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Effect of UV Irradiation on the Expression of Vaccinia Virus Gene Products Synthesized in a Cell-Free System Coupling Transcription and Translation

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The effect of UV irradiation on the expression of the vaccinia virus genome was investigated in a cell-free system coupling transcription with translation. Exposure of vaccinia virus to an increasing dose of irradiation resulted in differential reduction in the syntheses of virus-specified polypeptides in the coupled system, with sensitivity being proportional to the size of the gene product. This suggests that each translationally functional mRNA species produced in vitro by vaccinia virus cores is synthesized from an individual promoter site.

Vaccinia virus is a large, DNA-containing virus with a genome complexity of about 1.2×10^8 daltons (4, 6, 7, 9). Synthesis of viral RNA proceeds readily under in vitro conditions at a level which indicates that 40 to 50% of the entire genome is transcribed (19). This result suggests that the RNA synthesized under in vitro conditions could easily code for as many as 150 to 200 proteins in the size range of 10^4 to 10^5 daltons.

Although in vitro-synthesized vaccinia virus RNA has generally been described as a family of molecules with an average sedimentation value of between 8 and 12S (11, 26), the purification and partial characterization of a virion-associated species of high-molecular-weight vaccinia RNA have recently been described (17, 18). The results derived from a number of different experimental approaches are consistent with a precursor-product relationship between the high-molecular-weight RNA and the virion-released 8 to 12S mRNA (16-19).

The technique of UV transcription mapping developed by Sauerbier and colleagues (22, 23) originally with bacterial systems has recently been successfully employed to determine the order of transcription of several animal virus genomes (1, 3, 8). We decided to apply this technique to the vaccinia in vitro transcription system in an effort to detect the transcription of multiple genes from a common promoter, one of the possible modes of transcription suggested by the synthesis of a high-molecular-weight presumptive precursor to vaccinia mRNA (16-19). Since good resolution of individual in vitro-synthesized viral mRNA's has not been achieved, we coupled the transcription of the viral genome to translation in a wheat germ protein-synthesizing system and resolved the resulting polypeptides by polyacrylamide gel electrophoresis. In this report we describe the effects of increasing UV irradiation of vaccinia virus upon the final expression of individual viral gene products in the coupled transcription-translation system.

MATERIALS AND METHODS

UV irradiation. Purified vaccinia virus particles (strain WR), prepared as described by Joklik (10), at a concentration of 45 optical density (OD_{260}) units per ml in 10 mM Tris (pH 7.5) were exposed to incident UV irradiation of 5 ergs/s per mm^2 (supplied by a 253.7-nm-wavelength source situated 30 cm above the sample). Exposures were conducted with continuous stirring at room temperature in a plastic cap 7 mm in diameter and 2.5 mm in depth, with the thickness of the virus particle suspension measuring less than 1 mm.

In vitro RNA and protein synthesis. A cell-free protein-synthesizing system was prepared from Niblack wheat germ by the method of Roberts and Paterson (21). RNA synthesis was followed by determining the incorporation of [^{3}H]UTP (New England Nuclear Corp.) into trichloroacetic acid-precipitable material which was collected on Whatman GF/C glass-fiber filters (14). Conditions for in vitro RNA synthesis in the absence of the translation system were as follows. A 400-μl reaction mixture contained 50 mM Tris-hydrochloride (pH 8.4); 10 mM $MgCl_2$; 10 mM dithiothreitol; 0.05% Nonidet P-40; 2 mM each ATP, GTP, and CTP; 0.2 mM UTP; 40 μl of 0.5-mCi/ml [^{3}H]UTP; and 0.72 OD_{260} unit equivalent of virus. Incubation was at 37°C. Conditions used for the syntheses of RNA and protein in the coupled transcription-translation system are described in detail in Results. Protein synthesis was monitored in this system by determining the incorporation of [^{35}S]methionine (New England Nuclear Corp.) into polypeptides as described by Mans and Novelli (13). Globin and reovirus mRNA's were obtained from C. Baglioni and A. J. Shatkin, respectively.

