Structural Proteins and Cell-Free Translation Products of Total RNA and Hybrid-Selected RNA from Two DNA Variants of Vaccinia Virus

DONNA L. BEDARD†

Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201

Received 1 November 1982/Accepted 10 February 1983

Two major variants of vaccinia virus, large (L) and small (S), differ by a deletion of 9.7 kilobase pairs. The structural proteins and the translation products of RNA transcribed in vitro from each of these variants were analyzed by gel electrophoresis. Recombinant plasmids were used to select RNA transcribed from the L variant sequences corresponding to the deletion. This RNA yielded translation products indicating that a minimum of 11 polypeptides, including two structural proteins, map within the deletion.

Serially passaged vaccinia virus strain WR is composed of two DNA variants, S (small) and L (large), which differ by a deletion of 9.7 kilobase pairs (approximately 5% of the genome) located 10.2 kilobase pairs from the left end of the genome (17). Neither the infectivity nor the replication of the S variant is impaired under laboratory conditions; however, L variant sequences which bridge the deletion are transcribed into unique sequences both in vivo and in vitro (17). To determine whether these unique transcripts encode polypeptides, I compared both the structural proteins of the whole virus and the cell-free translation products of transcripts of both the L and S variants. RNA transcribed in vitro from purified virus was used for the translation studies because it is a pure population of viral RNA which is capped, polyadenylated, and highly efficient in programming translation. It is well established that proteins translated in cell-free systems from in vitro transcripts of vaccinia faithfully reflect the polypeptides synthesized in vivo by early and immediate early RNAs (7, 12, 18, 20).

Figure 1 shows the results of a translation of RNA transcribed from stock, L, or S variant vaccinia virus. At least 38 protein bands were resolved and six protein differences were consistently noted between the S and L variants. Four of these proteins, 21.1K, 18.7K, 17.1K, and 12.2K, were translated from L but not from S variant RNA, whereas two, 21.7K and 19.6K, were translated from S but not from L variant RNA. A faint protein band at 21.7K was also apparent in the L variant population. Hence,

either another protein comigrates with the S variant 21.7K protein or a single 21.7K protein is present but expressed to a greater extent in the S variant, perhaps owing to a change in regulation in the S variant. An important question is to determine whether the structural genes for these polypeptides reside in the deletion or whether some of the differences could be attributed to the loss of regulatory sequences which affect the expression of genes outside the deletion. After fine mapping, three restriction fragments which span the deleted sequence were cloned in the *Escherichia coli* plasmid pBR322. A map of the appropriate fragments is shown in Fig. 2. The recombinant clones were used to select transcripts from stock, L, or S variant RNA or from a posttranscriptional mix of L and S variant RNA. Figure 3 shows the translation products of these hybrid-selected RNAs. L-specific polypeptides of approximately 12.2K and 15.8K mapped in the region corresponding to pDB104 and extended into the region included in pDP202 (Fig. 3B and C). Five additional L-specific polypeptides ranging from 17.1K to 67K mapped in the region corresponding to the center of the deletion (Fig. 3C), and four polypeptides ranging from 18.7K to 21.5K mapped in the right end of the deletion (Fig. 3D). Polypeptides 21.1K, 18.7K, 17.1K, and 12.2K had been previously identified as L-specific proteins on the basis of translation of total RNA (Fig. 1). Hybrid-select translation resolved seven additional polypeptide differences which could not be seen on translation of total RNA owing to comigration of other polypeptides. Indeed, polypeptide 27K was barely detectable even under conditions which produced abundant amounts of other proteins.

† Present address: Biological Sciences Branch, General Electric Corporate Research and Development Center, Schenectady, NY 12301.

FIG. 1. Cell-free translation products of RNA transcribed in vitro from stock (W), L variant (L), or S variant (S) vaccinia virus. Lane V, Migration of [35 S]methionine-labeled vaccinia virus structural proteins; lane E, translation in the absence of added RNA; lane M, combined translation products of separate translations of L variant and S variant RNA; lane L+S, translation of a 1:1 mixture of RNA transcribed separately from L and from S variant virus. The region between 14K and 16K is somewhat distorted by the migration of excess globin contained in the reticulocyte lysate. Vaccinia virus strain WR was originally obtained from B. Moss of the National Institutes of Health, Bethesda, Md. Stock and plaque-purified S and L variant vaccinia viruses (17) were maintained by serial passage in HeLa cells. Purified virus was prepared essentially as described in the legend to Fig. 4. RNA was synthesized by purified virus in a standard in vitro transcription reaction (3). The size of the RNA ranged up to 28S, with a peak at 8S to 12S. A total of 80 to 90% of the RNA was polyadenylated. Crude reticulocyte lysate prepared by the method of Safer et al. (21) was purchased from Clinical Convenience Products, Madison, Wis., and rendered mRNA dependent by treatment with micrococcal nuclease (19). The optimal Mg^{2+} ion concentration for translation of vaccinia virus RNA was determined to be 0.1 mM, and potassium acetate was included at a concentration of 120 mM. RNA was heated to 100°C for 80 s and rapidly quenched in dry ice-methanol immediately before translation. The incubation mixture (30 μ l) contained RNA at a final concentration of 25 to 40 μ g/ml and 5 to 10 μ Ci of [35 S]methionine (Amersham Corp. or New England Nuclear). Incubation was carried out at 30°C for 40 min. Translation products were analyzed by

