Mapping of the genes coding for the two major vaccinia virus core polypeptides

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ABSTRACT

We have mapped the genes coding for two major structural polypeptides of the vaccinia virus core by hybrid selection and transcriptional mapping. First, RNA was selected by hybridization to restriction fragments of the vaccinia virus genome, translated in vitro and the products were immunoprecipitated with antibodies against the two polypeptides. This approach allowed us to map the genes to the left hand end of the largest Hind III restriction fragment of 50 kilobase pairs. Second, transcriptional mapping of this region of the genome revealed the presence of the two expected RNAs. Both RNAs are transcribed from the leftward reading strand and the 5'-ends of the genes are separated by about 7.5 kilobase pairs of DNA. Thus, two genes encoding structural polypeptides with a similar location in the vaccinia virus particle are clustered at approximately 105 kilobase pairs from the left hand end of the 180 kilobase pair vaccinia virus genome.

INTRODUCTION

Vaccinia virus is the best-studied member of the poxvirus family. The virions, which are characterized by their large size and typical brick shape, contain a double-stranded DNA molecule of 180 kilobase pairs which is flanked by long inverted terminal repeats. Theoretically, vaccinia virus is thus capable of encoding close to 200 average-sized polypeptides. Expression of this large amount of genetic information is temporally well regulated. Shortly after penetration into the host cell, the viral DNA is uncoated in two steps. After a first partial uncoating, early genes are transcribed within subviral particles (= cores) by the RNA polymerase contained in the virions (1, 2). Following complete uncoating, the DNA is replicated and late genes encoding predominantly structural polypeptides are then expressed.

The molecular basis for the switch from early to late gene expression is

not understood. As an attractive hypothesis one may speculate that gene expression is regulated by early and late specific DNA sequences in the vicinity of the genes. As a first step to identify such regulatory elements, putative early and late promoters will have to be sequenced and compared to each other. Such sequence information is currently available for four early genes (3, 4, 5), but not for late genes.

We therefore have started to map late genes coding for major late virion polypeptides to provide a basis for a detailed fine structure analysis. In such a study we have already mapped the gene coding for a structural polypeptide with a molecular weight of 11,000 (6). In this communication we describe the mapping of two further genes encoding the major polypeptides of the vaccinia virus core. These two structural proteins have been designated 4a and 4b and comprise about 25% of the total protein mass of the virion (7).

MATERIALS AND METHODS

Virus and cells

The WR strain of vaccinia virus was obtained from B. Moss, National Institutes of Health, Bethesda. Rabbit kidney cells (RK-13) were grown and infected with vaccinia virus as described (6).

Isolation of the core proteins and antibody induction

Purified vaccinia virions were dissociated by boiling for 4 min in 70 mM Tris-HCl (pH 6,8), 3% SDS and 5% 2-mercaptoethanol (at a final protein concentration of 3 mg/ml). Samples corresponding to 3 - 4 mg of total virion protein were loaded onto preparative SDS-polyacrylamide gels. After short staining with coomassie blue the upper part of band 4a and the lower part of band 4b was excised. Electrophoretic elution and further processing of samples has been described (8). For immunization the two separated core proteins were injected both subcutaneously and intraperitonally into rabbits. The first injections contained complete Freund adjuvant, while booster injections at 3-week intervals contained incomplete adjuvant. A total of 500 µg protein was given per animal, divided into three or four injections. For some experiments, the obtained antisera were submitted to affinity chromatography on either polypeptide 4a or 4b covalently coupled to sepharose beads.

RNA extraction

Total cytoplasmic RNA was purified from vaccinia virus infected cells as described (6). To obtain early RNA, infected cells were maintained in medium containing 100 µg/ml cycloheximide and RNA was extracted at 4 h post infection. Late RNA was isolated from cells that were not treated with the inhibitor at 6 h after infection.

Hybridization selection of RNA

The conditions for selecting RNA by hybridization to cloned DNA restriction fragments of the vaccinia virus genome have been described (6).

In vitro translation and immunoprecipitation

In vitro translation of RNA in a message-dependent reticulocyte lysate and immunopreciptations were performed exactly as described (6).

S1 nuclease analysis

RNA transcripts were mapped by S1 analysis (9) using 5' end-labelled DNA fragments as hybridization probes (10) as described (6).

