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Colinearity of RNAs with the Vaccinia Virus Genome: Anomalies with Two Complementary Early and Late RNAs Result from a Small Deletion or Rearrangement Within the Inverted Terminal Repetition

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The colinearity of RNA transcripts with the vaccinia virus genome was investigated. Cytoplasmic RNA from infected cells was annealed to a cloned DNA segment that extended from 9 to 15.6 kilobase pairs from the left end of the genome and contained approximately 800 base pairs of the inverted terminal repetition (ITR). Remaining unhybridized single strands of DNA were digested with nuclease S1, and the lengths of the protected DNA fragments were determined by agarose gel electrophoresis under neutral and alkaline conditions. Uniformly ^{32}P-labeled cloned DNA insert, separated recombinant DNA strands, and smaller restriction fragments, as well as 3′ and 5′ end-labeled DNA, were employed to map five early RNAs and one late RNA. One of the early RNAs hybridized to sequences within the ITR, and the other four hybridized to sequences proximal to the ITR. The late RNA was initiated proximal to the ITR but extended into it. Interestingly, the 3′ portion of this late RNA was complementary to the early RNA transcribed from the opposite strand of the ITR. From a comparison of the lengths of the protected DNA fragments on neutral and alkaline gels, all except the complementary early and late RNAs appeared to be colinear with the genome. Although the anomalous nuclease S1 data obtained with the latter RNAs mimicked splicing, they were shown by DNA-DNA hybridization to result from a small deletion or rearrangement within the ITR. Thus far, no true examples of spliced vaccinia virus RNAs have been found.

Vaccinia virus provides a unique model system for studying the synthesis and posttranscriptional modification of mRNA encoded by a DNA genome. RNA synthesized by enzymes packaged within the virus particle (9, 13) contains a 5′ cap structure (17) and a 3′ polyadenylate tail (8). Because of these eucaryotic features, there is considerable interest in determining whether vaccinia virus RNAs are spliced. No evidence of splicing was found for three early mRNAs encoded within the long inverted terminal repetition (ITR; 5, 18, 19) or in another DNA fragment (6). To extend these studies, we have examined by the Berk and Sharp hybridization and nuclease S1 protection procedure (1, 2) several additional RNAs encoded within the left penultimate 6.6-kilobase pair (kbp) *Eco*RI fragment. The results described here confirm RNA size and map positions determined by other methods (20). In addition, we detected a late RNA that is complementary in sequence to one of the early RNAs. Although the majority of the transcripts examined appeared to be colinear with the genome, the two complementary RNAs were exceptions. Further analysis of the DNA, however, demonstrated that the appearance of splicing resulted from a small deletion or sequence rearrangement within a large subpopulation of the plaque-purified virus.

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MATERIALS AND METHODS

Isolation of RNA. Procedures for infection of cells with the WR strain of vaccinia virus and purification of cytoplasmic RNA by centrifugation through CsCl have been described (19).

In vivo labeling of recombinant λ phage DNA. Infected LE392 cells were labeled with 30 to 50 μCi of $^{32}P_i$ per ml in modified MOPS medium, and the phage were purified as described (19). The specific activity of the DNA was about 50,000 cpm/μg.

In vitro labeling of DNA fragments. The 5′ ends of DNA fragments were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase essentially as described by Maxam and Gilbert (10). Labeling of recessed 3′ ends was accomplished by using the Klenow fragment of

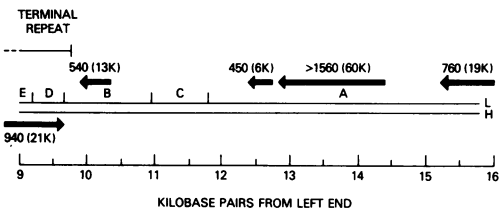

FIG. 1. Transcriptional map of the 6.6-kbp left penultimate *Eco*RI fragment of the vaccinia virus genome. Arrows indicate the direction of transcription, numbers refer to the sizes of the polyadenylated RNAs in bases, and the numbers in parentheses are the sizes of the polypeptide translation products. The *Hpa*II digestion products are indicated by letters. H, Heavy strand of the recombinant DNA; L, light strand of the recombinant DNA.

Escherichia coli DNA polymerase to add α-^{32}P-labeled deoxyribonucleoside triphosphates.

Nuclease S1 analysis. Nuclease S1 analysis was performed by the methods of Berk and Sharp (1, 2). Hybrids between vaccinia RNA and the purified ^{32}P-labeled vaccinia virus DNA fragment were formed in 30 μl of 80% formamide–0.4 M NaCl–0.04 M PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid), pH 6.4]–0.001 M EDTA. The samples were denatured by incubation at 60°C for 10 min and hybridized for 16 h at 42°C. Alternatively, hybridization of labeled separated strands and vaccinia RNA was performed in 0.6 M NaCl–0.06 M sodium citrate for 16 h at 60°C. Hybridizations were terminated by diluting the reaction mixtures with 10 volumes of 0.25 M NaCl, 0.03 M sodium acetate (pH 4.5), 0.001 M $ZnSO_4$, 5% glycerol, and 375 U of S1 nuclease (Miles) per ml. Samples were incubated at 45°C for 30 min, and S1-resistant material was precipitated by the addition of 3 volumes of ethanol.

