JOURNAL OF VIROLOGY, Jan. 1981, p. 284-294
0022-538X/81/010284-11$02.00/0

Vol. 37, No. 1

Hybridization Selection and Cell-Free Translation of mRNA's Encoded Within the Inverted Terminal Repetition of the Vaccinia Virus Genome

JONATHAN A. COOPER,† RICCARDO WITTEK, AND BERNARD MOSS*

Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

Early polypeptides encoded within the 10,000-base pair terminally repeated region of the vaccinia virus genome were mapped by cell-free translation of mRNA that was selected by hybridization to restriction fragments and to separated strands of a recombinant λ phage. The results, which were confirmed by hybrid arrest of translation, indicated that polypeptides of 7,500 (7.5K), 19,000 (19K), and 42,000 (42K) daltons mapped at approximately 3.2 to 4.3, 6.5 to 7.2, and 7.2 to 8.3 kilobase pairs from the end of the genome, respectively. mRNA's for the 42K and 7.5K polypeptides were transcribed towards the end of the genome, whereas mRNA for the 19K polypeptide was transcribed in the opposite direction. Including polyadenylic acid tails, the lengths of the mRNA's for the 7.5K, 19K, and 42K polypeptides, determined by gel electrophoresis of denatured RNA, hybridization selection, and cell-free translation, were approximately 1,200, 680, and 1,280 nucleotides, respectively. mRNA's for the 42K and 19K polypeptides were only about 100 nucleotides longer than the minimums required to code for their respective polypeptides, whereas mRNA for the 7.5K polypeptide contained 900 nucleotides of untranslated sequence. This long untranslated portion of the latter mRNA was probably located near the 3′ end, because this gene was only inactivated by high doses of UV irradiation. This small target size also excluded certain models for RNA processing involving formation of the mRNA's for the 42K and 7.5K polypeptides from a common promoter. Rabbitpox virus, which has an inverted terminal repetition approximately half that of vaccinia virus, was also shown to encode mRNA's that hybridized to the cloned terminal segment of vaccinia virus DNA.

Vaccinia virus has a large double-stranded DNA genome capable of encoding more than 150 polypeptides (4, 22). The difficulty in mapping them by classical genetic methods led us to undertake an alternative biochemical approach. First, early and late mRNA's from vaccinia virus-infected cells were shown to be accurately translated in a message-dependent reticulocyte cell-free system (8). Specific mRNA's were then selected by hybridization to purified restriction fragments, and their translation products were analyzed. In this manner, major polypeptides encoded within three large regions of the genome were determined (9). To facilitate more detailed analyses necessary for fine-structure mapping and for studying the mechanisms involved in the synthesis and processing of mRNA, we cloned fragments of the vaccinia virus genome in bacteriophage λ (31). One of the cloned fragments contains nearly the entire 10-kilobase pair (kbp) inverted terminal repetition, which is divided into a terminal nontranscribed portion containing tandem repetitions of a 70-bp sequence (35) and a longer region that encodes three early polypeptides of 42,000 (42K), 19,000 (19K), and 7,500 (7.5K) daltons (31).

In this communication, we describe further in vitro translation experiments with mRNA selected by hybridization to restriction fragments and separated strands of the cloned terminal repetition. These have enabled us to map the three genes precisely and to determine their directions of transcription. Additional experiments providing information on the physical sizes of the translatable mRNA's and the functional sizes of their genes are also summarized. Complementary studies involving the direct analysis of RNA species encoded in this region have been described elsewhere (32).

MATERIALS AND METHODS

Isolation of RNA from virus-infected cells. HeLa cells were grown in suspension cultures and

† Present address: Tumor Virology Laboratory, The Salk Institute, San Diego, CA 92138.

infected with 1,500 particles (30 PFU) of purified vaccinia virus (WR strain) per cell as described previously (19). Total cytoplasmic RNA from infected cells that were treated at the time of infection with cycloheximide (100 μg/ml) or cytosine arabinoside (40 μg/ml) was isolated at 4 h after infection by sedimentation through a CsCl cushion as described previously (4, 14). Efficient synthesis of rabbitpox virus mRNA required a modified infection procedure (17). HeLa cells were concentrated to 10^7 cells per ml of Puck saline containing 1% horse serum, 10 mM $MgCl_2$, and 100 μg of cycloheximide per ml, and 30 PFU of purified rabbitpox virus (strain Utrecht) per cell was added. After 30 min at 37°C, the cells were diluted to 5×10^5 per ml of Eagle medium containing 5% horse serum and 100 μg of cycloheximide per ml, and RNA was extracted after 3.5 h.

DNA preparation. The construction, isolation, purification, and characterization under P1 conditions of a recombinant λgtWES·λB bacteriophage designated λA7/1 containing the terminal *Eco*RI fragment of vaccinia virus (WR) DNA and methods for the extraction of DNA have been described previously (31, 35). Strands were separated by equilibrium centrifugation of denatured DNA in the presence of polyuridylic acid-polyguanylic (1:1, P-L Biochemicals) as described previously (29). The strands were harvested, incubated in 0.25 M NaOH for 2 h at 37°C to hydrolyze the polyuridylic acid-polyguanylic acid and dialyzed exhaustively.

Hybridization selection of RNA for translation. Approximately 40 μg of recombinant DNA was denatured in alkali. The solution was then neutralized, made 6× SSC (0.90 M NaCl–0.090 M sodium citrate), and immediately filtered through a 2.5-cm-diameter nitrocellulose membrane and then baked at 80°C for 2 h. To prepare filters carrying individual restriction fragments from the vaccinia virus insert, the recombinant DNA was cleaved with *Eco*RI and then with a second specified restriction enzyme, and the fragments were separated by electrophoresis on an agarose gel. Generally, 0.4 pmol of DNA (11 to 12 μg) was digested and loaded on a 5-cm-wide track in a 3-mm-thick 1% or 1.4% agarose gel. After electrophoresis, the gel was stained and then blotted onto nitrocellulose sheets by the procedure of Southern (28) or the modified procedure of Wahl et al. (30)

Total cytoplasmic RNA (210 μg) was hybridized to strips of nitrocellulose filters carrying 0.2 pmol of recombinant DNA as described previously (31). The hybridization conditions were 80% formamide–0.4 M NaCl–0.04 M sodium-PIPES [sodium piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4)–0.001 M EDTA–0.1% sodium dodecyl sulfate at 37°C for 18 h. At the end of the hybridization, the filters were stringently washed to remove unhybridized RNA (31). This consisted of three washes at room temperature with 4 ml of 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA–0.1% sodium dodecyl sulfate and then three washes with 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA. The filters were then washed twice with 0.4 ml of hybridization solution lacking sodium dodecyl sulfate at 37°C for 15 min and finally in 0.4 ml of 80% formamide–40 mM PIPES (pH 6.4)–1 mM EDTA at 37°C for 15 min. Hybridized RNA was eluted in 0.2 ml of water at 100°C for 2 min and then alcohol precipitated with 20 μg of calf liver tRNA (Boehringer Mannheim). The precipitate was washed twice with 70% ethanol and dried in vacuo.