Polyacrylamide gel electrophoresis. Samples containing [^{35}S]methionine-labeled polypeptides from the coupled transcription-translation system were prepared for analysis by adding an equal volume of water and 2 volumes of 2× sample buffer so that the final concentrations of sample buffer ingredients were 0.1 M dithiothreitol, 2% sodium dodecyl sulfate, 0.08 M Tris-hydrochloride (pH 6.8), 10% glycerol, and 0.4% bromophenol blue. The samples were boiled for 2 min and applied to 15% sodium dodecyl sulfate-polyacrylamide slab gels prepared according to Anderson et al. (2) by the method of Laemmli (12). After electrophoresis, gels were fixed and stained with 0.025% (wt/vol) Coomassie brilliant blue in 40% (wt/vol) methanol–7.5% (wt/vol) acetic acid, usually overnight. Gels were destained in several changes of 40% (wt/vol) methanol–7.5% (wt/vol) acetic acid and then dried in vacuo and autoradiographed on Kodak BB54 medical X-ray film. An appropriate exposure time was chosen in which the film gave a linear response to the amount of radioactivity. Autoradiographs were scanned with a Beckman model R-112 scanning densitometer. The radioactivity incorporated into individual polypeptides was estimated by determining the area under the peaks in the autoradiograph tracings or measured directly by cutting out the polypeptide bands and determining the amount of radioactivity as previously described (15).

RESULTS

Syntheses of vaccinia virus RNA and polypeptides in the wheat germ cell-free system. In considering the coupling of vacinia virus-directed RNA synthesis to translation, we first determined the level and rate of RNA synthesis under standard conditions used for protein synthesis in the wheat germ translation system. RNA synthesis began slowly and then proceeded linearly under these conditions for over 4 h (Fig. 1). Although the initial rate of RNA synthesis in the translation system at 22.5°C and pH 7.6 was approximately 50% that observed under standard transcription conditions at 37°C and pH 8.4 (data not shown), the final level of RNA synthesis was greater in the translation system. This is because RNA synthesis normally ends within 30 to 40 min when transcription proceeds in the absence of an energy-regenerating system (18).

[^{35}S]methionine incorporation into polypeptides under conditions which support both transcription and translation in the wheat germ system was dependent upon the addition of either mRNA or vaccinia virus (Fig. 2). After addition of virus particles to the reaction mixture, there was a 30- to 45-min lag in the incorporation of [^{35}S]methionine into polypeptides, followed by a linear increase in protein synthesis for approximately 2 h, at which time [^{35}S]methionine incorporation ended abruptly. The lag phase was not a characteristic of the translation system,