RESULTS

Preparation and characterization of antisera

When the structural polypeptides of purified vaccinia virions are separated by SDS polyacrylamide gel electrophoresis, a large number of bands are observed (Fig.1, lane a). Among the most intense is a small polypeptide of molecular weight 11,000 (11 K) for which we have recently mapped the gene on the vaccinia virus DNA (6). Two further polypeptides that are present in large amounts in the virion run as closely spaced bands with apparent molecular weights of 58 K and 62 K. These polypeptides have been designated 4a (upper band) and 4b (lower band) and represent major constituents of the virus core (7). The two polypeptides were purified by polyacrylamide gel electrophoresis and injected into rabbits to raise antibodies. Antibody specificities were then determined by reacting the antisera against total virion polypeptides immobilized on nitrocellulose membranes (Fig.1). In these immuneblotting experiments, each antiserum reacted with one of the core polypeptides only and was thus strictly monospecific.

Identification of the precursors of polypeptides 4a and 4b synthesized in a cell-free system.

Both mature polypeptides 4a and 4b are formed by cleavage of higher-

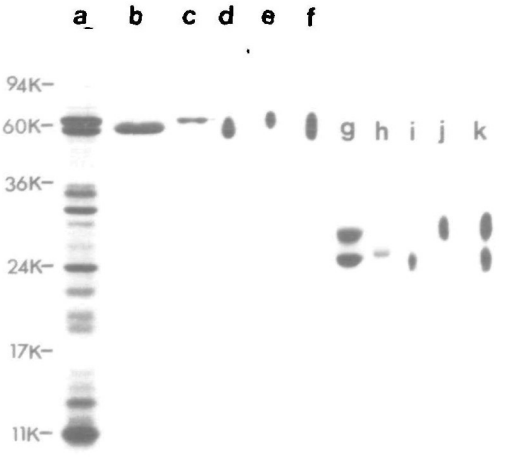

Figure 1. Analysis of purified virion polypeptides used for antibody induction and characterization of antibody specificities. Structural proteins were separated on SDS-polyacrylamide gels. a: coomassie blue-stained total vaccinia virus polypeptides; b, c : the two major core proteins 4b and 4a (7) after purification (coomassie blue-stained); d, e, f: reactivities of the antisera raised against separated 4a and 4b bands on complete patterns of virus structural proteins immobilized on nitrocellulose membranes. Antibody decoration is detected by bound ^{125}J - Protein A. d : anti 4b; e: anti 4a; f : mixture of anti 4a and anti 4b. By the use of a longer gel (24 cm) a wider separation of bands 4a and 4b could be achieved (g-k; only relevant molecular weight region shown). g : coomassie blue-stained bands 4a, b; h : catalase as molecular weight marker (MW 60,000); i, j, k : immune blots as in d, e, f. For all immune blots affinity-purified antibodies were used at 3 ug/ml.

molecular-weight precursors (11, 12, 13, 14). This cleavage is not performed in rabbit reticulocyte lysates on newly synthesized proteins. The ability of the antibodies to recognize the cell-free translation products, however, was a prerequisite in our mapping procedure. Total RNA from vaccinia virus infected cells was therefore translated in vitro and the products were immunoprecipitated with either the anti 4a or anti 4b antiserum or with water as a control (Fig.2). The antibody against 4b precipitated a major polypeptide with a molecular weight of 66 K (lanes a, b). This value is in agreement with the previously estimated size of the 4b precursor (14). Unexpectedly, the antibodies directed against 4a precipitated not only a polypeptide of 86 K which is a good candidate for the precursor of 4a (14)

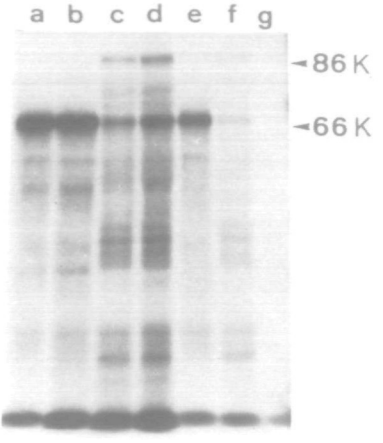

Figure 2. Identification of the precursors of polypeptides 4a and 4b in in vitro translation mixtures. Total RNA from vaccinia virus infected cells was translated in vitro and the products were immunoprecipitated with either the anti 4b (lanes a, b) or anti 4a (lanes c, d) sera or with water as a control (lane g). Immunoprecipitation was performed with either 2 ul (a, c) or 5 µl (b, d) of crude antiserum, or with affinity-purified antibodies (lane e : anti 4b; lane f : anti 4a). The fluorograph of a 12,5% polyacrylamide gel is shown.

