or BUdR added at the start of infection (○); FUdR added at the time of infection and actinomycin D added 10 hr later (- - -); alkaline DNase activity in uninfected cells (□).

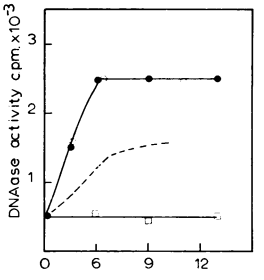

FIG. 3.—Increase in acid DNase in CP-infected cells. No analogues added (●); FUdR plus 10^{-4} M thymidine added at the start of infection (○); FUdR or FUdR plus 10^{-4} M uridine or aminopterin added at the start of infection (□); BUdR added at the time of infection (- - -).

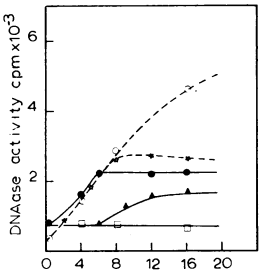

FIG. 4.—Failure to establish acid DNase repression in the presence of FUdR. Increase in acid DNase in CP-infected cells (●); increase in acid DNase (□), and alkaline DNase (○) in cells infected in the presence of FUdR; increase in acid DNase (▲), and alkaline DNase (☆) after infecting cells in the presence of FUdR and then removing FUdR 6 hr postinfection.

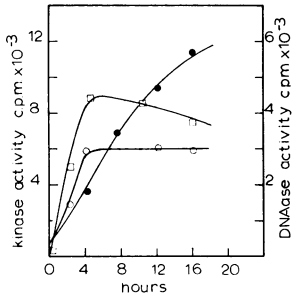

FIG. 5.—Kinetics of the increase in kinase activity (●); acid DNase (○); or alkaline DNase (□), in CL-R-infected cells.

were infected with CP in the presence or absence of FUdR. After the increase in acid DNase activity had terminated in the cells that had been infected in the absence of FUdR, both test and control cultures were washed twice in warm growth medium, and thymidine (10^{-5} *M*) was added to ensure prompt reversal of viral DNA synthesis in the test culture. FUdR prevented the increase in induced acid DNase but after removing FUdR, acid DNase activity increased (Fig. 4). This indicates that a mechanism for terminating the increase in acid DNase activity was not established during the delay in initiating DNA synthesis.

Induction and control of enzymes in CL-R-infected cells: The termination of the synthesis of induced thymidine kinase in HeLa cells infected with CL-R (input multiplicity of infection 10 PFU per cell) is considerably delayed, compared with the time that it occurs in cells infected with CP or RP (cf. McAuslan[8]). In most experiments thymidine kinase synthesis continued for more than 15 hr after infection (Fig. 5). Addition of FUdR or BUdR at the time of infection does not alter the final level of kinase activity nor does preirradiation of the virus with a dose of UV that abolishes the repression of CP- or RP-induced thymidine kinase. The course of viral DNA synthesis in the CL-R-infected HeLa cells was found by experiment to be similar to that reported by Salzman *et al.*[14] for the WR-infected HeLa system. Experiments were conducted to see if the alkaline and acid DNase activities were induced by the CL-R strain and if so, if these increases were terminated as they are in CP-infected cells. Assays were conducted on the same extracts used to follow thymidine kinase induction. The results (Fig. 5) indicate that although the termination of kinase is delayed, the increase in the activities of the alkaline and acid DNase activities are terminated sharply at about 4 hr post-CL-R-infection. Either FUdR or BUdR added at the time of infection prevented the cessation of alkaline DNase synthesis (Fig. 5).

In vaccinia (WR strain)-infected HeLa cells the termination of increases in thymidine kinase, DNA polymerase, and neutral DNase occurs about 5 hr post-infection.[3] Using the same system, Becker and Joklik[11] observed little if any further viral messenger RNA synthesis at 7 hr postinfection, and they noted that most of the polysomes of the infected cells had been broken down to 70*S* monomers at this time. In CL-R-infected cells the repression of acid, neutral, and alkaline DNases occurs at 4 hr postinfection. By pulse-labeling with C^{14}-uridine, appreciable amounts of radioactive messenger RNA were found to be associated with polyribosomes as late as 12 hr postinfection.

A summary of some experiments on the rate of synthesis of polyribosome-bound CL-R messenger RNA is presented in Figure 6. The data indicate that the repression of viral-induced DNase synthesis is not a reflection of a total inhibition of the transcription of the viral genome.