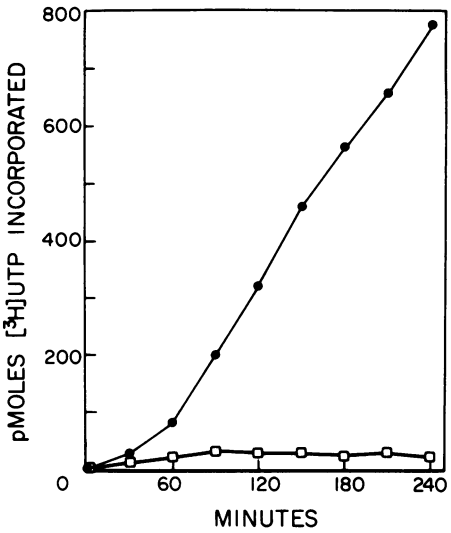

FIG. 1. *RNA synthesis directed by vaccinia virus in a wheat germ cell-free translation system. The reaction mixture contained 20% wheat germ extract, 15 mM creatine phosphate, 11 U of creatine phosphokinase per ml, 16 mM N-2-hydroxyethyl piperazine-N'-2-ethanesulfuric acid (pH 7.5), 10 mM dithiothreitol, 110 mM potassium acetate, 0.3 mM spermidine, 0.01% Nonidet P-40, 0.01 mM S-adenosyl-L-methionine, 4 mM magnesium acetate, 0.05 mM each amino acids minus methionine, 1.7 mM ATP, 0.75 mM GTP, 0.5 mM CTP, 200 mCi of [^{3}H]UTP per μmol at a final concentration of 0.1 mM; and 1 OD_{260} unit of purified vaccinia virus per ml of reaction mixture. Incubation was at 22.5°C, and [^{3}H]UTP incorporation was determined as described in the text. Symbols: □, [^{3}H]UTP incorporation in the absence of virus; ●, [^{3}H]UTP incorporation directed by added vaccinia virus particles.*

since no delay was observed in the synthesis of proteins directed by exogenous vaccinia RNA (Fig. 2). Rather, the lag probably reflects the time required for the energy-dependent extrusion of RNA from the virus cores (11).

The abrupt cessation in [^{35}S]methionine incorporation after 2 h of linear virus-directed protein synthesis appears to be due to the consumption or inactivation of some factor(s) in the wheat germ extract itself. Of all the reaction ingredients tested, only the wheat germ extract permitted protein synthesis to resume. This effect cannot be attributed to RNase contamination of the system since addition of fresh RNA failed to stimulate protein synthesis and ^{3}H-labeled vaccinia mRNA was stable, as determined by measuring acid-insoluble radioactivity, for several hours in the translation system (data not shown).

In preliminary experiments, a comparison was made of the level of translation directed by

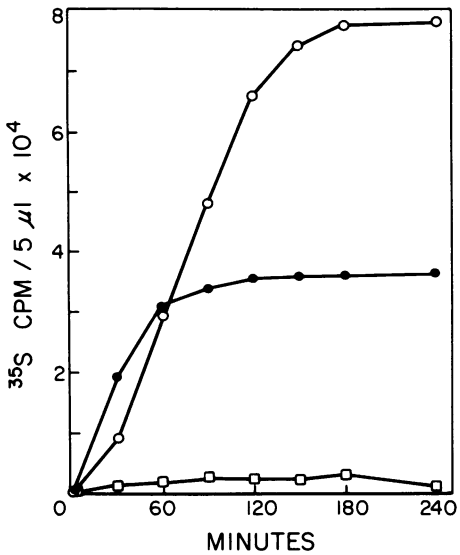

FIG. 2. *Protein synthesis in the wheat germ cell-free translation system directed by vaccinia virus or exogenous vaccinia RNA. [35S]methionine incorporation into protein was determined, as described in the text, under reaction conditions similar to those described in the legend to Fig. 1, except that unlabeled UTP was present at a concentration of 0.5 mM, and the reaction mixture contained 45 µCi of [35S]-methionine. Vaccinia virus RNA was extracted from an in vitro transcription reaction by phenol extraction of the virus-free supernatant after removal of virus particles by centrifugation. The RNA was further purified by gel filtration on Sephadex G-50 equilibrated with 0.01 M sodium acetate (pH 5.0), 0.05 M NaCl, and 0.5% sodium dodecyl sulfate, followed by three ethanol precipitations (as the sodium salt) to remove traces of sodium dodecyl sulfate. Symbols: ○, [35S]methionine incorporation directed by added vaccinia virus particles; ●, [35S]methionine incorporation directed by exogenous vaccinia RNA; □, level of [35S]methionine incorporation observed in the absence of added virus or RNA.*

vaccinia RNA extracted from an in vitro transcription reaction with the level of translation obtained with other mRNA species, e.g., globin and reovirus mRNA's. In vitro-synthesized vaccinia mRNA formed functional initiation complexes efficiently in both the wheat germ and the reticulocyte cell-free system (25). Moreover, the level of amino acid incorporation obtained per microgram of input RNA suggested that exogenous vaccinia RNA was approximately 70% as efficient as globin mRNA in stimulating protein synthesis (data not shown). From calculations of the amount of RNA synthesized in the coupled system, based on the level of [3H]-UTP incorporation into RNA (Fig. 1), it appears that the RNA synthesized directly in the cell-free translation system is only slightly more efficient in directing protein synthesis than vaccinia RNA purified from the standard in vitro transcription system. Thus, there is no indication that gross quantities of translationally inactive mRNA are synthesized and released by vaccinia virus cores in the coupled system. At the same time, these measurements do not rule out the possibility that a fraction of these RNA species are nonfunctional.