Nuclease S1-treated samples were analyzed by electrophoresis in either 1.5% neutral or alkaline agarose gels (19). The latter were cast in 0.03 M NaCl–0.002 M EDTA and pre-electrophoresed in 0.03 M NaOH–0.002 M EDTA for 1 to 2 h before loading the samples.

RESULTS

The 6.6-kbp left penultimate *Eco*RI fragment of the vaccinia virus genome was cloned in λgtWES·λB and used to locate the map positions of encoded mRNAs (20). The representation in Fig. 1 was derived from "Northern blot" analysis, hybridization of cap-labeled RNA to "Southern blots," and cell-free translation of RNA selected by hybridization to separated DNA strands and restriction fragments. In this manner, four early mRNAs were shown to hybridize to the light (L) strand, and one was found to hybridize to the heavy (H) strand. Similar results were obtained with early RNAs made in the presence of cycloheximide, an inhibitor of protein synthesis, and cytosine arabinoside, an inhibitor of DNA replication. Further work was necessary to analyze early and late transcription products by the Berk and Sharp hybridization and nuclease S1 protection procedure (1, 2) to confirm and improve the map and determine the colinearity of RNA and DNA. In particular, we were searching for evidence of splicing.

Total cytoplasmic RNA from vaccinia virus-infected cells was hybridized to the cloned vaccinia virus DNA fragment purified from uniformly ^{32}P-labeled recombinant DNA. The concentration of formamide and the temperature were selected previously to minimize DNA·DNA reannealing (19). Unhybridized single-stranded DNA was then digested with nuclease S1, and the resistant DNA·RNA hybrids were resolved by agarose gel electrophoresis. Under neutral conditions, the bands detected by fluorography are RNA·DNA hybrids (Fig. 2). Except for the highest-molecular-weight band, which represents reannealed DNA, none of the bands was detected in the absence of added RNA. The sizes of the hybrids indicated in Fig. 2 are averages obtained from several similar experiments. When immediate early RNA from cells infected in the presence of cycloheximide was used, the sum of the sizes of the RNA·DNA hybrids exceeded the 6.6-kbp coding capacity of the DNA fragment, indicating overlapping or complementary transcripts. To rule out artifacts resulting from limiting amounts of DNA, a 10-fold-higher DNA concentration was used. Although the same bands were detected, their relative intensities were quite different. In particular, the two smallest hybrids of approximately 360 and 440 bp were now most intense, reflecting their true abundance.

Similar results were also obtained with early RNA made in the presence of cytosine arabinoside (Fig. 2). Because of the low amounts of viral RNA made under these conditions, however, the bands were faint, and the relative abundance of the short transcripts was evident with either concentration of DNA. When late RNA was used for hybridization, a prominent band of 1,290 bp was found (Fig. 2). Faint bands corresponding to the early sequences also could be detected.

Under the neutral conditions of electrophoresis used above, interrupted segments of DNA that hybridize to a single RNA species would migrate as a unit. Under alkaline conditions, however, the DNA segments would be separated. This type of analysis was developed by Berk and Sharp and used to demonstrate splicing (2). In a similar manner, we sought to obtain information regarding the colinearity of vaccinia virus RNA and DNA by alkaline gel electrophoresis of nuclease S1-protected DNA fragments. With early viral RNAs made in the presence of cycloheximide or cytosine arabinoside, similar bands were obtained under both electrophoretic conditions, and the sizes of the DNA segments

