Vaccinia virus DNA polymerase will utilize a substrate consisting of φX174 DNA primed with a strand of a unique restriction fragment, but the reaction is inefficient. Examination of the reaction products by alkaline agarose gel electrophoresis revealed a few discrete fragments, each corresponding to an extended primer strand. This result implies that specific barriers exist on the φX174 template which impede, but do not completely halt, the progress of the enzyme. Only a few percent of the template molecules were completely copied. Similar findings were reported by Sherman and Gefter using Escherichia coli DNA polymerase II and fd DNA (J. Mol. Biol. (1976) 103, 61-76). Several observations suggest that the barriers are regions of template secondary structure. Some barriers are more effective than others, and they increase in both effectiveness and number as the temperature is decreased. The same barriers are observed with T4 DNA polymerase, but none are detected with E. coli DNA polymerase I. Finally, the major barriers are located in regions of the φX174 sequence known to contain hairpin structures of relatively high stability. The exact stopping point at one of the major barriers is within the duplex stem of a hairpin structure. These results show that DNA polymerases are a useful probe of the secondary structure of a single-stranded DNA.

When vaccinia virus infects susceptible cells, it induces the synthesis of many, if not all, of the proteins involved in the replication of its DNA. We are currently engaged in investigating the enzymatic mechanisms involved in vaccinia DNA replication, and as a step towards reconstructing vaccinia replication in vitro, we have begun a study of the virus-induced DNA polymerase. In the preceding report we described the purification and initial characterization of this enzyme (1), and in this paper we describe the effect of template secondary structure on its activity.

In common with many DNA polymerases, the most effective DNA substrate for vaccinia polymerase is partly degraded duplex DNA containing many short single-stranded sequences which serve as template. The enzyme is inactive on a fully duplex DNA containing nicks, and it has low activity on a long single-stranded template. In a study of *Escherichia coli* DNA polymerase II, which has template requirements similar to those of the vaccinia enzyme, Sherman and Gefter (2) used a homogenous DNA substrate consisting of coliphage fd viral DNA which was primed with a complementary strand from a unique restriction enzyme fragment. Their studies showed that 1) chain elongation by polymerase II is inhibited by regions of secondary structure in the template, and 2) E. coli helix destabilizing protein stimulates the polymerase by facilitating DNA synthesis through the sites of template secondary structure.

In this study we employed a similar defined substrate to study the properties of vaccinia DNA polymerase. Instead of primed fd DNA, we used φX174 DNA because its nucleotide sequence is known (3). We showed that, in common with DNA polymerase II, chain elongation by vaccinia polymerase is impeded by uniquely located structures on the DNA template. We mapped the location of these sites on the φX174 genome and correlated them with regions of potential secondary structure in the primary sequence of the DNA. These findings not only reveal features of the polymerase mechanism, but also provide a useful method for mapping sites of secondary structure on single-stranded DNAs.

**MATERIALS AND METHODS**

*Nucleic Acids and Nucleotides—* Stocks of coliphage φX174 am 3 were from Dr. Nancy Shaper of The Johns Hopkins University. φX174 am 3 viral DNA was prepared from virions by three extractions with neutralized phenol followed by dialysis against 20 mM Tris-HCl, pH 8, 1 mM EDTA. φX174 am 3 RF DNA was prepared by the procedure of Godsen and Boyer (4).

φX174 RF DNA was digested with EndoR. *Hae* III in an incubation mixture (1.0 ml) containing 20 mM Tris-HCl, pH 7.2, 7 mM MgCl₂, 300 to 350 μg of φX174 RF DNA, and 75 units of EndoR- *Hae* III (from Dr. Michael Mann of The Johns Hopkins University). Digestion was complete in 4 h at 37°C. The reaction was stopped by the addition of 0.2 ml of 30% glycerol, 0.1 M EDTA, 2% sodium dodecyl sulfate, 0.5% bromphenol blue; 0.6 ml of the resulting mixture (containing about 150 μg of DNA) was layered onto an 8% polyacrylamide slab gel (15 cm x 40 cm x 0.45 cm). Electrophoresis was in 40 mM Tris-HCl, pH 7.5, 0.5 mM sodium acetate, 1 mM EDTA at room temperature for 14 to 16 h at 150 V. After staining with ethidium bromide (0.4 μg/ml) for 30 min, DNA bands were visualized under long wavelength UV light. The segments of the gel containing each DNA band were cut out and eluted by the method of Maxam and Gilbert (5). DNA was dissolved in 0.5 ml of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and dialyzed against that buffer.