In vitro translation. Translation of RNA in a message-dependent reticulocyte lysate was as described previously (8, 31). RNA was translated in 5- or 10-μl reactions containing 2 μCi of [^{35}S]methionine per μl. Control reactions contained tRNA only or tRNA and 0.5 or 1 μg of total cytoplasmic RNA (final concentration, 100 μg/ml).

Samples of the in vitro translations were analyzed by electrophoresis on dodecyl sulfate–20% polyacrylamide gels (7). Radioactive proteins were detected by fluorography, using preexposed Kodak XR-2 film, after impregnation with PPO (2,5-diphenyloxazole) (3) or with Enhance, a product of New England Nuclear Corp.

RNA fractionation on methylmercury gels. Agarose gels, 1.5% containing 5 mM methylmercuric hydroxide (Alfa Div., Ventron Corp.), were made according to Bailey and Davidson (2). The samples were prepared as follows: 650 μg of total cytoplasmic RNA and 5′-end-labeled [^{32}P]DNA markers was incubated in 0.28 ml of half-concentration E buffer (2) containing 20% glycerol, 50 μg of bromophenol blue per ml, and 10 mM methylmercuric hydroxide at 60°C for 5 min and loaded on a 13-cm-wide slot in a 3-mm-thick, 16-cm-long agarose gel which was then run at 30 V for 16 h. After electrophoresis, the gel was detoxified by washing it three times in 0.5 M ammonium acetate and then exposed to X-ray film for 2 h at 4°C (using an intensifying screen) to determine the position of the internal ^{32}P-labeled DNA size markers. Gel slices (4 mm thick) were frozen, and the RNA was extracted with phenol (10, 16) and ethanol precipitated with 20 μg of carrier tRNA. The RNA was dissolved in 1 ml of 0.2 M NaCl–10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA, and a 50-μl sample of each fraction was reprecipitated and translated in a 10-μl reaction. Samples of RNA from each fraction were also used for hybridization to recombinant DNA immobilized on filters: 200-μl samples of each fraction were ethanol precipitated, dissolved in 100 μl of hybridization buffer, and hybridized with 0.2 pmol of recombinant DNA as described above. Thus, each hybridization contained RNA fractionated from 130 μg of total cytoplasmic RNA. The RNA species separated by electrophoresis on methylmercury gels were also identified by blotting to diazotized paper (1) and probing with recombinant [^{32}P]DNA. The results were similar to those previously obtained by electrophoresis of glyoxylated vaccinia virus RNA (32).

Hybridization arrest of translation. The locations of transcripts coding for certain polypeptides were confirmed by hybrid arrest of translation, using specific restriction fragments (23). The procedure was modified by first purifying RNA that hybridized to immobilized total recombinant DNA. For each hybridization arrest experiment, RNA purified from approximately 0.07 pmol of recombinant DNA was ethanol precipitated with approximately 0.2 pmol of a purified DNA fragment and 25 μg of tRNA. The precipitate was dissolved in 45 μl of 89% formamide–10 mM sodium-PIPES (pH 6.4) and heated at 100°C for 2 min

to denature the DNA. The sample was quenched in ice-cold water, and 5 μl of 4 M NaCl–0.4 M sodium-PIPES (pH 6.4)–10 mM EDTA was added. After hybridization at 37°C for 3 h, 350 μl of ice-cold water was added, and 200 μl of the solution was heated at 100°C for 2 min. Both the hybridized and the melted solutions were ethanol precipitated and translated in 10-μl reactions.

RESULTS

Translation of RNA selected by hybridization to total recombinant DNA. Previous studies (31) demonstrated that early RNA made in the presence of cycloheximide (an inhibitor of protein synthesis) or cytosine arabinoside (an inhibitor of DNA synthesis) and selected by hybridization to the cloned terminal 9-kbp *Eco*RI fragment of the vaccinia virus genome directed the synthesis of 42K, 19K, and 7.5K major polypeptides in the rabbit reticulocyte cell-free system. Greatly reduced amounts of these polypeptides and no additional ones were detected when late mRNA, made in the absence of inhibitors, was analyzed in this manner. These three polypeptides, as well as nonspecific products which migrated at 48,000 daltons and at the hemoglobin front (just above the 7.5K polypeptide), are seen in Fig. 1, track 7. Additional minor bands at 38,000, 24,200, and 22,800 daltons were seen upon longer exposure of this autoradiograph and in subsequent figures.

The hybridization selection and washing procedure effectively removed translatable cellular mRNA's and viral mRNA's encoded within other parts of the genome. However, significant amounts of specifically hybridized mRNA's were also lost. Relatively more of the mRNA's for the 42K and 19K polypeptides than for the 7.5K polypeptide were eluted during washing. This may have been due to differences in the stabilities of the DNA·RNA hybrids as was shown by translating the eluant obtained upon washing the filter at 37°C in 80% formamide with successively decreasing concentrations of salt (Fig. 1). The mRNA for the 42K polypeptide also appeared to be more easily degraded during purification or translation. For these reasons, the relative amounts of the three major polypeptides did not necessarily reflect the relative starting quantities of their mRNA's. Nevertheless, we doubt that any major translatable RNAs encoded predominantly within the cloned DNA segment were lost entirely, since the same polypeptide pattern was seen with less stringent hybridization conditions (50% formamide–0.4 M NaCl, 42°C).

The 42K, 19K, and 7.5K polypeptides were also detected when selected RNA was translated in a wheat germ cell-free system (data not shown). Differences in the minor polypeptides, however, were noted. Instead of discrete 38K, 24.2K, and 22.8K polypeptides, there were a larger number of other labeled minor polypeptides up to 42K, which probably represented premature termination products (15).