It was anticipated that all L-specific proteins would be translated from wild-type (stock) RNA and from an artificial mix of L and S transcripts as well as from L variant RNA. This was not the case. Polypeptide 20.1K was translated from L variant RNA or from a mix of L and S variant RNA but not from wild-type RNA. Equally puzzling was the observation that the proteins migrating at 18.7K and 21.5K could be translated only from wild-type RNA when the RNA was hybrid selected before translation, even though the 18.7K protein was translated from wild-type, L variant, and a mix of L and S variant RNA when the RNA was not hybrid selected before translation (Fig. 1). This translation pattern was seen in all of four separate experiments. Although these observations are very interesting, I have no explanation for them at present.

Two S-specific proteins, 21.7K and 19.6K (Fig. 1), which were translated from total in vitro transcribed RNA did not map in the region flanking the deletion. It is interesting to speculate that these might represent proteins which are released from the control of a protein encoded within the deleted region.

Figure 4 shows an analysis of the structural proteins of purified stock, L, and S vaccinia virus. Four L-specific proteins were detected in L variant virus and in an artificial mix of L and S variant virus (data not shown) but not in the stock virus. Two of these (68.4K and 29.8K) migrate nearly identically to two polypeptides translated in vitro which map in the center of the deletion (67K and 28.6K; Fig. 3). The other structural protein differences noted do not parallel any of the protein differences which map in the deleted region. However, both of these proteins, 86.4K and 74.6K, migrate very closely to vaccinia proteins which are known to be

electrophoresis on a 10 to 15% polyacrylamide gradient gel in the presence of sodium dodecyl sulfate (14). Samples contained a maximum of 3.5 μ l of the translation reaction in a final volume of 35 μ l. Equivalent disintegrations per minute of each translation were analyzed, and an appropriate volume of unlabeled reticulocyte lysate was added to each translated sample so that the perturbation in migration caused by the hemoglobin present applied equally to all samples. Each sample was boiled for 5 min before gel loading. Radioactive proteins were visualized by autoradiography. Apparent molecular weights of vaccinia virus structural proteins were determined by electrophoresing in parallel with the molecular weight standards described in the legend to Fig. 4 and are virtually identical to the molecular weights reported by Oie and Ichihashi (16). In subsequent experiments, vaccinia virus structural proteins labeled with [35 S]methionine (7) were included as a standard on each gel. The molecular weights assigned to each polypeptide represent the mean of four separate determinations.

FIG. 2. Map of the left termini of S and L variant vaccinia virus DNAs showing the regions included in each clone. The *Xho*I and *Ava*I cleavage sites and the location of the deletion are from Panicali et al. (17), with the exception of the *Sal*I site and the position of the *Ava*I-R fragment, which were mapped during this study (data not shown). The *Xho*I-H and *Xho*I-F fragments were isolated from agarose gels by binding to glass powder (23) as described previously (17) and inserted into pBR322 (1). The *Ava*I-A fragment (L variant vaccinia virus) was eluted from an agarose gel as described above, digested with *Sal*I, and inserted into pBR322. After transformation (9) of *E. coli* HB101 (4) with the recombinant DNAs, the cloned plasmids were analyzed by restriction analysis (11).

glycosylated (88K and 74K) (16), and the differences seen may reflect differences in glycosylation.