but also a polypeptide of 66 K (lanes c, d). These two high molecular weight polypeptides (86 K and 66 K) in addition to several smaller ones which probably represent premature termination products, were consistently seen in all experiments with the anti 4a antiserum. Mapping studies (see below) showed that the presence of the 66 K polypeptide in lanes c, d, f is due to cross-reactivity of the antiserum with the precursor of 4b. The use of affinity purified antibodies instead of the crude antisera did not alter the composition of the immunoprecipitates (lanes e, f).

Mapping of the genes coding for polypeptides 4a and 4b

To map the genes coding for the two core polypeptides 4a and 4b, total RNA isolated from vaccinia virus infected cells was selected by hybridization to DNA restriction fragments as indicated by numbers 1-5 on the upper part of Fig.3 which represents a Hind III cleavage map of the vaccinia genome. The Hind III C fragment was not included since previous transcriptional mapping of this region of the vaccinia virus genome did not reveal any major late transcripts (15). The selected RNA was translated in vitro and the precursors of polypeptides 4a and 4b were identified by immunoprecipitation and subsequent analysis by polyacrylamide gel electrophoresis (Fig.3). As seen in lanes 4, both polypeptides were made from RNA selected on the largest Hind III fragment (fragment A) of approximately 50 kilobase pairs.

The Bam HI and Sal I cleavage sites were therefore mapped on the Hind III

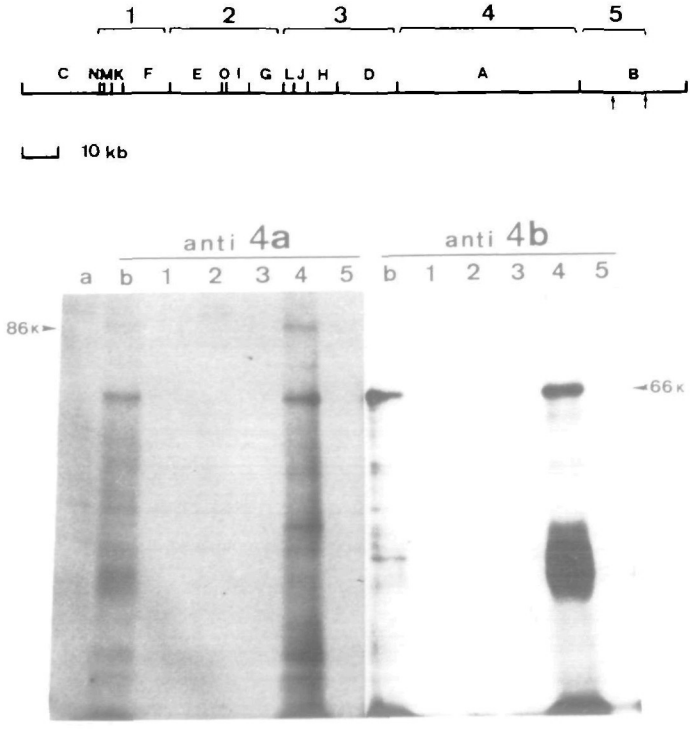

Figure 3. Gel analysis of immunoprecipitated cell-free translation products. Total RNA from infected cells was translated in vitro and the products were immunoprecipitated with the indicated antisera (lanes b) or with water as a control (lane a). Lanes 1-5 show the immunoprecipitated polypeptides made by RNA selected on different regions of the vaccinia virus genome as indicated in the upper part of the figure. A Hind III map of the vaccinia virus DNA is shown. The arrows indicate the positions of two Sal I sites which were used to subclone the unique sequences of the Hind III B fragment.