[35S]methionine incorporation in the virus-directed coupled system was optimized with respect to each reaction ingredient (Table 1). As previously determined for the translation of exogenous vaccinia RNA (25), the translation of vaccinia virus RNA synthesized directly in the coupled system was also dependent upon the methyl donor *S*-adenosylmethionine being present during RNA synthesis. Nonidet P-40 was required for RNA synthesis to proceed; however, it had little effect on the subsequent translation of the RNA over a concentration range of 0.01 to 0.1%. RNA synthesis was also dependent upon the presence of Mg^{2+}, with the optimum concentration for protein synthesis in the coupled system being 2 mM above the total nucleoside

TABLE 1. *Optimal conditions of protein synthesis in the wheat germ cell-free system coupling transcription by vaccinia virus particles with translation*[a]

Reaction mixture constitutents	Concn
Vaccinia virus[b]	1 OD_{260} unit/ml
ATP[c]	1.70 mM
GTP[c]	0.75 mM
CTP[c]	0.50 mM
UTP[c]	0.10 mM
Total nucleoside triphosphates[c]	3.05 mM
Nonidet P-40[c]	0.01–1.1%
S-adenosyl-L-methionine[b]	0.01 mM
Creatine phosphate[c]	15.00 mM
Creatine phosphokinase[c]	11.00 U/ml
HEPES-KOH (pH 7.6)[c,d]	20.00 mM
Mg acetate[b]	5.00 mM
K acetate[b]	135.00 mM
Amino acids minus methionine[c]	0.05 mM each
Spermidine[b]	0.03 mM
Dithiothreitol[b]	13.00 mM
Wheat germ extract[b]	20.0 OD_{260} units/ml

[a] The effect of varying the concentration of individual reaction constituents on the level of [35S]methionine incorporation in the coupled system programmed by vaccinia virus particles was determined.

[b] Reagent which showed a clear concentration optimum.

[c] Reagent for which no clear optimum was observed, but the concentration shown is saturating for protein synthesis.

[d] HEPES, *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid.

triphosphate level in the reaction mixture. Addition of nucleoside triphosphates or amino acids to concentration levels in excess of that shown in Table 1 resulted in no increase in protein synthesis. The salt optimum for protein synthesis in the coupled system was established as 135 mM potassium acetate. When the chloride salt of potassium was used, however, the optimum was much lower, presumably due to the inhibitory effect of the chloride ion (23, 25). Distinct optima were also observed for the amounts of virus particles and wheat germ extract added to the reaction mixture.

Analysis of polypeptides synthesized in the coupled system. ^{35}S-labeled polypeptides synthesized in the coupled system were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as shown in Fig. 3. Polypeptides synthesized in the wheat germ extract, directed by RNA purified from the in vitro transcription reaction, are shown in lane 1. Approximately 30 to 35 prominent bands are observed, ranging in molecular weight from 10,000 to 50,000. When protein synthesis was directed by RNA resulting from transcription by virus particles directly in the translation system, the same polypeptides bands were made, but a greater proportion of the higher-molecular-weight polypeptides was observed with molecular weights in excess of 65,000. The relative synthesis of individual polypeptides changed dramatically when the system was programmed by the virus rather than by exogenous RNA; e.g., notice the change in the levels of synthesis of p-17.5, p-20.2, p-22.6, and p-45. (cf. lane 1 with lanes 2 to 5). We also observed that the virus concentration affected the relative synthesis of individual proteins (cf. lanes 2 through 5). This phenomenon appeared to be dependent upon the actual virus particle concentration and not upon the relative amounts of viral RNA made in the system. A similar effect on the synthesis of individual polypeptides was not observed when the amount of exogenous vaccinia mRNA was varied over a concentration range similar to the level of RNA synthesized in the coupled system (data not shown). Also, the gel profiles of polypeptides synthesized in the coupled system did not change with time during the reaction, i.e., as mRNA synthesis increased, for reactions containing a given concentration of virus particles. Thus, it appears that the relative synthesis and/or relative translation of individual mRNA's is affected by the virus particle concentration rather than the mRNA concentration. Whether this effect has any relation to a control mechanism normally operating in the infected cells is not presently known.