In the case of RP-infected HeLa cells, the synthesis of thymidine kinase is repressed at 7–8 hr postinfection. Synthesis of RP messenger RNA can be detected in the polyribosome fraction even after 12 hr postinfection. Furthermore, an

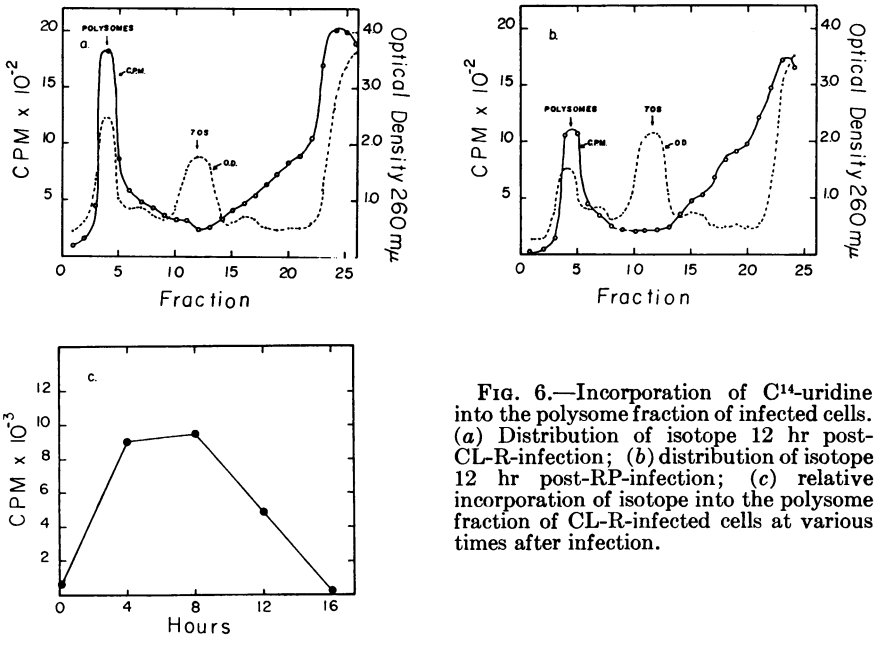

FIG. 6.—Incorporation of C^{14}-uridine into the polysome fraction of infected cells. (*a*) Distribution of isotope 12 hr post-CL-R-infection; (*b*) distribution of isotope 12 hr post-RP-infection; (*c*) relative incorporation of isotope into the polysome fraction of CL-R-infected cells at various times after infection.

appreciable portion of polyribosomes relative to 70*S* monomers is present at that time (Fig. 6). Thus, even in the case of an enzyme with a stable messenger RNA,[5] it appears unlikely that a random breakdown of polyribosomes is responsible for its repression.

Effect of UV-irradiated virus on the regulation of deoxyribonucleases: It has been shown that if poxvirus is irradiated with UV light prior to infecting cells, viral-induced thymidine kinase may be synthesized at the same rate as it is in cells infected with live virus, but the usual repression of kinase synthesis that takes place is abolished.[8] We examined the effect of preirradiating poxvirus on its capacity to induce deoxyribonucleases. Purified CP virus was irradiated with various doses of UV light and then used to infect HeLa cells at an input multiplicity of infection equivalent to 10 PFU per cell of nonirradiated virus. The results (Fig. 7) indicate that although the induction of alkaline DNase is more sensitive to UV irradiation than is thymidine kinase, repression of both enzymes is abolished by the same UV dose.

Discussion.—The evidence presented suggests that there are at least three mechanisms for the regulation of poxvirus-induced enzymes. The termination of both thymidine kinase synthesis and alkaline DNase synthesis can be prevented by blocking DNA synthesis with FUdR, or by permitting viral DNA synthesis to proceed in the presence of BUdR, or by preirradiation of the virus particles. The short postinfection time (about 2 hr), after which addition of either FUdR or BUdR is ineffectual, suggests that a small amount of progeny DNA is sufficient to trigger the subsequent repression of kinase and alkaline DNase. Since thymidine kinase has a stable messenger[5, 3] and alkaline DNase has an unstable messenger,[2] there are three possible situations concerning the termination of synthesis of these enzymes: (1) a common mechanism blocks both the transcription of the unstable