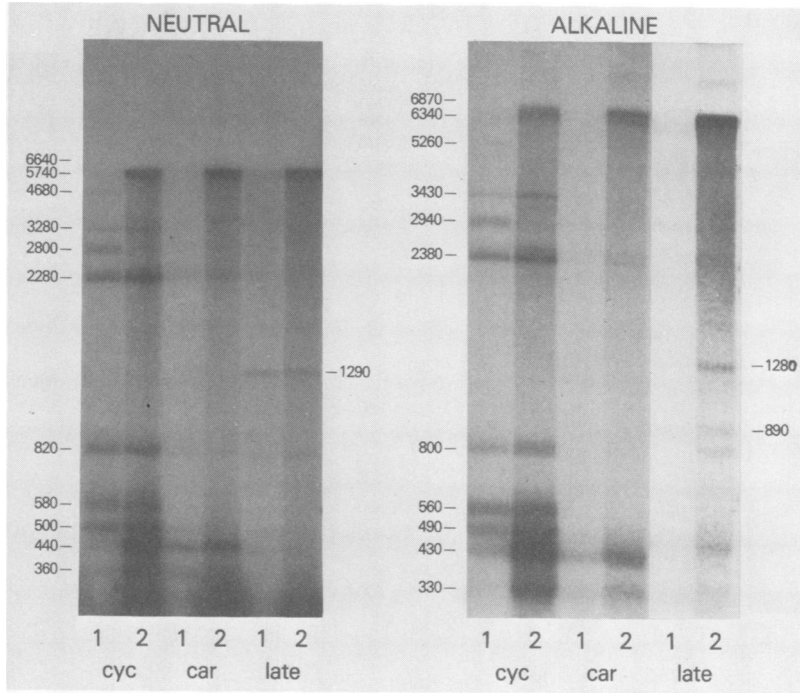

FIG. 2. Autoradiograph of nuclease S1-resistant RNA·[^{32}P]DNA hybrids analyzed by electrophoresis on neutral and alkaline agarose gels. A 10-μg sample of total cytoplasmic RNA from vaccinia virus-infected cells treated with cycloheximide (cyc), cytosine arabinoside (car), or no inhibitors (late) was hybridized to the 6.6-kbp cloned DNA fragment isolated from uniformly ^{32}P-labeled recombinant phage. In all tracks numbered 2, a 10-fold greater amount of DNA was used than for tracks labeled 1. Unhybridized single-stranded DNA was digested with nuclease S1 (375 U/ml), and resistant material was resolved by electrophoresis on either neutral or alkaline 1.5% agarose gels. The indicated lengths in nucleotides of the bands are averages from several gels in which end-labeled λ *Hin*dIII and φX174 RF *Hae*III fragments were co-electrophoresed as markers.

agreed within the error of the method. As will be shown later, however, the large number of protected DNA fragments obscured real differences in the lower part of the gel. With late RNA an additional band of 890 bases was resolved by alkaline gel electrophoresis and a band of about 430 bases appeared to increase slightly in intensity. However, a band of approximately 1,280 bases was still present, making the result somewhat puzzling.

Because the RNAs of greater than 2,000 nucleotides have not been shown to be translatable, appear to be overlapping in sequence, and are of unknown significance, we will make very little further comment regarding them in this communication.

Strand assignment of RNAs. To further our analysis, RNA was hybridized to the separated strands of uniformly labeled λ recombinant DNA and the unhybridized material was digested with nuclease S1. Upon neutral agarose gel electrophoresis, a single band of 820 bp was detected when viral RNA made in the presence of cycloheximide was annealed to the heavy (rightward reading) DNA strand (Fig. 3). Since previously (20) only a single mRNA of 940 nucleotides was shown to hybridize to the heavy strand, we surmised that the band contained the message for the 21,000-dalton (21K) polypeptide (Fig. 1). Further evidence to support this contention and to explain the apparent discrepancy in RNA size will be presented in a later section.

The remaining nuclease S1-protected hybrids were detected by using the light DNA strand (Fig. 3). The two smallest hybrids of 360 and 440 bp were apparent on longer exposure. The faintness of these bands evidently was due to the limiting amounts of separated DNA strands available for hybridization. This view was supported by results obtained with viral RNA made in the presence of cytosine arabinoside. Because of the low amounts of viral RNA and the consequently higher DNA/RNA ratio, the abundant low-molecular-weight hybrids were predominant (Fig. 3). The specific late 1,290-bp hybrid was also formed with the light DNA strand (Fig. 3).