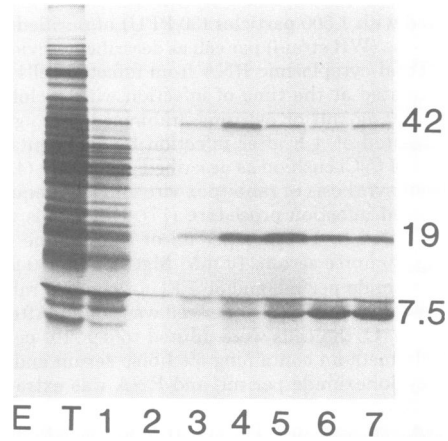

FIG. 1. *Effect of stringent washing on elution of translatable RNAs from filters containing recombinant DNA. Approximately 210 μg of cytoplasmic RNA from cells infected in the presence of cycloheximide was hybridized with 0.2 pmol of recombinant DNA immobilized on a nitrocellulose filter. After brief washing in 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA–0.1% sodium dodecyl sulfate at room temperature, the filter was incubated for successive 5-min periods at 37°C with 40 mM PIPES (pH 6.4)–1 mM EDTA–80% formamide plus: 0.4 M NaCl (track 1), repeat with 0.4 M NaCl (track 2), 0.12 M NaCl (track 3), 0.08 M NaCl (track 4), 0.04 M NaCl (track 5), or no NaCl (track 6). The filter was then incubated for 2 min at 100°C with water (track 7). In each case, the eluted RNA was translated in the reticulocyte cell-free system, and the polypeptides were resolved by electrophoresis on a 20% polyacrylamide gel. E, Endogenous translation products without added RNA, T, translation products of total unselected RNA.*

Sizes of translatable RNAs. To determine the sizes of the mRNA's coding for the 7.5K, 19K, and 42K polypeptides, total cytoplasmic RNA from cells infected in the presence of cycloheximide was denatured with methylmercuric hydroxide and electrophoresed on agarose gels. RNA was then eluted from each slice, and one portion was translated directly, whereas another portion was first selected by hybridization to the recombinant DNA.

The translation products of total unselected RNA are shown in Fig. 2A. The relationship between mRNA size and polypeptide size was similar to that observed previously on nondenaturing sucrose gradients (8). For each size

class of mRNA, a number of different polypeptides were made, the largest of which used most of the coding capacity of an mRNA of that size range.

The translation products of RNA selected by hybridization to recombinant DNA are shown in Fig. 2B. The results indicated that the RNAs coding for the 42K and 7.5K polypeptides were very similar in size, although that coding for the 7.5K polypeptide was slightly smaller. We estimated the sizes of the RNAs for the 42K and 7.5K polypeptides as 1,280 and 1,200 nucleotides, respectively. The synthesis of minor amounts of 7.5K polypeptide by smaller RNAs down to the size of approximately 350 nucleotides could have resulted from translation of degraded RNA, since the coding sequence necessary for this polypeptide was so small. RNA coding for the 19K polypeptide had an average size of about 680 nucleotides.

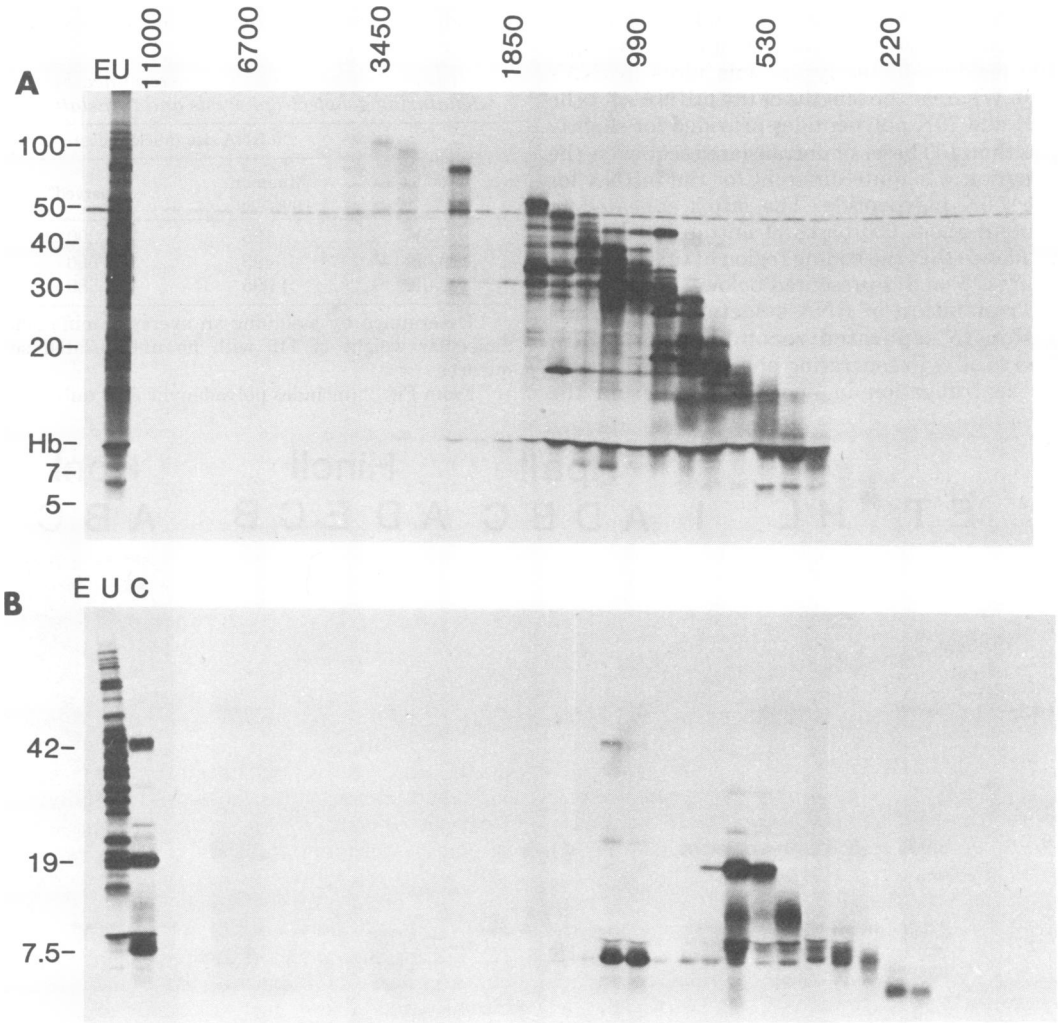

FIG. 2. *Translation of RNAs separated by gel electrophoresis. Total cytoplasmic RNA from cycloheximide-treated infected cells was fractionated by electrophoresis on an agarose gel containing methylmercuric hydroxide. Radioactive DNA restriction fragments were included as internal length standards. RNA from each gel slice was either translated directly (A) or first hybridized to recombinant DNA (B). Translation products were analyzed by electrophoresis on a 20% polyacrylamide gel. E, Endogeneous products of the cell-free system; U, products of unfractionated, unhybridized RNA; C, products of unfractionated, hybridized RNA. For (A), RNA sizes (in bases) are indicated at the top and polypeptide sizes (in kilodaltons) are on the left, Hb indicates the position of globin. For (B), RNA sizes correspond to those of part A except that the gel tracks are displaced by one to the right.*

The synthesis of additional minor polypeptides was also detected: a 22.8K polypeptide was made from RNA the same size as that coding for the 42K polypeptide; 24.2K to 38K polypeptides were made from RNA the same size as that coding for the 19K polypeptide. An additional diffuse radioactive band migrating with globin also appeared when RNAs of about 500 to 600 nucleotides were translated.