A fragment (upper part of Fig.4) and various restriction fragments were subcloned in pBR 322. The DNA fragments (numbered 1-11) were immobilized on nitrocellulose membranes and used to select RNA by hybridization. After cell-free translation, polypeptides again were immunoprecipitated with either the anti 4a or anti 4b serum (Fig.4). Each lane shows the products obtained from RNA selected on the corresponding DNA indicated in the upper part of the figure. With the antiserum directed against 4b, a polypeptide of precursor size was immunoprecipitated from the in vitro translation products

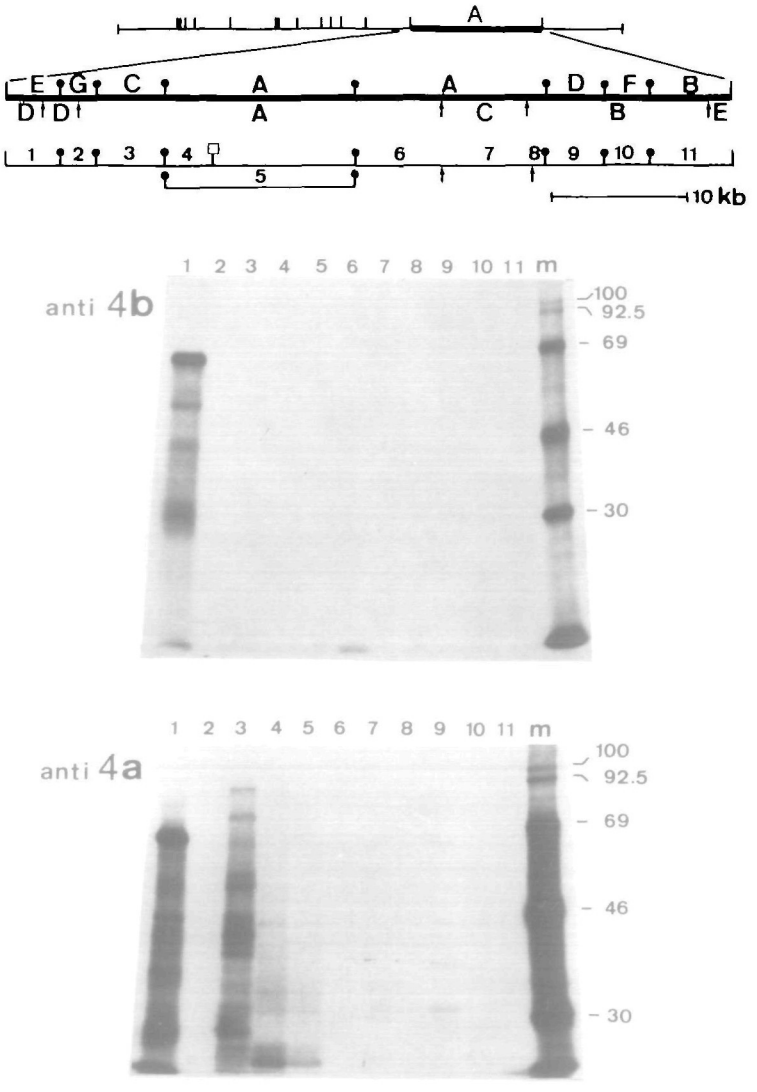

Figure 4. Polyacrylamide gels of immunoprecipitated polypeptides synthesized in vitro in response to selected RNA. RNA was selected by hybridization to immobilized DNA segments of the Hind III A fragment. Aliquots of each sample were precipitated with either the anti 4a or anti 4b serum. Each lane (1-11) shows the products obtained from RNA selected on the corresponding DNAs shown in the upper part of the figure. A Bam HI ( ) and Sal I ( ) cleavage map of the Hind III A fragment is shown. A Pvu II site ( ) of particular interest is also indicated. m, molecular weight markers and their sizes (x 1000).

of RNA selected on the leftmost Hind III - Bam HI fragment of 4.1 kilobase pairs (lane 1). The antiserum against 4a precipitated polypeptides made from RNA selected on two different fragments. First, the same polypeptide that was also detected with antiserum against 4b was immunoprecipitated (lane 1). Second, the 86 k polypeptide was precipitated from the cell-free translation products of RNA selected on the Bam HI C fragment of 5 kilobase pairs (lane 3).