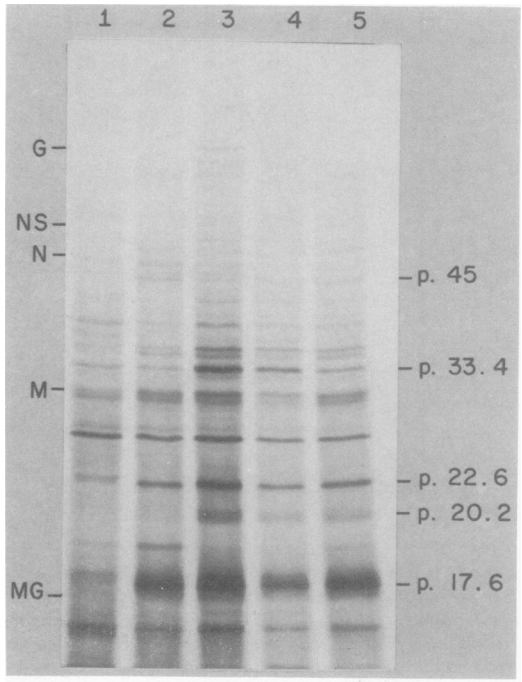

FIG. 3. *Sodium dodecyl sulfate-polyacrylamide gel analysis of ^{35}S-labeled translation products programmed by exogenous vaccinia RNA or by purified virus particles. The autoradiograph shows the polypeptides resulting from translation of exogenous vaccinia RNA (lane 1) and the translation products synthesized in the coupled system at increasing virus concentrations: (lane 2) 0.5 OD_{260} unit/ml; (lane 3) 1 OD_{260} unit/ml; (lane 4) 1.5 OD_{260} units/ml; and (lane 5) 2 OD_{260} units/ml. The letters on the left denote the locations of protein markers, which include the vesicular stomatitis virus glycoprotein (G), minor protein (NS), nucleocapsid protein (N), and membrane matrix protein (M) with respective molecular weights of 66,000, 52,000, 47,000, and 30,000. MG denotes the position of myoglobin (molecular weight, 17,000). The numbers on the right indicate the calculated molecular weights of several polypeptide bands of interest.*

Effect of UV irradiation of vaccinia virus upon the expression of viral gene products in the coupled system. High-molecular-weight RNAs are synthesized in vitro by vaccinia virus, and these RNAs have a number of properties consistent with their being precursors to 8 to 12S mRNA (17, 18). This finding stimulated us to investigate the mechanism of vaccinia virus transcription by using the coupled transcription-translation system as a means of resolving and quantitating the viral gene products after exposure of the virus to UV irradiation. Consequently, we first determined the effect of UV irradiation on the infectivity of the virus (Fig. 4A). Infectivity decreased at an exponential rate, with a 37% survival dose of 60 ergs/mm^2. Simi-

larly, RNA synthesis, as measured either in the standard in vitro transcription reaction or in the coupled system, decreased with single-hit kinetics after exposure to UV irradiation, as did protein synthesis in the coupled system directed by virus particles exposed to increasing doses of irradiation (Fig. 4B). Similar results were obtained whether or not exposure of virus particles to UV irradiation was preceded by sonic oscillation to disrupt aggregates.