The minimum coding lengths and the observed lengths of the mRNA's for the 7.5K, 19K, and 42K polypeptides are summarized in Table 1. The observed lengths include a nontranscribed 3'-polyadenylic acid tract which averages 100 residues for early vaccinia virus mRNA's (20). Whereas the lengths of the mRNA's for the 42K and 19K polypeptides provided for slightly less than 100 bases of untranslated sequence, the situation was quite different for the mRNA for the 7.5K polypeptide. The latter appeared to contain about 900 bases of untranslated RNA. Evidence that the coding region of this RNA lies near its 5' end is presented below.

Translation of RNA selected by hybridization to separated recombinant strands. The facility in separating phage λ DNA strands by centrifugation in density gradients in the presence of polyuridylic acid-polyguanylic acid (29) was a significant factor in our selection of this cloning vehicle. We found that the recombinant DNA strands also separated under these conditions. Moreover, hybridization of the separated strands of wild-type λ DNA to ^{32}P-labeled strands of the recombinant demonstrated that they had the same polarity (data not shown).

Early viral RNA that hybridized to the heavy recombinant DNA strand programed the in vitro synthesis of the 19K and faint 24.2K polypeptides (Fig. 3, track H). The 7.5K and 42K polypeptides, as well as other minor polypeptides,

TABLE 1. *Sizes of mRNA's determined by denaturing gel electrophoresis and translation*

Polypeptide product	mRNA size (nucleotides)	
	Minimum possible[a]	Observed[b]
7.5K	195	1,200
19.0K	495	680
42.0K	1,095	1,280

[a] Determined by assuming an average amino acid molecular weight of 115 with no untranslated sequences.

[b] From Fig. 2 (includes polyadenylic acid tail).

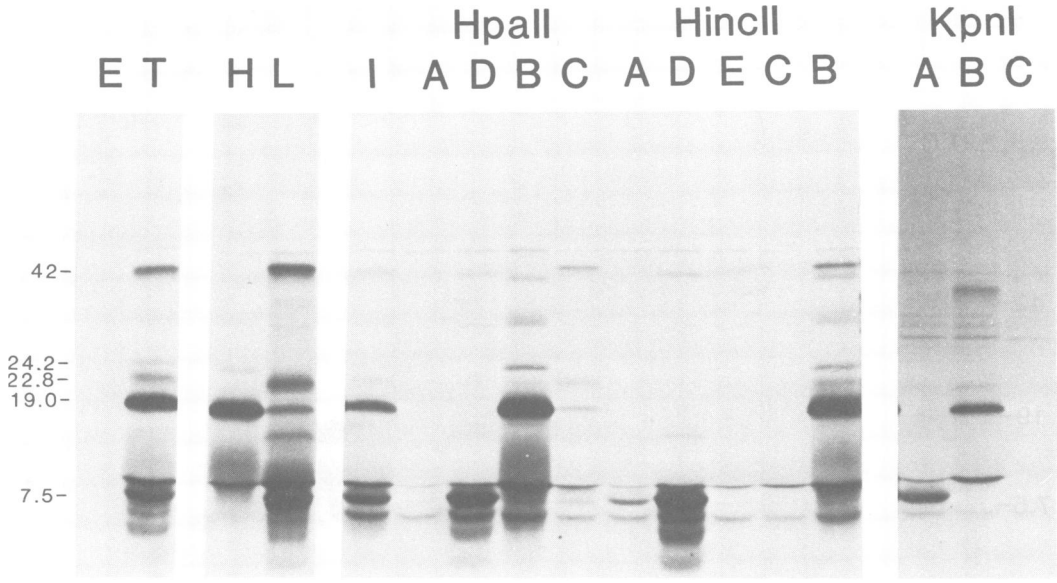

FIG. 3. *Fluorogram of [^{35}S]methionine-labeled in vitro translation products of RNAs purified by hybridization selection. Total cytoplasmic RNA from vaccinia virus-infected cells treated with cycloheximide was hybridized to DNAs immobilized on nitrocellulose membranes and then translated in a reticulocyte cell-free system. The sizes (in kilodaltons) of the polypeptides are shown on the left. Polypeptides translated with RNA selected by hybridization to total recombinant DNA (T), the heavy strand of the recombinant DNA (H), the light strand of the recombinant DNA (L), purified vaccinia virus DNA insert (I), and indicated HpaII, HincII, and KpnI restriction fragments which are arranged in correct map order with the following exception. The HincII E fragment is a mixture of two fragments: one is in the correct sequence, and the other follows the HincII B fragment at the end of the cloned segment. E, Endogeneous products of the cell-free system.*

were made by using RNA selected by hybridization to the L strand (Fig. 3, track L). The light strand was slightly contaminated with heavy strand, however, accounting for the synthesis of some 19K polypeptide. Since the light strand of phage λ is the template for leftward-reading transcripts, DNA encoding the mRNA's for the 42K and 7.5K polypeptides is transcribed to the left (toward the end of the genome) and DNA for the 19K polypeptide is transcribed to the right.

Translation of RNA selected by hybridization to restriction fragments. Polypeptides were mapped by translating mRNA selected by hybridization to restriction fragments immobilized on nitrocellulose (18, 26) or by a solution hybridization procedure (9). The sites where the restriction enzymes *Xho*I, *Kpn*I, *Hpa*II, and *Hinc*II cut the cloned terminal *Eco*RI segment of the vaccinia virus genome were mapped by conventional procedures (32) and are shown in Fig. 4. The positions of these sites agreed well with those determined for DNA obtained directly from virus particles and enabled us to determine that the left end of the map corresponded to the terminus of the vaccinia virus genome (31).

RNA coding for the 7.5K polypeptide was initially selected with the *Xho*I A fragment, using solution hybridization procedures (not shown). Synthesis of the same polypeptide was also programed by RNA hybridizing to *Hpa*II D, *Hinc*II D, and *Kpn*I A fragments (Fig. 3). Reduced but significant synthesis was detected with mRNA hybridizing to the *Hinc*II A fragment but not to the *Hpa*II A fragment, placing its 3′ end approximately 3.2 kbp from the end of the genome. Since this RNA was about 1,100 nucleotides long exclusive of polyadenylic acid, its 5′ end should have mapped at about 4.3 kbp (Fig. 4). In agreement with this, the RNA did not hybridize to the *Hinc*II E fragment. Hybridization to the *Hpa*II E and *Hinc*II F fragments, however, was not tested for technical reasons.