Thus, with respect to the in vitro translation products, the antiserum against 4b was strictly monospecific, but the antiserum against 4a cross-reacted strongly also with the precursor of polypeptide 4b.

Mapping of the 5'-ends of the 4a and 4b specific RNAs

S1 analysis was used to map precisely the 5'-ends of the RNAs and also to determine their direction of transcription. A DNA segment from an XhoI site located at 1.3 kilobase pairs to the left of the Hind III site to a Pvu II site at 15.5 kilobase pairs to the right was included in this analysis. Various DNA fragments (Fig.5, part C) labelled at their 5'-ends were used as hybridization probes. Each probe (numbered 1-8) was analyzed either directly (lanes p) or after hybridization to tRNA as a control (lanes t), or to early (lanes e) and late (lanes l) RNA isolated from vaccinia virus infected cells. The map positions of the most likely messages of polypeptides 4a and 4b which were derived from the S1 analysis are shown in Fig.5, part C. In this map, only major late transcripts were considered since the core polypeptides are not made in cells treated with cytosine arabinoside (G.

Figure 5. S1 mapping of RNA transcripts. A DNA segment shown in part C of the figure from an Xho I site ( ) at 1.3 kb to the left of the Hind III site to a Pvu II site ( ) within the Bam HI A fragment (see also Fig.4) was used for transcriptional mapping. An Eco RI site ( ) of particular interest is indicated. Various fragments derived from this region were labelled at the 5'-end (solid circle) and used as hybridization probes. Each probe (1-8) was analyzed either directly (lanes p) or after hybridization to tRNA as a control (lanes t), or after hybridization to early (lanes e) and late (lanes l) RNA isolated from infected cells. S1 resistant material was analyzed on neutral agarose gels (part D) or on sequencing polyacrylamide gels (parts E, F). Temperatures for hybridization and S1 treatment are indicated at the bottom of the autoradiographs. The map position and direction of transcription of the mRNAs for polypeptides 4a and 4b are indicated (part C). A : Hind III map of the vaccinia virus DNA. B : Bam HI ( ) and Sal I ( ) map of the Hind III A fragment.

Hiller, unpublished), which inhibits viral DNA replication and thus arrests the infection cycle in the early phase. In addition, based on the size of the polypeptides, the coding sequences of both mRNAs exceed 1700 bases but each RNA was selected on a single fragment. We therefore considered only RNAs of which a large portion is transcribed from the fragments that also selected the mRNA in the hybridization selection procedure and thus expected only short, if any, overlaps into the neighboring fragments.

A major late RNA which protected the entire fragment was detected with probe 2. When hybridization was performed with the uncleaved Bam HI E fragment labelled at both ends, an RNA again protecting the entire probe was observed (not shown). This RNA is a good candidate for the mRNA of polypeptide 4b and its 5'-end must map close to the Bam HI site since the Bam HI G fragment did not select it. The putative 5'-end of this RNA was detected with a mixture of probes 4 and 5 which yielded a fragment of approximately 100 bases. However, a similar band was also obtained with early RNA. To determine whether the two RNAs indeed have common 5'-ends, hybridizations were performed with probes 4 and 5 individually and at two different temperatures and S1 resistant material was analyzed on a high resolution sequencing gel. Bands were only observed with probe 4 (Fig.5, part C), but not with probe 5 (not shown). This analysis clearly showed that the two RNAs have different initiation sites, since early RNA protected a fragment of 120 bases whereas a fragment of 85 bases was seen with late RNA. Similarly, a late RNA transcribed from the same DNA strand and which protected the entire fragment was observed with probe 6. This is a very likely candidate for the mRNA of polypeptide 4a since it had been selected on the same fragment. To map its 5'-end, a Bam HI - Pvu II fragment labelled at the Bam HI site (probe 8) was used as the hybridization probe. Hybridization and S1 treatment were again performed at 42°C and 37°C and resistant material was analyzed on a sequencing gel. No fragment in the expected size range was obtained when hybridization and S1 treatment were performed at 42°C. At 37°C however, a fragment of 180 bases was protected with late RNA. Since we routinely perform hybridization selection at 42°C this may explain why the Bam HI - Pvu II fragment had not selected the mRNA for polypeptide 4a.