The nomenclature used for restriction fragments of φX174 RF is essentially that of Sanger et al. (3), except that restriction enzymes are denoted by a three-letter (6) rather than a one-letter code. The approximate size and position of each of the fragments used in this study are shown in Fig. 7. The *Hpa* I-3 restriction enzyme fragment was prepared by digesting the φX174 RF DNA sequentially with EndoR- *Hae* III and EndoR- *Hpa* I. Double digestion was necessary to separate the *Hpa* I-2 fragment (640 base pairs, containing an EndoR- *Hae* III site) from the *Hpa* I-3 fragment (614 base pairs, not containing an EndoR- *Hae* III site).
III site) (3). 4x174 RF DNA (150 ng in 0.5 ml) was digested with EndoR-Hae III as described above. After 2 h at 37°C, 6 µl of 5 M NaCl, 12 µl of 1.0 M mercaptoethanol, and 30 units of EndoR-Hha I (New England Biolabs) were added, and the solution was incubated for an additional 2 h at 37°C. The digest was then fractionated by polyacrylamide gel electrophoresis as described in the preceding paragraph. The Hha I-3 fragment is the largest DNA species on the gel and is well separated from all other fragments. The DNA was eluted and redissolved as described above.

4x174 DNA primed by a strand of a restriction fragment was prepared by mixing 4x174 DNA single strand circles (about 25 µg/ml) and the restriction fragment (about 1/10 the molar concentration of circles) in a total volume of 60 µl of 20 mM Tris-HCl, pH 8.0, 1 mM EDTA. The DNA was denatured by the addition of 5 ml of 1.0 M NaOH. After 10 min at room temperature, the solution was neutralized by the addition of 5.0 ml of 2.0 M NaH2PO4 and incubated at 60°C for 10 min. After dialysis with an equal volume of water, the solution was filtered through a hydroxylapatite column (1.2 cm2 x 2.60 cm) which had been equilibrated with 0.14 M sodium phosphate, pH 6.8, at room temperature. The column was then washed with successive volumes (10 ml) of 0.14 M sodium phosphate, pH 6.8, at room temperature, 0.14 M sodium phosphate, pH 6.8, at 60°C, and finally with 0.4 M sodium phosphate, pH 6.8, at room temperature. Fractions (1.0 ml) were collected and DNA was assayed by its absorbance at 260 nm. The DNA eluting in 0.4 M sodium phosphate included the strand of the restriction fragment, and reannealed fragment, in an approximate molar ratio of 10:1. After dialysis against 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, the DNA (4 ml) was stored at 4°C. The recovery of DNA from the hydroxylapatite column was 80 to 90%.

Activated salmon DNA was prepared as described previously (1). Restriction fragments of 4x174 RF or T7 DNA (7), to be used as size markers, were either uniformly labeled with 32P or were 5’ terminally labeled with [32P]dATP (8).

Deoxyribonucleoside triphosphates were purchased from Schwarz/Mann (60). Deoxyribonucleoside triphosphates were either synthesized (8) or purchased from New England Nuclear.

Protein and Enzymes—Vaccinia virus DNA polymerase was Fraction VI (specific activity, 19,000 units/mg). The concentration of enzyme molecules was calculated using a molecular weight of 115,000 (1). T4 DNA polymerase was Fraction VII (10) (specific activity, 49,000 units/mg). E. coli DNA polymerase I was Fraction VI (11) (specific activity, 15,000 units/mg).