When parallel samples of nuclease S1-resis-

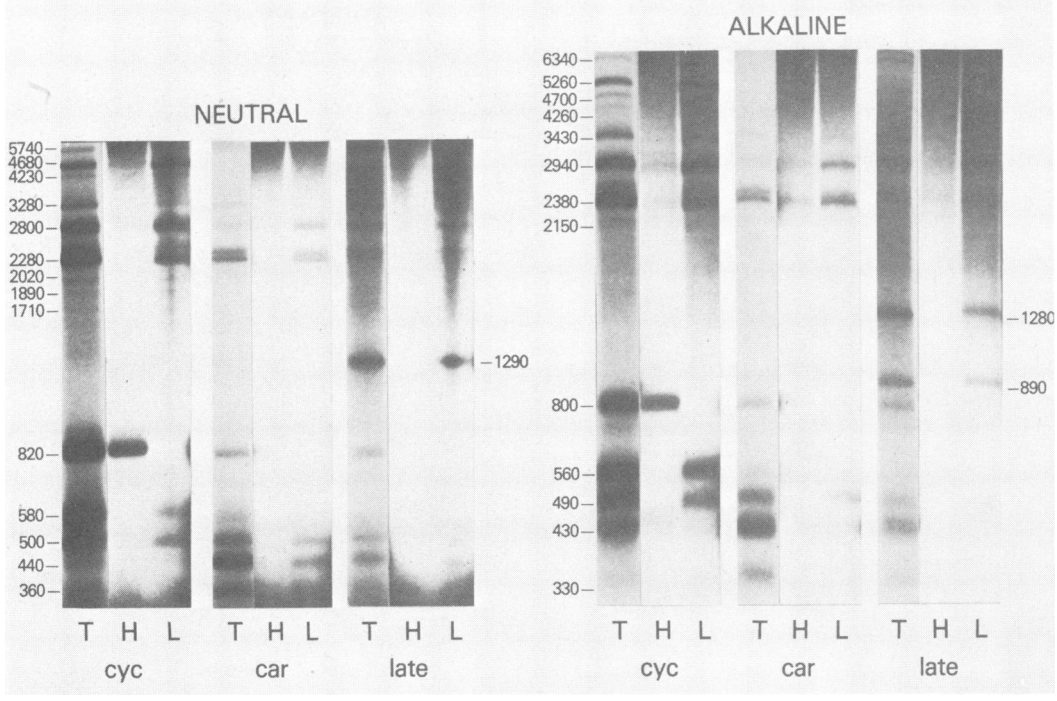

FIG. 3. Autoradiograph of nuclease S1-resistant material formed by hybridization of RNA to ^{32}P-labeled separated recombinant DNA strands and analyzed by neutral and alkaline agarose gel electrophoresis. A 25-μg sample of total cytoplasmic RNA, isolated from vaccinia virus-infected cells treated with cycloheximide (cyc), cytosine arabinoside (car), or no inhibitors (late), was hybridized to uniformly ^{32}P-labeled, cloned 6.6-kbp DNA fragment (T) and to purified heavy (H) and light (L) strand of recombinant DNA. Treatment with nuclease S1 and gel electrophoresis under neutral and alkaline conditions were carried out as described in the legend to Fig. 2.

tant material formed by hybridization of RNA to separated DNA strands were analyzed on alkaline gels, essentially the same pattern was obtained (Fig. 3). One exception, however, was the presence of a faint 430-base band in addition to the major 800-base band when RNA made in the presence of cycloheximide was hybridized to the heavy DNA strand. Although the faint band was seen on repeated experiments, we considered initially that it might have resulted from a slight contamination with light strand DNA. Subsequent experiments, however, demonstrated that this was not the correct explanation.

As before, we observed the band of 890 bases as well as a faint band of 430 in addition to the one of approximately 1,290 bases when late RNA·DNA hybrids were examined by alkaline gel electrophoresis. Moreover, these products were formed by hybridization to the light strand.

Mapping of early RNAs by hybridization to smaller restriction fragments. Additional experiments were designed to map the RNAs detected by hybridization and nuclease S1 analysis. Uniformly ^{32}P-labeled recombinant DNA was digested with *Eco*RI, and the vaccinia virus DNA segment was isolated. The latter was then digested with *Hpa*II, and the resulting fragments, indicated in Fig. 1, were purified. These labeled *Hpa*II fragments were then annealed to immediate early RNA, and the unhybridized single strands were digested with nuclease S1. We considered that RNAs entirely encoded within individual *Hpa*II fragments would give hybrids identical in size to those obtained with the larger *Eco*RI fragment. On the other hand, RNA species that hybridize to two or more adjacent *Hpa*II fragments will form truncated hybrids by this procedure. Apparently, unshortened hybrids of 360, 440, 580, and 2,280 bp were detected when the large *Hpa*II A fragment was used, and one of 500 bp was found by using the *Hpa*II B fragment (Fig. 4); the remaining bands appeared to be truncated to some extent. The 500-bp band obtained with the *Hpa*II B fragment was evidently the mRNA for the 13K polypeptide (Fig. 1). The bands detected by using the *Hpa*II A fragment were candidates for the 6K, 19K, and 60K early polypeptides.

Identification of early RNAs mapping at the left and right ends of the cloned *Eco*RI DNA segment.

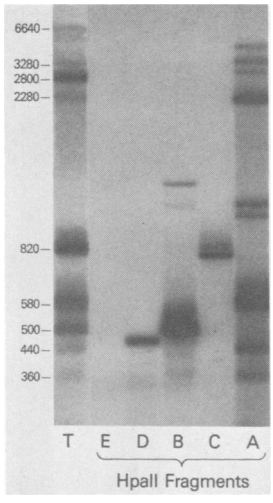

FIG. 4. Autoradiograph of nuclease S1-resistant material formed by hybridization of RNA to ^{32}P-labeled *Hpa*II restriction fragments and analyzed by neutral agarose gel electrophoresis. Total cytoplasmic RNA from virus-infected cells treated with cycloheximide was hybridized to uniformly ^{32}P-labeled, cloned 6.6-kbp *Eco*RI DNA fragment (T) and to the indicated *Hpa*II subfragments (see Fig. 1). Treatment with nuclease S1 and gel electrophoresis under neutral conditions were carried out as described in the legend to Fig. 2.

Our previous studies (5, 16, 20) suggested that mRNAs for the 21K and 19K polypeptides were initiated to the left and right, respectively, of the cloned penultimate *Eco*RI fragment studied here (Fig. 1). Accordingly, those RNAs should hybridize to the 3′ ends of the *Eco*RI fragment and protect the terminal nucleotide from nuclease S1 digestion. To confirm this, [α^{32}-P]dATP and [α^{32}-P]dTTP were used with DNA polymerase to label the staggered ends of the DNA. The *Eco*RI fragment was then cleaved at the single *Xho*I site, and the two resulting pieces were separated by agarose gel electrophoresis. RNA, from cells infected in the presence of cycloheximide, was then hybridized to the labeled DNA fragments, and the remaining single-stranded DNA was digested with nuclease S1. The labeled bands were then identified by autoradiography after neutral gel electrophoresis. With the left fragment, a prominent band of 820 bp and a faint one of 2,280 bp was observed (Fig. 5). With the right fragment, an intense band of 580 bp was detected (Fig. 5). Hybrids of 820 and 580 bp were among those detected with uniformly ^{32}P-labeled DNA (Fig. 5). The map in Fig. 1 suggests that the 820-bp and 580-bp bands are derived from the mRNAs for the 21K and 19K polypeptides, respectively. It should be realized that the sizes of the protected DNA fragments do not represent the full lengths of the mRNAs since the latter are initiated in adjacent *Eco*RI fragments. By contrast, the values determined directly by gel electrophoresis of denatured RNA (shown in Fig. 1) represent full lengths plus a polyadenylate tail of approximately 100 nucleotides. Thus, there is no discrepancy in the results obtained by the two methods.