RNA coding for the 19K major polypeptide, as well as some minor ones, including 24.2K and 38K polypeptides, was selected by hybridization to the *Hpa*II B, *Hinc*II B, and *Kpn*I B fragments (Fig. 3). Since this RNA did not hybridize to the *Hinc*II C fragment, the 5′ end of the message must lie to the right of 6.5 kbp. A faint 19K band was detected with RNA selected by hybridization to the *Hpa*II C fragment, suggesting that the 3′ end was at approximately 7.2 kbp. The distance between these points was just long enough to accommodate a single RNA of the measured size. Since the region was not long enough to code separately for the 19K major polypeptide and the minor polypeptides, it is possible that all were products of in vitro translation of the same message.

RNA coding for the 42K polypeptide appeared to be particularly susceptible to degradation as well as most easily lost during stringent washing, and in this experiment, only small amounts of this polypeptide were made (Fig. 3, track I). Nevertheless, it can be seen that RNA encoding the 42K polypeptide, as well as the RNA for the 22.8K polypeptide, hybridized to the *Hpa*II C and *Hinc*II B fragments. Its failure to hybridize to the *Hpa*II B fragment indicated that its 3′ end must start near 7.2 kbp (Fig. 4). The failure of the RNA to hybridize to *Hinc*II E fragment and its size place the 5′ end at about 8.3 kbp.

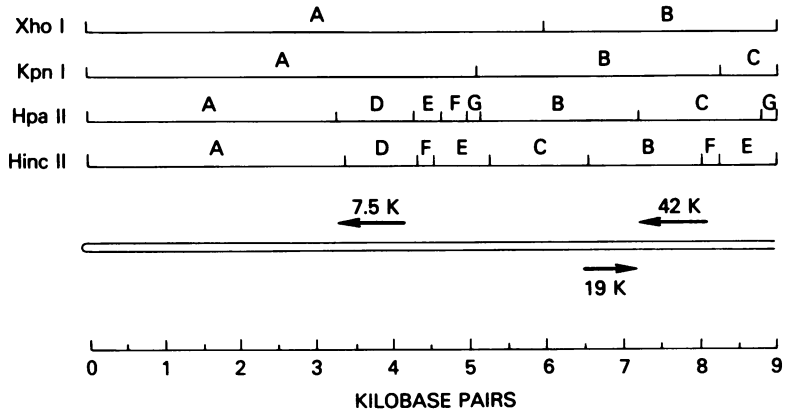

FIG. 4. *Restriction maps of the terminal EcoRI fragment of vaccinia virus DNA cloned in bacteriophage λ. Cleavage sites of XhoI, KpnI, HpaII, and HincII are shown in the upper portion of the figure. The map positions and directions of transcription of the mRNA's coding for the 7.5K, 19K, and 42K polypeptides are superimposed on a representation of the terminal 9 kbp of vaccinia virus DNA, the end of which is cross-linked.*

Since this region is just long enough for a single RNA, we cannot rule out the possibility that the 22.8K polypeptide, which was always made in minor amounts, was an artifact of in vitro translation.

Mapping of mRNA's by hybridization arrest. The map positions of the RNAs coding for the 19K, 42K, and 7.5K polypeptides were also established by the hybridization arrest procedure of Paterson et al. (23). The procedure was modified by first selecting RNA by hybridization to immobilized total recombinant DNA. The eluted RNA was then hybridized in solution to purified restriction fragments. The mixture was then split into two portions: one was translated directly, and the other was first denatured. The translation products are shown in Fig. 5. In agreement with results obtained above, synthesis of the 19K polypeptide was arrested by the *Hpa*II B and *Hinc*II B fragments, synthesis of the 42K polypeptide was arrested by the *Hpa*II C and *Hinc*II B fragments, and synthesis of the 7.5K polypeptide was arrested by *Hpa*II D and *Hinc*II D fragments. Note that the failure of the *Hinc*II C fragment to arrest the synthesis of any polypeptide is also consistent with previous experiments.

Functional UV target sizes. Thymine dimers, produced by UV irradiation of DNA, cause premature termination of transcription. Therefore, the inhibition of synthesis of a given mRNA is proportional to the distance between its promoter and the 3′ end of the cistron, providing of course that all DNA is equally susceptible to UV damage. The relative functional UV target sizes of the three polypeptides encoded within the inverted terminal repetition were investigated for several reasons. Most importantly, high-molecular-weight RNAs were found to be transcribed from the same DNA strand as the mRNA for the 42K and 7.5K polypeptides (32). Moreover, these RNAs appeared to map between the 42K and 7.5K mRNA's, raising the possibility of a large common precursor. We considered that if mRNA is processed from the 3′ end of the higher-molecular-weight RNAs, then the target size for the 7.5K polypeptide should be several times larger than that of the 42K polypeptide.

Vaccinia virus particles were irradiated with UV light for various amounts of time. The effectiveness of the irradiation was tested by incubating the virus particles in a coupled transcription-translation system (7). The translation products are shown in Fig. 6A. It is evident that with increasing times of irradiation, the synthesis of smaller and smaller polypeptides was progressively inhibited. The relative rates of synthesis of representative polypeptides were determined from densitometer scans, and we confirmed previous in vitro findings that the rate constant for inactivation was directly proportional to the size of the polypeptide (5, 24).

The same preparations of UV-irradiated virus

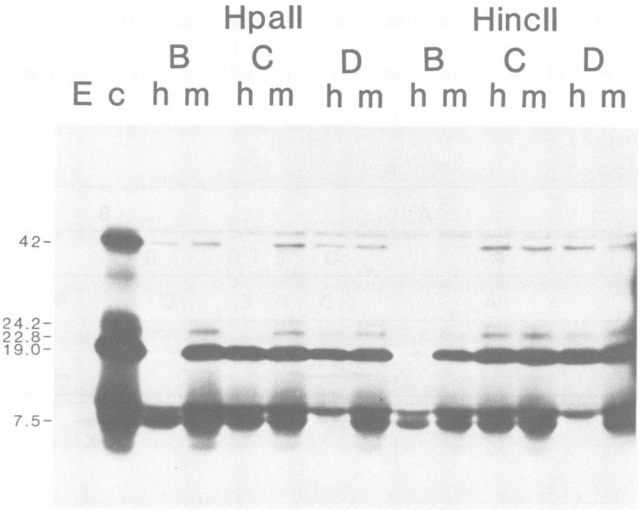

FIG. 5. *Hybridization arrest of translation. RNA encoded within the inverted terminal repetition was purified by hybridization to the total recombinant DNA. The selected RNA was then hybridized with excess amounts of isolated HpaII B, C, or D or HincII B, C, or D restriction fragments. One portion of the hybridized RNA was translated directly (h), and the other was heated to melt the hybrids (m). A fluorograph of a 20% polyacrylamide gel of the products is shown. The sizes (in kilodaltons) of the polypeptides are shown on the left. E, Endogeneous products of the reticulocyte lysate; c, control translation with selected RNA.*