When the viral polypeptide products which were synthesized in the coupled system programmed by irradiated virus particles were analyzed by polyacrylamide gel electrophoresis, it was observed that the individual polypeptides were differentially inhibited in their synthesis (Fig. 5). This experiment was repeated a total of five times, and in each case visual inspection suggested that the sensitivity of the individual proteins was directly related to their molecular weights. On several occasions, a diffuse band of approximately 55,000 molecular weight appeared to be considerably more resistant to UV inactivation than did other polypeptide bands in that molecular weight range. When gel electrophoresis conditions were altered to give maximum resolution in this region, the band was resolved into three polypeptide bands having sensitivities similar to those of their immediate neighbors; i.e., they failed to show the resistance exhibited by the unresolved band. The nature and significance of this puzzling phenomena are

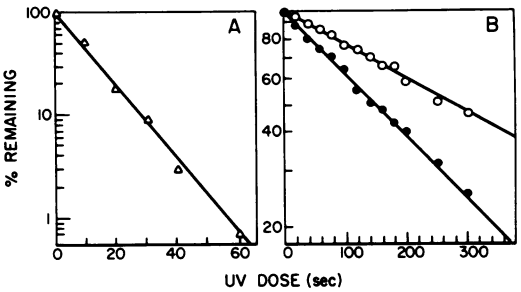

FIG. 4. *Effect of UV irradiation on vaccinia virus infectivity and on the rates of RNA synthesis and protein synthesis in the coupled system directed by UV-treated virus particles. (A) Effect of UV irradiation on infectivity. Purified virus particles were exposed to UV irradiation, as described in the text, and the infectivity was subsequently determined on L-cell monolayers. (B) Reduction in RNA synthesis (●) and protein synthesis (○) programed by virus particles which had been exposed to UV irradiation under identical conditions.*

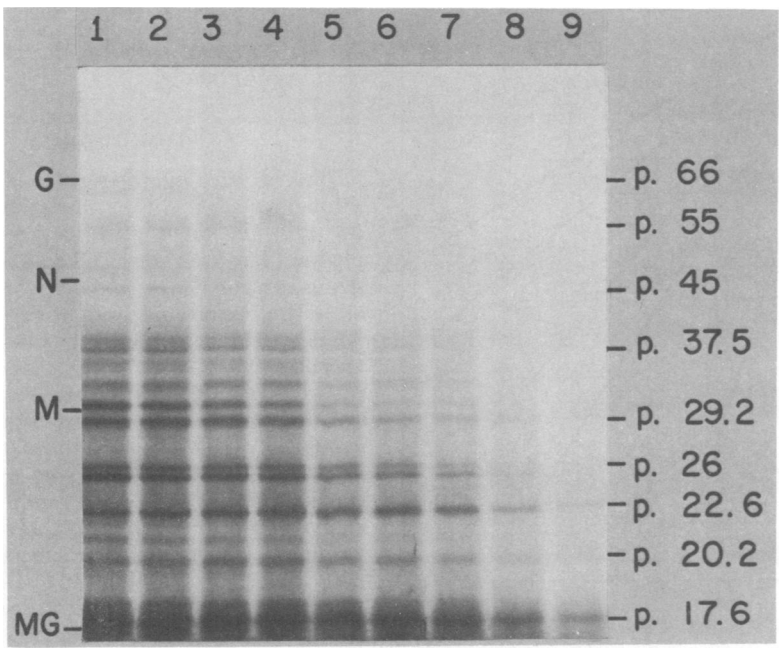

FIG. 5. *Sodium dodecyl sulfate-polyacrylamide gel analysis of translation products synthesized in the coupled system programmed by virus particles exposed to increasing doses of UV irradiation. As in Fig. 3, the letters on the left of this autoradiograph show the positions of the marker polypeptides, and the numbers on the right denote the calculated molecular weights of several prominent polypeptide bands. Lane 1, Translation products programmed by virus particles which received no UV irradiation. Lanes 2 to 9, Translation products programmed by virus particles exposed to the following UV doses: 40, 100, 140, 200, 300, 400, 600, and 1,000 s. Each well received identical amounts of the coupled cell-free extract.*

unclear since the differences in the relative sensitivities of all other polypeptide bands remained the same under the varying conditions of gel analysis. In addition, it should be mentioned that exposure of virus to UV irradiation resulted neither in the appearance of new polypeptides synthesized in the coupled system, nor in any evidence of increased early quitting (Fig. 5).