Parallel nuclease S1-treated samples were also examined by electrophoresis on an alkaline gel. When the hybridization products obtained with the right-end-labeled DNA fragment were analyzed, the size of the single band was similar to that obtained on a neutral gel (Fig. 5, track 2). In contrast, a remarkable difference was noted when the products obtained with the left-end-labeled fragment on neutral and alkaline gels were compared (Fig. 5, track 1). Whereas a single band of approximately 820 bp was found on neutral gels, two bands of 800 and 430 bases were resolved under alkaline conditions, suggesting the possibility of splicing (Fig. 5, track 1). Identical results also were obtained when RNA made in the presence of cytosine arabinoside was hybridized to end-labeled DNA (not shown). Although a faint 430-base band had been detected on alkaline gels when uniformly

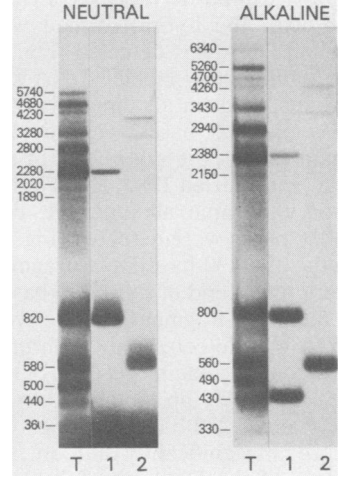

FIG. 5. Autoradiograph of nuclease S1-resistant material formed by hybridization of RNA to 3′ end-labeled DNA fragments and analyzed by neutral and alkaline agarose gel electrophoresis. A 25-μg sample of total cytoplasmic RNA, isolated from vaccinia virus-infected cells that were treated with cycloheximide, was hybridized to uniformly labeled, 6.6-kbp cloned *Eco*RI DNA fragment (T) and to the left (1) and right (2) *Xho*I subfragments 3′ end-labeled at the *Eco*RI restriction sites. Nuclease S1 digestion and agarose gel electrophoresis were carried out as described in the legend to Fig. 2.

labeled heavy strand DNA was used for hybridization (Fig. 3), the presence of a similar size band derived from the light strands obscured the difference between neutral and alkaline gels when uniformly labeled DNA that was not strand separated was used.

Next, we determined that none of the early RNAs that are initiated within the penultimate *Eco*RI fragment extends into adjacent fragments. To do this, the cloned *Eco*RI fragment was labeled at its 5′ ends with polynucleotide kinase and [γ-^{32}P]ATP and then cleaved at the single *Xho*I site. The two DNA fragments were purified and hydridized to RNA made either in the presence of cycloheximide or cytosine arabinoside. However, in neither case did RNA hybridize to the terminally labeled nucleotide and protect it from nuclease S1 digestion.

Identification of a late RNA mapping at the left end of the cloned *Eco*RI DNA segment. Experiments similar to those outlined in the previous section were performed with late RNA obtained 6 h after vaccinia virus infection. When 3′ end-labeled DNA was used, no labeled bands were obtained; negative results also were obtained with the right *Xho*I subfragment labeled at the 5′ *Eco*RI end (Fig. 6, track 1). However, when late RNA was hybridized to the left *Xho*I subfragment labeled at the 5′ *Eco*RI end, nuclease-protected bands were resolved. On neutral agarose gels, a 1,290-bp RNA·DNA hybrid that comigrated with the hybrid formed with uniformly labeled DNA was detected (Fig. 6, track 2). When the uniformly labeled DNA·RNA hybrids were analyzed on alkaline gels, bands of 1,280, 890, and 430 bases were detected as before, again raising the possibility of splicing. When the 5′ end-labeled DNA·RNA hybrid was analyzed on the same alkaline gel, bands of 1,280 and 430 bases were detected (Fig. 6, track 2). Evidently, the 430-base DNA fragment was derived from the 5′ end of the 1,290-base DNA. Since the 890-base fragment was derived from the 3′ end, it was detected only with uniformly labeled DNA on the alkaline gel (Fig. 6, track T). The values of 1,290 bp obtained on neutral agarose gels and 1,280 bases obtained on alkaline gels were not significantly different, suggesting that some of the DNA was fully protected from nuclease S1.

The 5′ end of this late transcript must map about 500 nucleotides to the right of the ITR (Fig. 1). Since the RNA extends past the *Eco*RI site of the cloned DNA segment, its 3′ end must be in the adjacent DNA fragment. Thus, the full length of the transcript is greater than 1,280 bases. Furthermore, a large portion of this late RNA must be complementary in sequence to the early RNA for the 21K polypeptide (Fig. 1). The map position of the RNA for the 13K polypeptide is not sufficiently refined to know whether it partially overlaps the late transcript.