DNA Synthesis—Incubation mixtures for fragment-primed synthesis of 4x174 DNA (60 µl) contained 50 mM potassium phosphate, pH 7.5, 5 mM MgCl2, 400 µg/ml bovine serum albumin, 0.1 mM dithiothreitol, 30 µM each of dATP, dCTP, and dGTP, 20 µM [α32P]dTTP (106 to 108 cpm/pmol), about 0.05 pmol of 4x174 DNA circles primed with a strand of a restriction enzyme fragment, and enzyme (0.35 to 7 units). Unless noted otherwise, incubations were at 37°C. Reactions were terminated by addition of 5 µl of 0.5 M EDTA, and acid-insoluble radioactivity was determined (12). Samples were prepared for gel electrophoresis by extracting once with an equal volume of neutralized phenol and then dialyzing, first against 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1.0 M NaCl, and then against 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

Determination of Fragment Size by Gel Electrophoresis—Single-stranded DNA fragments greater than 300 nucleotides in length were fractionated by electrophoresis on agarose gels under alkaline conditions (7). The gels were vertical slabs (15x40 x 0.16 cm) prepared from a solution of 1.4% agarose, 30 mM NaOH, and 2 mM EDTA. Just prior to electrophoresis, 6 µl of 30% glycerol, 0.1 M NaOH, 0.3% bromphenol blue (7) was added to the samples (25 µl), which were then layered onto the top of the gel. Electrophoresis was in 30 mM NaOH, 2 mM EDTA at a constant voltage of 130 V for 14 to 15 h. The gels were dried under vacuum onto Whatman 3MM filter paper, and DNA bands were located on the dried gel by autoradiography on Kodak XR-1 x-ray film in the presence of an Ilford fast-tungstate intensifying screen at -70°C. Molecular weight standards were restriction fragments of T7 (7) or 4x174 RF DNA (3).

DNA fragments smaller than 300 nucleotides were electrophoresed on a 20% polyacrylamide gel in the presence of 7 M urea (6). Size markers in these gels were the dyes bromphenol blue and xylenecyanol FF, which migrate together with fragments of 10 and 28 nucleotides, respectively (6). The spacing between oligonucleotides differing in length by one nucleotide was determined from a partial pancreatic DNase digest of T7 [32P]DNA.

RESULTS

Utilization of a 4x174 Template—Some DNA polymerases are sensitive to the length of the single-stranded template being copied and are incapable of efficiently utilizing templates which contain long single-stranded regions without the addition of other protein factors such as helix destabilizing...
proteins or elongation factors (2, 13, 14). Others, such as E. coli DNA polymerase I, can efficiently copy long templates (15). To test the efficiency of vaccinia DNA polymerase on a long single-stranded template we compared the synthesis on activated salmon DNA, which presumably contains many nicks and short gaps, to that on φX174 single-stranded DNA primed with a single unique restriction fragment. The results in Table I show that the primed φX174 DNA is a poor substrate for vaccinia polymerase, with an amount of synthesis about 0.1% that on activated salmon DNA.

One possible explanation for the low activity on primed φX174 DNA is that many enzyme molecules might bind nonproductively at internal single-stranded sequences on the template. Nonproductive binding of this type has been suggested as an explanation for the low activity of polymerase-associated exonucleases on long single-stranded DNA substrates (16). However, the experiment in Table II shows that addition of a 6-fold excess of single-stranded DNA, in the form of unprimed φX174 circles, does not inhibit the polymerizing activity on primed φX174 DNA.