Quantitation of the major discrete polypeptide bands over a molecular weight range revealed that the reduction in synthesis of these polypeptides after exposure to UV irradiation exhibited single-hit kinetics (Fig. 6) for several representative polypeptides. In addition, when the rates of inactivation for the synthesis of 17 polypeptides were plotted versus the molecular weights of these polypeptides, a linear relationship resulted (Fig. 7). This relationship suggests that the mRNA's coding for the individual viral translation products synthesized in the coupled system are transcribed from individual promoter sites; i.e., they appear not to be derived from polycistronic mRNA precursor molecules which are the result of transcription of several genes from a single promoter site.

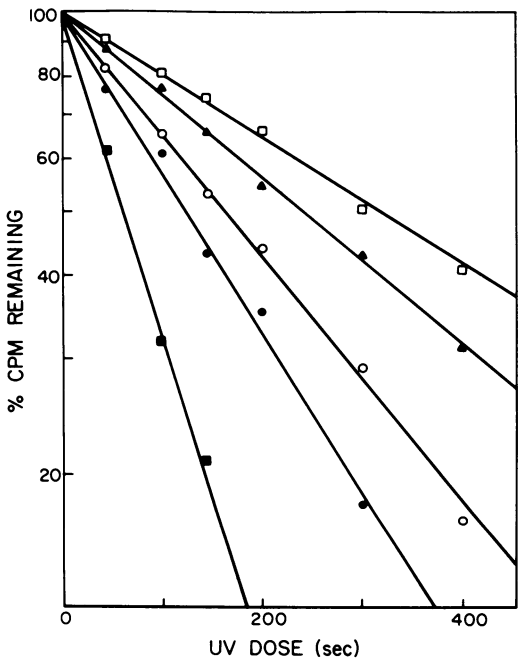

FIG. 6. *Survival curves for several representative virus-programed polypeptides. This figure shows the effect of increased UV irradiation of virus particles on the syntheses in the coupled system of five polypeptides of different molecular weights. The calculated molecular weights of the five polypeptides are 17,600 (□), 22,600 (▲), 29,000 (○), 45,000 (●), and 80,000 (■).*

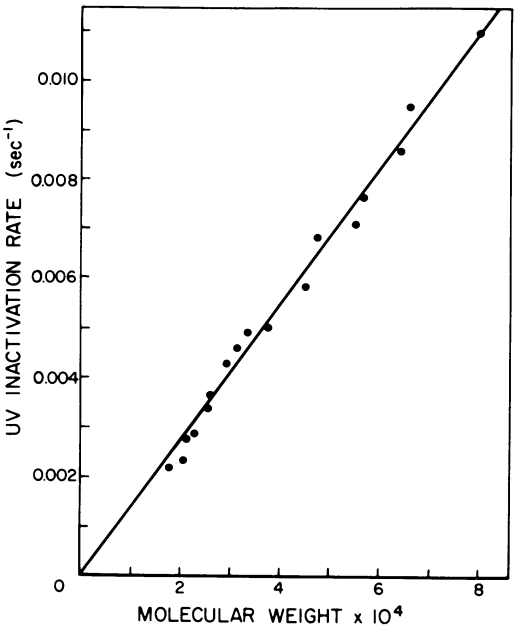

FIG. 7. *Relationship between the rates of inactivation and the molecular weights of translation products synthesized in the coupled system programmed by UV-treated virus particles. From survival curves such as shown in Fig. 6, the inactivation rates for 17 polypeptide bands were determined and are plotted against the calculated molecular weights of the respective polypeptides.*