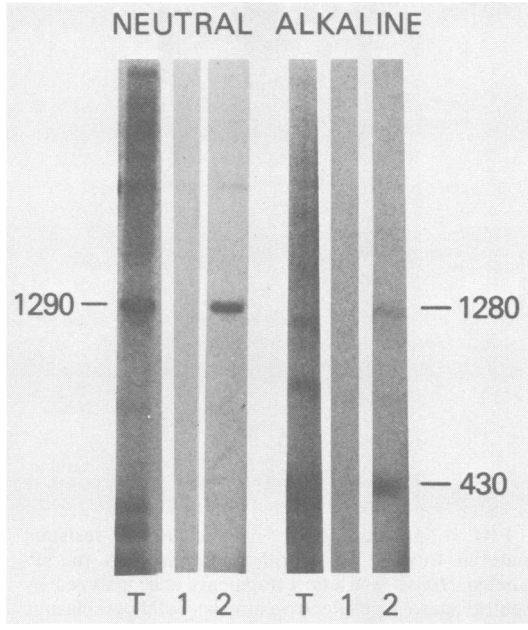

FIG. 6. Autoradiograph of nuclease S1-resistant material formed by hybridization of late RNA to 5′ end-labeled DNA fragments and analyzed by neutral and alkaline agarose gel electrophoresis. A 25-μg sample of total cytoplasmic RNA, isolated 6 h after infection, was hybridized to uniformly labeled, 6.6-kbp cloned *Eco*RI fragment (T) and to the left (1) and right (2) *Xho*I subfragments 5′ end-labeled at the *Eco*RI sites. Nuclease S1 digestion and agarose gel electrophoresis were carried out as described in the legend to Fig. 2.

Interestingly, the DNA hybrids formed with the late RNA and the RNA for the 21K polypeptide were incompletely protected against nuclease S1 as indicated by the smaller fragments obtained on alkaline agarose gels. Furthermore, in each case, the smaller end-labeled fragment was 430 bases. Their identical electrophoretic mobilities were confirmed by analysis on the same gel (not shown). This coincidence led us to look for an explanation, other than RNA splicing, for the anomalous nuclease S1 results.

Evidence for a deletion or sequence rearrangement in the genome. We considered that the probable cause for the above results was the occurrence of a small deletion or sequence rearrangement, approximately 430 bp from the *Eco*RI site within either the virion or cloned DNA. Evidence supporting the above hypothesis came from DNA·DNA hybridization studies. Total virion DNA, from the virus stock used for infections, was digested with *Eco*RI and incu-

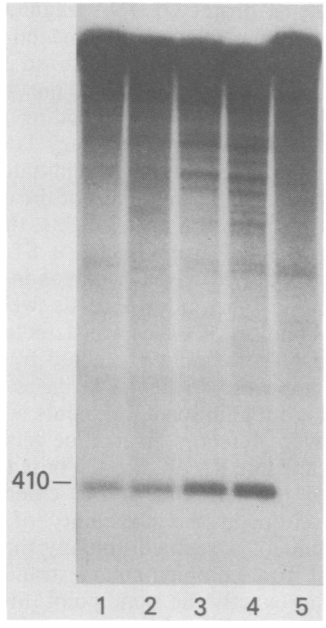

FIG. 7. Autoradiograph of nuclease S1-resistant material formed by hybridization of unlabeled virion DNA to ^{32}P-labeled cloned DNA and analyzed by alkaline agarose gel electrophoresis. A constant amount of uniformly ^{32}P-labeled, 6.6-kbp cloned *Eco*RI DNA insert was annealed to 0 μg (track 5), 2 μg (track 1), 4 μg (track 2), 6 μg (track 3), and 8 μg (track 4) of unlabeled, *Eco*RI-digested total DNA from vaccinia virions. Nuclease S1 digestion and alkaline agarose gel electrophoresis were carried out as described in the legend to Fig. 2.

bated under suitable conditions with the cloned, uniformly ^{32}P-labeled DNA segment. After nuclease S1 digestion, the hybrids were analyzed on an alkaline gel. With increasing amounts of total vaccinia DNA, an increasingly intense band of approximately 400 bases was found (Fig. 7, tracks 1 to 4). This band was not detected upon self-hybridization of the cloned DNA (Fig. 7, track 5). The size of this band was in good agreement with the anomalous ones detected by alkaline gel electrophoresis after RNA·DNA hybridization (Fig. 5 and 6). Similar results were also obtained by hybridizing the *Eco*RI-digested left terminal *Hin*dIII fragment to the ^{32}P-labeled cloned fragment. Moreover, in that experiment we not only detected the 400-base band but also demonstrated a small reduction in size of the remaining approximately 6,000-bp fragment (not shown).

To locate the precise position of the deletion or rearrangement, *Eco*RI-digested total vaccinia virus DNA was hybridized to the 3′ end-labeled left *Xho*I subfragment of the cloned DNA segment, and the products were treated with nuclease S1. Upon neutral agarose gel electrophoresis, a very intense band corresponding to the entire left portion of the cloned segment was found, as well as some very minor bands of unknown significance (Fig. 8, track 2). More importantly, an additional 430-base band was detected under alkaline conditions (Fig. 8, track 2). Moreover, this band comigrated with the product of RNA·DNA hybridization (Fig. 8, track 1).