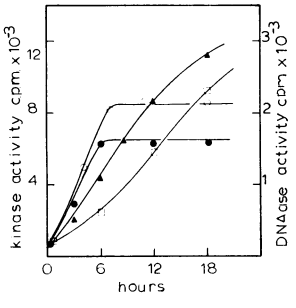

FIG. 7.—Increase in thymidine kinase (●) and alkaline DNase (○) after infection of cells with live CP; increase in thymidine kinase (▲) and alkaline DNase (□) after infection of cells with CP irradiated to $10^{-3.5}$ of its original titer.

messenger for alkaline DNase as well as the translation of stable kinase messenger; (2) one mechanism blocks the translation of both unstable and stable messengers; or (3) there are separate mechanisms involved for the regulation of each enzyme. We incline to the third possibility because of the observations that the synthesis of alkaline DNase in CL-R-infected cells is sharply terminated many hours in advance of any possible termination of kinase synthesis and repression of alkaline DNase is established during puromycin inhibition,[2] whereas repression of thymidine kinase is not (cf. McAuslan[5]). The regulation of induced DNA polymerase, which also has a comparatively stable messenger,[3] may be mediated in the same way as that for thymidine kinase. It remains to be shown if the neutral DNase has a stable or unstable messenger and, therefore, if its regulation should be grouped for consideration with the induced kinase and polymerase or with the induced alkaline DNase. However, it is clear that this neutral DNase is an enzyme distinct from the acid DNase because of the difference in their induction in the presence of FUdR; induced acid DNase is remarkable in that the control of the increase in its activity is dependent on viral DNA synthesis. An increase in the acid DNase activity of FUdR-treated CP-infected cells appears when FUdR is removed well after the time that termination of the increase in activity of this enzyme would normally occur (Fig. 4). Apparently, transcription of unstable messenger for this acid DNase is started and stopped when viral DNA synthesis starts and stops. It will be of interest to see if the induced acid DNase is representative of a class of viral-coded proteins whose synthesis takes place only after progeny DNA is synthesized. Possible candidates for this class are the protein or proteins involved in thymidine kinase repression and perhaps the maturation protein mentioned by Salzman and co-workers.[14]

Summary.—The control of three poxvirus-induced enzymes (thymidine kinase, "alkaline" DNase, and "acid" DNase) involves three different mechanisms. FUdR or BUdR blocks the repression of both thymidine kinase which has a stable messenger, and the repression of alkaline DNase which has an unstable messenger. The times at which thymidine kinase synthesis and the increase in alkaline DNase activity are arrested in one cell-virus system can be clearly distinguished. The mechanism responsible for the termination of the increase in alkaline DNase activity is not dependent on protein synthesis, whereas repression of thymidine kinase synthesis requires protein synthesis. In contrast to the induction of thymidine kinase or alkaline DNase, the viral induction of acid DNase is completely inhibited by inhibiting viral DNA synthesis with FUdR. The FUdR effect is reversible.

Abbreviations: DNase, deoxyribonuclease; FUdR, 5-fluorodeoxyuridine; TdR, thymidine; BUdR, 5-bromodeoxyuridine; Ur, uridine; CP, cowpox; RP, rabbitpox; CL-R, Connaught Laboratories' strain of poxvirus; PFU, pock-forming units.

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† Recipient of USPHS postdoctoral fellowship.

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*POLYAMINES AND RNA SYNTHESIS IN A POLYAUXOTROPHIC STRAIN OF E. COLI**

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Increasing evidence suggests that the naturally occurring polyamines, putrescine, spermidine, and spermine, play a role in the stability of cellular organelles, membranes, nucleic acids, and particles containing nucleic acid, e.g., ribosomes (see ref. 1). In addition to stabilizing cellular polyanions, polyamines also might affect directly the rate and extent of nucleic acid synthesis, probably by combining with nucleic acid products. Such a mechanism does appear to play a part in the stimulation by polyamines of bacterial DNA-directed RNA polymerase.[2–4] Further, in regenerating rat liver there is a close correlation between the concentration of RNA and polyamines, as well as in the stimulation of synthesis of these compounds, as measured by incorporation of labeled precursors.[5–7] A correlation in the accumulation of polyamines and nucleic acids has also been reported to occur in the developing chick embryo.[8]

In the present work we have used the polyauxotrophic *E. coli* strain 15 TAU[9] to study the relationship between polyamines and nucleic acids in various nutritional conditions. It was shown earlier that by depriving this organism of an essential amino acid, the rate of RNA synthesis is reduced to about 10–15 per cent of normal.[10, 11] Under these conditions the addition of chloramphenicol or streptomycin results in a stimulation in RNA synthesis.[11, 12]

In this communication it will be shown that deprivation of an essential amino