Further insight into the mechanism of the vaccinia polymerase on the primed φX174 substrate came from an investigation of the rate and extent of synthesis as a function of enzyme concentration (Fig. 1A). At a saturating level of enzyme (about 30 enzyme molecules per Hae III-2 primer), a maximum of about 500 nucleotides/φX174 circle were incorporated. This level is only about one-eighth of that expected if the entire template had been copied. Addition of more enzyme after 1 h did not significantly increase the amount of synthesis. However, the level of about 500 nucleotides incorporated/primer is not an intrinsic property of the polymerase, since use of a different primer, a strand of Hae III-6b, resulted in an extent of synthesis which was 2 to 3 times higher (Fig. 1B). In contrast to these results, E. coli DNA polymerase I incorporated greater than 5000 nucleotides per primer on φX174 DNA primed with a strand of Hae III-2.

An interpretation of these results, which was suggested by the experiments of Sherman and Gefter (2), is that during synthesis the polymerase encounters uniquely located barriers on the template strand. Thus the maximum level of incorporation of nucleotides is defined by the location of these barriers relative to the 3' terminus of the restriction fragment primer. Support for this interpretation is presented below.

**Gel Electrophoresis of the Product Made on Primed φX174 DNA**—To test whether there are uniquely located barriers to polymerization on φX174 DNA, we analyzed the products of synthesis on primed φX174 DNA by alkaline agarose gel electrophoresis. The results with four different primers are shown in Fig. 2. The two primers shown in Panel A were tested at two different enzyme levels; the two shown in Panel B were tested at two times of incubation. In all cases, the products consist of a few major discrete species and several minor species, indicating that progress of the enzyme along the template was delayed by barriers at specific locations. Synthesis between the barriers must be relatively rapid, and passage through the barriers appears to be the limiting step in the overall rate of synthesis. As shown in Fig. 2A, an increase in the ratio of polymerase molecules to primer molecules from 7 to 34 results in a relative increase in the amount of longer products.

In most experiments, a small amount of radioactivity (<10% of the total) migrated on the gels in the position expected for...

**Fig. 2 (left). Alkaline agarose gel electrophoresis of the products of synthesis by vaccinia DNA polymerase on φX174 DNA primed with restriction fragments.** Each reaction mixture (50 μl) contained 0.05 pmol of the φX174 DNA substrate. The reactions displayed in Panel A were incubated for 1 h with either 0.7 unit (0.35 pmol, “low enzyme”) or 3.5 units (1.7 pmol, “high enzyme”) of enzyme. The reactions in Panel B were incubated with 3.5 units (1.7 pmol) of enzyme for the indicated times. The lanes marked “std” contained a Hpa I digest of T7 [32P]DNA (Panel A) or the 5'32P-labeled Hae III fragments of φX174 RF (Panel B). The closed arrow indicates the position of a heterogeneous population of fragments slightly greater in size than a full-length φX174 strand. The open arrows indicate the position of primers (in the Hae III-6b experiment the primer migrated off the gel).

**Fig. 3 (right). Alkaline agarose gel electrophoresis of the products of synthesis with several DNA polymerases on Hae III-3-primed φX174 DNA.** The reaction mixtures (50 μl) contained 0.05 pmol of Hae III-3-primed φX174 DNA and 3.5 units of vaccinia polymerase (I), 0.5 unit of T4 DNA polymerase (II), or 0.5 unit of E. coli DNA polymerase I (III). Incubations were for 30 min.
a heterogeneous population of molecules slightly greater in size than full-length φX174 strands (see closed arrow on Fig. 2A). It is unlikely that these molecules are complete copies of the φX174 template, because they are often detectable after short incubations before some fragments of intermediate length are detected. Instead, it is likely that these molecules derive from the few template circles in each preparation which are randomly broken. These broken molecules could form hairpin template-primers (17), and the products synthesized would comprise a heterogeneous population of molecules slightly larger than a full-length strand of φX174 DNA.