DISCUSSION

Early and late RNAs encoded within the 6.6-kbp left penultimate *Eco*RI fragment of the vaccinia virus genome were analyzed by neutral and alkaline agarose gel electrophoresis of nuclease S1-digested RNA·DNA hybrids. By using uniformly ^{32}P-labeled cloned *Eco*RI fragment, separated recombinant DNA strands, and *Hpa*II restriction fragments as well as 3′ and 5′ end-labeled DNA for hybridization, it was possible to confirm and extend the transcriptional map

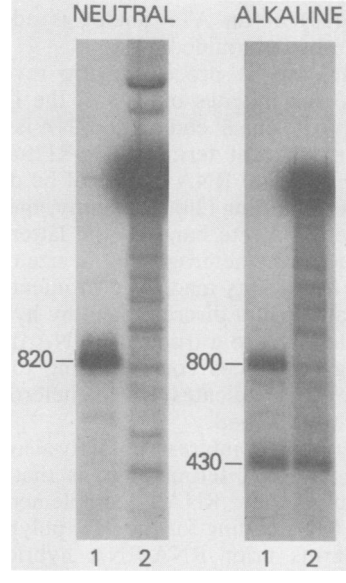

FIG. 8. Autoradiograph of nuclease S1-resistant material formed by hybridization of RNA and DNA to 3′ end-labeled DNA fragments and analyzed by neutral and alkaline agarose gel electrophoresis. The left subfragment, obtained by *Xho*I digestion of the 6.6-kbp 3′ end-labeled *Eco*RI fragment, was hybridized to total cytoplasmic RNA from vaccinia virus-infected cells treated with cycloheximide (1) and to 5 μg of *Eco*RI-digested total DNA from vaccinia virions (2). Nuclease S1 digestion and neutral and alkaline agarose gel electrophoresis were carried out as described in the legend to Fig. 2.

obtained by independent methods (20). mRNAs for the early 21K and 19K polypeptides were shown to extend into the left and right adjacent fragments, respectively. Early mRNA for the 13K polypeptide was encoded entirely within the *Hpa*II B fragment, whereas mRNAs believed to code for the early 6K and 60K polypeptides hybridized to sequences contained within the *Hpa*II A fragment. There was some ambiguity regarding the mRNA for the 6K polypeptide since two transcripts of 360 and 440 bases were detected by nuclease S1 analysis whereas only a single polyadenylated RNA of 450 bases was detected by the lower-resolution Northern blotting procedure (20).

The nuclease S1 procedure also resolved additional early high-molecular-weight RNAs that appeared very faintly on Northern blots (20). High-molecular-weight early RNAs encoded within other restriction fragments were noted previously (6, 19). In those studies, the possibilities that these RNAs are read-through products or higher-molecular-weight precursors that are destined to be trimmed at the 3′ ends were considered. Whether these in vivo high-molecular-weight RNAs are related to the high-molecular-weight RNAs formed in vitro when ATP is depleted (15) or an ATP analog is added (7) remains to be determined.

The nuclease S1 procedure also revealed a late RNA that initiates outside of the ITR and extends into it. The 3′ end of this RNA is located within the adjacent terminal *Eco*RI fragment. However, this late RNA could not be detected by Northern blotting (20). Evidently, the failure to resolve a discrete band by the latter procedure is due to the heterogeneity in size of many if not all transcripts made late in infection (6). That we obtained a discrete band by hybridization of late RNA to a truncated DNA fragment containing sequences corresponding to the 5′ end of the RNA indicates that the heterogeneity must be in the 3′ end.

Another point of interest, possibly also related to failure of RNA termination, is that a long segment of the late RNA is complementary to the early RNA coding for the 21K polypeptide. This confirms prior RNA·RNA hybridization studies indicating that some late RNAs are "anti-early" (3) as well as self-complementary (4). We do not know whether the late RNA analyzed in this study is translatable. Although a late 22K polypeptide is encoded within the cloned 6.6-kbp DNA fragment, its map position does not appear to correspond with that of this transcript.

The main purpose of this study was to examine the colinearity of the transcripts with the genome. In particular, we sought evidence of RNA splicing. For most RNAs, the lengths of the nuclease S1-protected DNA fragments were similar under neutral and alkaline conditions, indicating the absence of unhybridized intervening DNA sequences. This was not the case either for the early mRNA encoding the 21K polypeptide or the complementary late RNA. The early 940-nucleotide RNA is initiated in the *Eco*RI DNA fragment to the left of the one used in this study. In neutral agarose gels, the length of DNA protected from nuclease S1 by this RNA was about 820 bases, whereas in alkaline gels a band of the latter size as well as an additional band of 430 bases was detected. Similarly, the DNA fragment protected by the late transcript appeared to be 1,290 bases long on neutral gels, but additional fragments of 890 and 430 bases were detected on alkaline gels. Within both early and late RNAs, the 430-base fragment represented the left end of the cloned DNA segment. Although the possibility of splicing was entertained, it seemed unlikely that RNAs transcribed from complementary strands would be spliced at exactly the same point. Moreover, in both cases, considerable amounts of the full-size DNA segment remained under alkaline conditions regardless of the amount of nuclease S1 used. The cause of the anomalous nuclease S1 results was found to be a small deletion or sequence rearrangement within the genome. This was shown by hybridizing *Eco*RI-digested virion DNA to the cloned *Eco*RI fragment and digesting with nuclease S1. As predicted, a fragment of about 430 bases was formed. Thus, although the same plaque-purified virus had been used for cloning and preparing large virus stocks, a genomic variant arose in the latter. Consequently, RNA transcribed from the variant did not completely protect the cloned DNA segment from nuclease S1.