DISCUSSION

The in vitro synthesis of RNA by vaccinia virus particles and the translation of the resulting transcription products were successfully coupled in the wheat germ cell-free protein-synthesizing system. Since evidence for the synthesis of a high-molecular-weight virion-associated RNA species which may be the precursors to vaccinia virus 8 to 12S mRNA has recently been reported (17, 18), we decided to use this coupled system as a means of analyzing the effect of UV irradiation on the synthesis of the viral gene products. Although coupling of transcription to translation provides only an indirect method of analyzing and quantitating the survival of the viral gene transcripts after exposure of virus to UV irradiation, it is the only method immediately available for this task, since resolution of individual viral mRNA's has not been satisfactorily achieved. Inactivation of virus infectivity (Fig. 4A), transcription, and resulting translation in the coupled system (Fig. 4B) did follow single-hit kinetics after exposure of the virus to UV irradiation. Analysis of the polypeptides synthesized at the direction of virus particles exposed to increasing doses of irradiation showed a dif-

ferential inhibition of synthesis of individual polypeptides (Fig. 5 and 6). Finally, a linear relationship between the rates of UV inactivation and the molecular weights of individual polypeptides synthesized in the coupled system was observed (Fig. 7). We obtained no evidence which would suggest the transcription of multiple genes from a common promoter site. While this work was in progress, similar results were reported by Pelham (20) for a coupled system, using a messenger-dependent reticulocyte cell-free translation system.

There are, however, several limitations inherent in these systems. First, there is currently no way of determining the actual relationship between the relative sizes of the polypeptides and the corresponding mRNA molecules coding for those polypeptides; i.e., just because polypeptide A is twice the molecular weight of polypeptide B, it does not necessarily follow that the mRNA coding for polypeptide A is twice the size of the mRNA coded for polypeptide B. This uncertainty most probably affects the results plotted in Fig. 7. Second, we have little information concerning the relationship between the relative abundance of individual mRNA's synthesized by the virus particle and the relative synthesis of polypeptides in the coupled system as determined by quantitation after polyacrylamide gel electrophoresis. Boone and Moss (5) have reported that a small portion of the complexity of in vitro vaccinia mRNA is present at a concentration of about 150-fold in excess of other sequences (J. Virol., in press). This may explain why only 30 to 40 polypeptides, rather than a possible 200 to 300 vaccinia polypeptides, are detected in cell-free translation systems. Alternately, the polypeptides synthesized in the coupled system may represent the translation products of only a fraction of the bulk of transcripts normally synthesized by the virus particles, a portion of which may be translationally nonfunctional. In this regard, a comparison of initiation complex formation and the level of amino acid incorporation stimulated by vaccinia mRNA with that observed for other functional mRNA species does suggest that the RNA synthesized and released by vaccinia virus cores is not grossly contaminated with nonfunctional mRNA (25; unpublished data). However, we cannot rule out the possibility that the polypeptides expressed in the coupled system are the products of sequences found very near the 5′ end of several long transcriptional units, whereas other sequences that are normally expressed only after processing of these long transcripts are silent in our system. In this respect, we have recently translated purified virion-associated high-molecular-weight vaccinia RNA and found the translation products to be identical to the translation products of 8 to 12S in vitro mRNA (manuscript in preparation). Additional experiments in progress suggest that the sequences translated are located at the 5′ ends of these long transcripts. Third, a detailed comparison of the polypeptides synthesized in the coupled system programmed by virus particle with the virus-specific polypeptides synthesized in infected cells has not yet been completed. However, a comparison of the cell-free translation products of vaccinia in vitro-synthesized mRNA with the cell-free translation products of vaccinia mRNA isolated from polyribosomes of infected cells has revealed that many of the polypeptides have similar migration rates on polyacrylamide gels (J. Cooper and B. Moss, personal communication). Furthermore, since evidence for a high-molecular-weight presumptive precursor mRNA has so far been obtained only under in vitro transcription conditions (16, 17–19), we feel justified in using the system in its present state of characterization to investigate possible modes of transcription as it proceeds in the purified viral particles.

Additional studies are required both to characterize the coupled system, which should prove a useful tool in elucidating control mechanisms operative in infected cells, and to determine the functional significance of the virus-associated high-molecular-weight RNA.

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