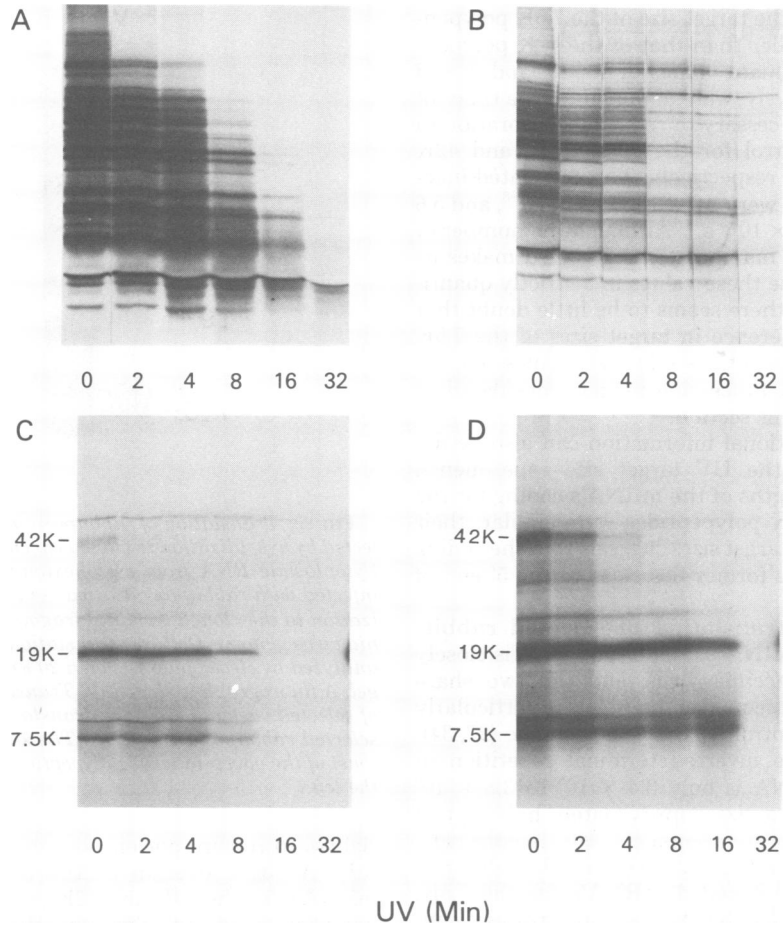

FIG. 6. *Effect of UV irradiation on transcription of functional mRNA by vaccinia virus. Purified vaccinia virus was UV irradiated for 0, 1, 2, 4, 8, 16, and 32 min as previously described (13) except that the lamp was 75 cm from the sample. (A) Virus was added to an in vitro coupled transcription-translation system, and the [^{35}S]methionine-labeled polypeptides were resolved on a dodecyl sulfate-polyacrylamide gel and fluorographed for 1 day. (B) Samples of the same irradiated virus stocks were used to infect cells in the presence of cycloheximide, and RNAs were purified 2 h after infection and translated in vitro at a final concentration of 50 μg/ml; a 3-day fluorograph of the translation products is shown. (C) Samples of RNA (70 μg) used in (B) were hybridized to 0.2 pmol of recombinant DNA immobilized on a filter, and one-third of the selected RNA was translated in 5-μl fractions; an 8-day fluorograph of the translation products is shown. (D) In a separate experiment, 210 μg of RNA used in (B) was similarly hybridized to recombinant DNA and the selected RNA was translated; a 10-day fluorograph of translation products is shown. Polypeptides are indicated on the left in (C) and (D).*

were then used to infect cells that had been treated with cycloheximide. After 2 h, the cells were harvested and the cytoplasmic RNA was purified. The in vitro translation products are shown in Fig. 6B. As expected, syntheses of some polypeptides, presumably of cellular origin, were not affected by the amount of irradiation of the virus particles. However, a progressive inhibition of viral polypeptide synthesis with increasing UV dose was evident, and rate constants for UV inactivation were directly proportional to polypeptide sizes, suggesting that the organizations of transcription units were similar in vivo and in vitro.

To determine the relative target sizes for the 7.5K, 19K, and 42K polypeptides, the mRNA's synthesized in vivo were selected by hybridization to recombinant DNA before translation. Two concentrations of RNA were used to ensure that it was not saturating for either hybridization or translation. Autoradiographs of the translation products are shown in Fig. 6C and D. It was

evident that the target size of the 7.5K polypeptide was smaller than that of the 42K polypeptide. Approximate values of 16, 10, and 2 to 3 min, respectively, were obtained for the times of irradiation necessary to reduce incorporation to 36.8% of control for the 7.5K, 19K, and 42K polypeptides, respectively. The calculated inactivation rates were 1.0×10^{-3}, 1.7×10^{-3}, and 5.6×10^{-3} to 8.3×10^{-3} s^{-1}. Although the number of experimental manipulations involved makes us hesitate to use these values in a strictly quantitative sense, there seems to be little doubt that the large difference in target sizes of the 7.5K and 42K polypeptides effectively rules out the specific RNA processing models considered at the start of this section.

Some additional information can also be deduced from the UV target size experiments. Since the lengths of the mRNA's coding for the 7.5K and 42K polypeptides were similar, their difference in target sizes suggests that the coding region for the former lies close to the 5′ end of its message.

Cell-free translation of selected rabbitpox virus mRNA's. Rabbitpox virus is closely related to vaccinia virus, and the two share considerable sequence homology, particularly within the central region of the genome (27, 34). However, the inverted terminal repetition in rabbitpox DNA is only 3.4×10^6 to 3.6×10^6 daltons (33), a size approximately half that of the repetition in vaccinia (11, 33). Accordingly, it was of interest to learn whether the rabbitpox virus genome encoded mRNA's for the 7.5K, 19K, and 42K polypeptides. Our strategy consisted of using the cloned terminal repetition of vaccinia virus to select RNA made in rabbitpox virus-infected cells. Cell-free translation (Fig. 7) indicated that rabbitpox did indeed synthesize mRNA's that shared sequence homology with vaccinia virus DNA and which encoded polypeptides similar in size to the 7.5K, 19K, and 42K polypeptides of vaccinia virus. Slight differences in the electrophoretic mobilities of the polypeptides, however, suggested some genetic variation.