Because the deletion or rearrangement occurred within the ITR, we also considered the possibility that the left and right ends of the DNA are not identical. Under these circumstances, similar but nonidentical mRNAs could be formed from the two ends. However, when DNA from the left side of the genome was hybridized to a cloned fragment from the left side, the DNA heterogeneity was still observed. We suspect that the region of the genome near the junction of the ITR is a "hot spot" for deletions or rearrangements. Moyer and coworkers found that large deletions within the rabbit poxvirus genome begin in this region (12), and similarly large deletions have been found in plaque isolates of vaccinia virus (11, 14).

In conclusion, no evidence for splicing of vaccinia virus mRNAs has been obtained thus far. Whether such a generalization will hold true for all vaccinia virus transcripts remains to be seen.

LITERATURE CITED

1. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNA's by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
2. **Berk, A. J., and P. A. Sharp.** 1978. Spliced early mRNA's of simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **75**:1274–1278.
3. **Boone, R. F., R. P. Parr, and B. Moss.** 1979. Intermolecular duplexes formed from polyadenylylated vaccinia virus RNA. J. Virol. **30**:365–374.
4. **Colby, C., C. Jurale, and J. R. Kates.** 1971. Mechanism of synthesis of vaccinia virus double-stranded ribonucleic acid in vivo and in vitro. J. Virol. **7**:71–76.
5. **Cooper, J. A., R. Wittek, and B. Moss.** 1981. Hybridization selection and cell-free translation of mRNA's encoded within the inverted terminal repetition of the vaccinia virus genome. J. Virol. **37**:284–294.
6. **Cooper, J. A., R. Wittek, and B. Moss.** 1981. Extension of the translational map of the left end of the vaccinia virus genome to 21 kilobase pairs. J. Virol. **39**:733–745.
7. **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.
8. **Kates, J. R., and J. Beeson.** 1970. Ribonucleic acid synthesis in vaccinia virus. II. Synthesis of polyriboadenylic acid. J. Mol. Biol. **50**:19–33.
9. **Kates, J. R., and B. R. McAuslan.** 1967. Messenger RNA synthesis by a "coated" viral genome. Proc. Natl. Acad. Sci. U.S.A. **57**:314–320.
10. **Maxam, A., and W. Gilbert.** 1980. Sequencing end labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
11. **Moss, B., E. Winters, and J. A. Cooper.** 1981. Deletion of a 9,000-base pair segment of the vaccinia virus genome that encodes nonessential polypeptides. J. Virol. **40**:387–395.
12. **Moyer, R. W., and C. T. Rothe.** 1980. The white pock mutants of rabbit poxvirus. I. Spontaneous host range mutants contain deletions. Virology **102**:119–132.
13. **Munyon, W., E. Paoletti, and J. T. Grace, Jr.** 1967. RNA polymerase activity in purified infectious vaccinia virus. Proc. Natl. Acad. Sci. U.S.A. **58**:2280–2287.
14. **Panicalli, D., S. W. Davis, S. R. Mercer, and E. Paoletti.** 1981. Two major DNA variants present in serially propagated stocks of the WR strain of vaccinia virus. J. Virol. **37**:1000–1010.
15. **Paoletti, E., and B. R. Lipinskas.** 1978. The role of ATP in the biogenesis of vaccinia virus mRNA *in vitro*. Virology **87**:317–325.
16. **Venkatesan, S., and B. Moss.** 1981. In vitro transcription of the inverted terminal repetition of the vaccinia virus genome: correspondence of initiation and cap sites. J. Virol. **37**:738–747.
17. **Wei, C.-M., and B. Moss.** 1975. Methylated nucleotides block 5'-terminus of vaccinia virus messenger RNA. Proc. Natl. Acad. Sci. U.S.A. **72**:318–322.
18. **Wittek, R., E. Barbosa, J. A. Cooper, C. F. Garon, H. Chan, and B. Moss.** 1980. The inverted terminal repetition in vaccinia virus DNA encodes early mRNAs. Nature (London) **285**:21–25.
19. **Wittek, R., J. A. Cooper, E. Barbosa, and B. Moss.** 1980. Expression of the vaccinia virus genome: analysis and mapping of mRNAs encoded within the inverted terminal repetition. Cell **21**:487–493.
20. **Wittek, R., J. A. Cooper, and B. Moss.** 1981. Transcriptional and translational mapping of a 6.6-kilobase-pair DNA fragment containing the junction of the terminal repetition and unique sequence at the left end of the vaccinia virus genome. J. Virol. **39**:722–732.