DISCUSSION

In considering strategies for gene mapping, the large size of the vaccinia virus genome and hence the large number of virus coded polypeptides has to be kept in mind. Conditional lethal virus mutants have proven to be particularly useful for the mapping of early genes encoding viral enzymes. Using such mutants and the marker rescue technique in combination with other biochemical procedures, the thymidine kinase (16) and DNA polymerase gene (17, 18) have been mapped on the vaccinia virus genome. Thymidine kinase mRNA translated in vitro yielded active enzyme, therefore hybrid selection of RNA and estimation of enzymatic activity offered on alternative procedure for gene mapping (19). Clearly, genes which specify products lacking measurable biological activities are more difficult to identify. If, however, antibodies are available for identification of such gene products, hybrid selection and in vitro translation combined with immunoprecipitation can be used (6). With this approach we have now mapped additional vaccinia virus late genes. The two core polypeptides 4a and 4b (7) were chosen mainly because of their high abundance in the virus particle which indicates that they are important structural elements. In addition, we expected the corresponding mRNAs to be present in large amounts in infected cells which would allow to purify sufficient amounts by hybrid selection for in vitro translation and immunoprecipitation. The disadvantage, however, of mapping genes coding for relatively large polypeptides is that long RNA molecules are more susceptible to degradation during hybrid selection. Also premature termination may occur more frequently during cell-free translation.

The antisera raised against the SDS-denatured core polypeptides were further purified by affinity chromatography on the immobilized antigens. Characterization of the purified antibodies in immunoblotting experiments demonstrated their strict monospecificity when tested on authentic polypeptides (see Fig.1) under partially denaturing conditions. Upon immunoprecipitation of samples containing the uncleaved precursor molecules, anti 4a precipitated not only the 4a precursor but apparently also the precursor of 4b (see Fig.2). Since no proteolytic cleavage occurs in the rabbit reticulocyte lysate used for cell-free translation we do not know whether anti 4a also recognizes authentic 4b under native conditions. Thus two

possibilities are open to explain the unexpected crossreactivity. Either the more stringent conditions of immunoblotting as compared to immunoprecipitation do not allow for cross-reaction, or the peptide of the 4b precursor which is lost upon proteolytic processing has an antigenic site which is also present in 4a. This crossreactivity of anti 4a did not hinder the mapping of both genes since anti 4b proved to be monospecific in both experimental conditions used.

Immunoprecipitation of the in vitro translation products of RNA selected by hybridization to different regions of the vaccinia virus genome allowed us to map the genes for the core polypeptides to the largest Hind III fragment of 50 kilobase pairs. Hybrid selection on subcloned fragments showed that the genes for polypeptides 4a and 4b map within DNA segments of 5 and 4.1 kilobase pairs, respectively, which are located at the left hand end of the Hind III A fragment. Transcriptional mapping of this part revealed the presence of two RNAs that are very likely candidates for the mRNAs for polypeptides 4a and 4b. Both RNAs are transcribed from the leftward-reading strand and the 5' ends of the genes are separated by 7.5 kilobase pairs. Interistingly, the two genes coding for polypeptides with a similar location in the virus particle are clustered on the vaccinia virus genome.

An interesting situation was observed during transcriptional mapping of the message for polypeptide 4b. Early in infection, an RNA is transcribed from a similar region of the genome. The late-specific transcript is initiated about 35 bases downstream of the initiation site of the early transcript. More work will be needed in order to determine whether this early RNA has any biological significance. Because of the enormous length heterogeneity of late RNAs (20) we have made no attempt to map the 3'-ends of the mRNAs for polypeptides 4a and 4b. The large size of the RNAs detected by S1 mapping may be the consequence of a failure to correctly terminate transcription late in infection.

Foreign genes have recently been introduced into the vaccinia virus DNA by homologous in vivo recombination (21, 22, 23, 24, 25) and the use of such recombinant vaccinia virus as live vaccines has been discussed (21). Expression of the inserted DNA was shown to depend on the use of vaccinia

virus promoter sequences (22). Since the two major core polypeptides together represent about one fourth (7) of the total protein mass present in the virion, the two genes probably have very strong promoters. These promoters should therefore be useful to obtain high levels of expression of genes cloned into the vaccinia virus genome.

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