Two polypeptides, 12.2K and 15.8K, overlap

the junction of pDB104 and pDP202, and five additional polypeptides map within pDP202. Assuming an average molecular weight of 115 per amino acid, the combined molecular weight of

FIG. 3. Polypeptides translated from RNA hybridized to cloned L variant DNAs spanning the S variant deletion. (A) [35 S]methionine-labeled vaccinia virus structural proteins used as a standard (V) and the endogenous translation products of reticulocyte lysate (E). Polypeptides translated from RNA hybridized to pDB104 (B), pDP202 (C), and pDB116 (D) are shown. The RNA was transcribed in vitro from stock (W), L variant (L), or S variant (S) vaccinia virus. The lanes designated L+S denote polypeptides translated from a posttranscriptional mix of L and S RNAs. Recombinant plasmid DNAs were prepared by a modification of the cleared lysate procedure of Clewell and Helinski (6) after chloramphenicol amplification (5) and were bound to diazobenzylloxymethyl paper prepared fresh from nitrobenzylloxymethyl paper (Transabind, NBM-2020; Schleicher & Schuell Co.) according to the instructions of the manufacturer. RNA was hybridized to diazobenzylloxymethyl paper filters and stringently washed as described by Cooper et al. (8). Translation products of the hybrid-selected RNAs were electrophoresed as described in the legend to Fig. 1 and visualized by fluorography after impregnation of the gel with 2,5-diphenyloxazole (2) or En 3 Hance (New England Nuclear Corp.).

FIG. 4. Structural proteins of stock (W), L variant (L), and S variant (S) vaccinia virus. Virus was extensively purified by a modification of the procedure described by Joklik (13) involving sedimentation through a sucrose cushion and banding in three successive sucrose gradients (25 to 40%). The purified virus was analyzed by electrophoresis on a 10 to 15% polyacrylamide gradient gel in the presence of sodium dodecyl sulfate (14). Proteins were stained with Coomassie brilliant blue. The unmarked lane shows the migration of molecular weight standards (Pharmacia Fine Chemicals, Inc. and Boehringer Mannheim Corp.), *E. coli* RNA polymerase (subunits 165,000, 155,000, and 39,000), phosphorylase B (subunit 94,000), bovine serum albumin (subunit 67,000), ovalbumin (subunit 43,000), carbonic anhydrase (subunit 30,000), soybean trypsin inhibition (subunit 20,100), and lysozyme (subunit 14,400). The molecular weight assigned to each protein represents the mean of three separate determinations.

all seven proteins, 187,300, requires a coding capacity of 4,900 nucleotides for all translated regions plus 100 to 200 nucleotides per RNA, representing untranslated regions. This figure is easily accommodated by the 6,800 base pairs spanned by pDP202. The remaining four proteins, which map in the region corresponding to pDB116, represent a combined molecular weight of 81,400 and require a coding sequence of 2,100 nucleotides. However, the portion of the deletion spanned by pDB116 is approximately 1,700 nucleotides. If all four of these polypeptides are encoded by unrelated RNAs, then at least one

RNA must map partially to the right of the deletion or overlapping transcription must occur.

Several laboratories have reported minor deletions at the termini of vaccinia virus DNA (for review, see reference 17), but there have been only two other reports of major deletions (10, 15). Drillien et al. (10) isolated a host range mutant of vaccinia virus after nitrous acid mutagenesis. The restriction pattern of the DNA of this variant resembled that of the WR strain of vaccinia virus and revealed a 12.6-megadalton deletion which appears to completely overlap the deletion in S variant vaccinia virus. The host range mutant was not tested on HeLa cells but was unable to grow in most human cell lines assayed. Furthermore, this mutant displayed altered plaque morphology, altered cytopathogenicity, and decreased infectivity on CV1 cells. These effects were not seen in S variant vaccinia virus (17).

While this work was in progress, Moss et al. reported a deletion mutant which is probably identical to that reported here (15). They established that eight polypeptides encoded by early RNAs transcribed *in vivo* and selected by hybridization to clones including the left and central part of the deletion are not encoded by the deletion mutant (15). The seven polypeptides reported here which map in the same region (pDB104 and pDP202) migrate comparably to those they have reported, although there is a variation in apparent molecular weights assigned to these proteins. The data presented here corroborate the map published by Moss et al. (15) and establish that these early RNAs are faithfully transcribed *in vitro* from vaccinia virus RNA. Furthermore, this report establishes that an additional four polypeptides are encoded in the right region of the deletion. Finally, this work indicates that 2 of the 11 polypeptides which map in the deleted region are structural proteins. This is a significant finding because these polypeptides are apparently not essential for replication or infection under laboratory conditions (15, 17). Moss et al. (15) have suggested that the gene products specified by the 6-megadalton deleted region may be required for extracellular dissemination of virus particles, a function which is not required for virus replication in tissue culture cells. If this is the case, it is not surprising that structural proteins are encoded in this region.

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