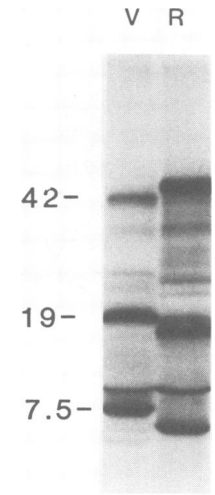

FIG. 7. *Translation of rabbitpox virus mRNA's selected by hybridization to cloned vaccinia virus DNA. Cytoplasmic RNA from cycloheximide-treated cells infected with rabbitpox virus was selected by hybridization to the cloned terminal fragment of the vaccinia virus genome. Cell-free translation products were analyzed by electrophoresis on a 20% polyacrylamide gel. A fluorograph is shown. V, Translation products of selected vaccinia RNA; R, translation products of selected rabbitpox virus RNA. The sizes (in kilodaltons) of the vaccinia virus polypeptides are shown on the left.*

DISCUSSION

We have used in vitro translation as an assay to map and size specific mRNA's encoded within the inverted terminal repetition of the vaccinia virus genome. mRNA's for polypeptides of 7.5, 19, and 42 kilodaltons were determined to be approximately 1,200, 680, and 1,280 nucleotides long, including the polyadenylic acid segments, and to map between 3.2 and 4.3, 6.5 and 7.2, and 7.2 and 8.3 kbp, respectively. Both strands of the genome are used; the leftward-reading one encodes the 7.5K and 42K polypeptides, and the rightward-reading one encodes the 19K polypeptide. A slight overlap of the 3′ ends of the mRNA's for the 19K and 42K polypeptides has not been ruled out. These results are summarized at the bottom of Fig. 4, and the sizes and map positions agree with a direct analysis of RNAs transcribed from this region of the genome (32).

Perhaps the most striking feature of the transcription map (Fig. 4) is the presence of long stretches of DNA that do not appear to encode translatable mRNA's. Indeed, the terminal 3 kbp of the genome is not transcribed at all (31, 32) and contains unusual structural features, including 30 repetitions of a 70-bp sequence (35). However, the region on the leftward-reading strand between the coding regions for the two mRNA's is transcribed into high-molecular-weight RNA species containing little or no polyadenylic acid (32). Whether our inability to detect translatable RNAs from this region reflects the small amounts of these transcripts, the conditions of in vitro translation, or a true lack of messenger function is not possible to determine at this time.

Paoletti (21) has suggested that vaccinia virus mRNA's are formed from high-molecular-weight precursors. We considered the possibility

that the high-molecular-weight RNA's transcribed from the inverted repetition represent excised fragments of a large precursor that contains coding sequences for both the 42K and the 7.5K polypeptides. According to this model, the UV target size of the 7.5K polypeptide should be several times larger than that of the 42K polypeptide. The results of such a determination, however, ruled out the possibility that the 7.5K mRNA is formed from the 3′ ends of a large precursor. This is consistent with the results of Pelham (24) and Bossart et al. (5), who studied the effects of UV inactivation on in vitro transcription of major unselected mRNA's from the entire genome. Our mapping of the cap sites of the mRNA's for the 7.5K, 19K, and 42K polypeptides and our finding that they do not appear to be spliced (32) are also consistent with initiation at the 5′ ends of the mature transcripts. None of the experiments, however, exclude the possibility that processing removes the 3′ end of some precursor RNA (12). Thus, the message for the 42K polypeptide may represent the 5′ end of a larger precursor, and the high-molecular-weight RNAs may represent nonpolyadenylated 3′ fragments of the latter.

The mRNA's for the 19K and 42K polypeptides are only about 100 nucleotides longer than absolutely necessary to code for their respective polypeptides. By contrast, the mRNA for the 7.5K polypeptide is about 900 nucleotides longer. The small UV target size of the 7.5K gene suggests that the coding sequences are located near the 5′ end of the message and that a long intron is not present within the coding region. These results also imply that UV damage within the noncoding 3′ portion of the gene has no effect on the synthesis of functional mRNA. Such a result agrees with previous studies which demonstrated that UV irradiation of vaccinia virus causes premature termination and that the shortened transcripts are capped and polyadenylylated (13).

In addition to the 42K, 19K, and 7.5K polypeptides, minor ones of 38, 24.2, and 22.8 kilodaltons were also detected. We are cautious regarding their significance, since the sizes and map positions of the 24.2K and 22.8K polypeptides are consistent with their synthesis from the same mRNA's that encode the 19K and 42K polypeptides, respectively. This might occur by suppression of termination in the case of the 24.2K polypeptide and premature termination in the case of the 22.8K polypeptide. In this regard, preliminary experiments indicated that at supraoptimal Mg^{2+} concentrations, the relative amounts of the 24.2K polypeptide increased and the 22.8K polypeptide decreased (not shown). These conditions suppress translation termination in both procaryotic and eucaryotic systems (6, 25). It is also possible that the 38K protein is an aggregate of the 19K polypeptide. As already mentioned, these minor polypeptides were not so evident when wheat germ extracts were used, but other minor translation products attributed to premature termination were seen.

When unfractionated RNAs were translated in vitro, a polypeptide of about 7.5 kilodaltons was detected, but the 42K and 19K polypeptides were not readily distinguished from the multitude of other labeled polypeptides. Similar problems were encountered when attempts were made to identify these polypeptides after in vivo labeling. Two-dimensional gel electrophoresis may be useful in resolving the polypeptides and in estimating their relative abundance in vivo. At this time, we have no information regarding the biological function of these polypeptides or the significance, if any, of their genome location within the inverted repetition.

The ability to use cloned segments of vaccinia virus DNA to select other poxvirus mRNA's may be useful in studying genetic variation. We found that mRNA's made in rabbitpox virus-infected cells hybridized to the cloned terminal segment of vaccinia virus DNA. Furthermore, polypeptides similar in size to the vaccinia virus 7.5K, 19K, and 42K polypeptides were synthesized in vitro by using selected rabbitpox mRNA's. These studies do not, however, indicate whether the coding sequences for these mRNA's are repeated at both ends of the rabbitpox virus genome. This is an interesting question, since the inverted terminal repetition of rabbitpox is approximately half that of vaccinia (27, 33).

We have cloned additional DNA segments representing nearly the entire vaccinia virus genome and are continuing hybridization selection and cell-free translation experiments to map additional early and late proteins.

ACKNOWLEDGMENTS

We thank N. Cooper for preparing RNA from infected cells, M. Rosenberg for a gift of purified strands of wild-type λ, B. Paterson for suggestions regarding hybridization selection of RNA, B. Roberts for recommending the procedure for extracting RNA from agarose gels, and J. Carolan and the National Institute of Allergy and Infectious Diseases Editorial Office for assistance with the preparation of the manuscript.

LITERATURE CITED

1. **Alwine, J. C., D. J. Kemp, and G. R. Stark.** 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc. Natl. Acad. Sci. U.S.A. **74**:5350–5354.
2. **Bailey, J. M., and N. Davidson.** 1976. Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. Anal. Biochem. **70**:75–85.
3. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection

method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.

4. **Boone, R. F., and B. Moss.** 1978. Sequence complexity and relative abundance of vaccinia virus mRNA's synthesized in vivo and in vitro. J. Virol. **26**:554–569.
5. **Bossart, W., E. Paoletti, and D. L. Nuss.** 1978. Cell-free translation of purified virion-associated high-molecular-weight RNA synthesized in vitro by vaccinia virus. J. Virol. **28**:905–916.
6. **Capecchi, M. R.** 1967. Polarity *in vitro*. J. Mol. Biol. **30**: 213–217.
7. **Cooper, J. A., and B. Moss.** 1978. Transcription of vaccinia virus mRNA coupled to translation *in vitro*. Virology **88**:149–165.
8. **Cooper, J. A., and B. Moss.** 1979. *In vitro* translation of immediate early, early, and late classes of RNA from vaccinia virus-infected cells. Virology **96**:368–380.
9. **Cooper, J. A., and B. Moss.** 1979. Translation of specific vaccinia virus RNAs purified as RNA-DNA hybrids on potassium iodide gradients. Nucleic Acids Res. **6**:3599–3612.
10. **Fuchs, E., and H. Green.** 1979. Multiple keratins of cultured human epidermal cells are translated from different mRNA molecules. Cell **17**:573–582.
11. **Garon, C. F., E. Barbosa, and B. Moss.** 1978. Visualization of an inverted terminal repetition in vaccinia virus DNA. Proc. Natl. Acad. Sci. U.S.A. **75**:4863–4867.
12. **Gershowitz, A., R. F. Boone, and B. Moss.** 1978. Multiple roles for ATP in the synthesis and processing of mRNA by vaccinia virus: specific inhibitory effects of adenosine (β-γ-imido)triphosphate. J. Virol. **27**:399–408.
13. **Gershowitz, A., and B. Moss.** 1979. Abortive transcription products of vaccinia virus are guanylylated, methylated, and polyadenylylated. J. Virol. **31**:849–853.
14. **Glisin, V., R. Crkvenjakov, and C. Byus.** 1974. Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry **13**:2633–2637.
15. **Hunter, A. R., P. J. Farrell, R. J. Jackson, and T. Hunt.** 1977. The role of polyamines in cell-free protein synthesis in the wheat-germ system. Eur. J. Biochem. **75**:149–157.
16. **Hunter, T., and J. I. Garrels.** 1977. Characterization of the mRNA's for α-, β- and γ-actin. Cell **12**:767–781.
17. **Joklik, W. K.** 1964. The intracellular uncoating of poxvirus DNA: 1. fate of radioactively labeled rabbitpox virus. J. Mol. Biol. **8**:263–276.
18. **McGrogan, M., D. J. Spector, C. J. Goldenberg, D. Halbert, and H. J. Raskas.** 1979. Purification of specific adenovirus 2 RNAs by preparative hybridization and selective thermal elution. Nucleic Acids Res. **6**: 593–607.
19. **Moss, B.** 1968. Inhibition of HeLa cell protein synthesis by the vaccinia virion. J. Virol. **2**:1028–1037.
20. **Nevins, J. R., and W. K. Joklik.** 1975. Poly(A) sequences of vaccinia virus messenger RNA: nature, mode of addition and function during translation *in vitro* and *in vivo*. Virology **63**:1–14.
21. **Paoletti, E.** 1977. High molecular weight virion-associated RNA of vaccinia. A possible precursor to 8 to 12S mRNA. J. Biol. Chem. **252**:872–877.
22. **Paoletti, E., and L. J. Grady.** 1977. Transcriptional complexity of vaccinia virus in vivo and in vitro. J. Virol. **23**:608–615.
23. **Paterson, B. M., B. E. Roberts, and E. L. Kuff.** 1977. Structural gene identification and mapping by DNA-mRNA hybrid-arrested cell-free translation. Proc. Natl. Acad. Sci. U.S.A. **74**:4370–4374.
24. **Pelham, H. R. B.** 1977. Use of coupled transcription and translation to study mRNA production by vaccinia cores. Nature (London) **269**:532–534.
25. **Pelham, H. R. B.** 1978. Leaky UAG termination codon in tobacco mosaic virus RNA. Nature (London) **272**:469–471.
26. **Ricciardi, R. P., J. S. Miller, and B. E. Roberts.** 1979. Purification and mapping of specific mRNAs by hybridization-selection and cell-free translation. Proc. Natl. Acad. Sci. U.S.A. **76**:4927–4931.
27. **Schümperli, D., A. Menna, F. Schwendimann, R. Wittek, and R. Wyler.** 1980. Symmetrical arrangement of the heterologous regions of rabbit poxvirus and vaccinia virus DNA. J. Gen. Virol. **47**:385–398.
28. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
29. **Szybalski, W., H. Kubinski, Z. Hradecna, and W. C. Summers.** 1971. Analytical and preparative separation of the complementary DNA strands. Methods Enzymol. **21D**:383–413.
30. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76**: 3683–3687.
31. **Wittek, R., E. Barbosa, J. A. Cooper, C. F. Garon, H. Chan, and B. Moss.** 1980. Inverted terminal repetition in vaccinia virus DNA encodes early mRNAs. Nature (London) **285**:21–25.
32. **Wittek, R., J. A. Cooper, E. Barbosa, and B. Moss.** 1980. Expression of the vaccinia virus genome: analysis and mapping of mRNA's encoded within the inverted terminal repetition. Cell **21**:487–493.
33. **Wittek, R., A. Menna, H. K. Müller, D. Schümperli, P. G. Boseley, and R. Wyler.** 1978. Inverted terminal repeats in rabbit poxvirus and vaccinia virus DNA. J. Virol. **28**:171–181.
34. **Wittek, R., A. Menna, D. Schümperli, S. Stoffel, H. K. Müller, and R. Wyler.** 1977. HindIII and SstI restriction sites mapped on rabbit poxvirus and vaccinia virus DNA. J. Virol. **23**:669–678.
35. **Wittek, R., and B. Moss.** 1980. Tandem repeats within the inverted terminal repetition of vaccinia virus DNA. Cell **21**:277–284.