Comparison with T4 DNA Polymerase and E. coli DNA Polymerase I—The barriers to polymerization by vaccinia polymerase could be regions of secondary structure on the template, or they could result from a unique interaction between the enzyme and specific sequences on the φX174 DNA. If they are regions of secondary structure, then the same barriers might be observed with another polymerase, such as that induced by bacteriophage T4, which cannot strand-displace (18) and therefore might be sensitive to secondary structure. The products of synthesis on φX174 primed with Hae III-3 were analyzed by alkaline agarose gel electrophoresis. The products of synthesis by the vaccinia polymerase (Fig. 3, Track I) are identical in size to those synthesized by the T4 enzyme (Fig. 3, Track II), indicating that the barriers are intrinsic properties of the template sequence rather than of the polymerase. In contrast to the vaccinia and T4 polymerases, the products synthesized by E. coli DNA polymerase I form a smear on the gel (Track III), indicating that this enzyme is insensitive to the barriers. The only discrete band observed is that corresponding to full-length φX174 molecules.

Effect of Temperature—Further support for the hypothesis that the barriers to polymerization are regions of secondary structure came from a study of the effect of incubation temperature on polymerization. The alkaline agarose gel in Fig. 4 shows that at low temperature, existing barriers become stronger and new barriers appear. The spacing between bands in the third track (10 min at 27°C) indicates that at 27°C the enzyme encounters a barrier on the average of every 100 nucleotides.

Identification of the Major Barriers on the φX174 Template—The experiments presented so far indicate that the barriers to polymerization may be regions of secondary structure on the φX174 template. From the location of the primer terminus, and from the sizes of the products of synthesis, it was possible to map the location of the barriers. We hoped that an inspection of the nucleotide sequences in the region of the barriers might reveal their nature. The location of the barriers detected with several primers are compiled, with reference to the genetic map of φX174, in Fig. 5. This map is derived from all bands detected after 10- and 60-min incubations. Since in all cases some bands were found at both times, it is unlikely that we have missed barriers which appeared transiently between 10 and 60 min. An estimation of the strength of barriers is complicated by the fact that the amount of radioactivity in a band varies as a function of both time and the distance between the barrier and the primer terminus. Taking these factors into consideration, we have designated the barriers in Fig. 5 as either strong (arrows) or weak (lines).

Although there are a number of minor barriers, some of which were not detected with every restriction fragment primer, there are three major barriers which show up as strong bands with nearly every primer. Barrier A is near nucleotide 4000 (near the junction of genes H and A), Barrier B is near nucleotide 3000 (within gene H), and Barrier C is near nucleotide 2000 (between genes F and G). Barrier C is by far the

Fig. 4. Effect of temperature on products of the reaction with Hae III-4-primed φX174 DNA. Incubation mixtures (50 μl) contained 0.05 pmol of Hae III-4-primed φX174 DNA and 3.5 units of vaccinia DNA polymerase. Incubation was at the indicated temperature for the indicated times. The products of the reaction were fractionated on a 1.4% alkaline agarose gel. The lane marked “std” contains a Hpa I digest of T7 [32P]DNA. The arrow indicates the position of the primer. Analysis of the product synthesized in 10 min at 37°C indicated that the smallest product detected contained about 2500 nucleotides (Fig. 3).

FIG. 5. Map of barriers on φX174 DNA. The location of each barrier was determined from the size of products of synthesis shown in Figs. 2 and 7A. Solid bars indicate the position of the primer; the 3′ terminus is at the left. Arrows denote the positions of strong barriers, and lines refer to weak barriers. The genetic map of φX174 is redrawn from published maps (3, 19). See text for discussions of the accuracy of this map and of the assignment of the strength of a barrier.
sequences to emphasize these "hairpin" regions. The numbers refer to the position of the indicated nucleotide relative to the single Pst I cleavage site. The vertical arrow in C indicates the position of the Hha I cleavage site in the complementary strand. The horizontal arrow indicates the position of the major stopping point during synthesis from the Hha I-3 primer (see Fig. 7).

The nucleotide sequence of φX174 DNA in the regions of the three major barriers are shown in Fig. 6. Each of these regions contains a sequence of nucleotides with the potential to form extensive secondary structure; we have drawn the sequences to emphasize these "hairpin" regions.

**The Precise Location of the Major Barrier on φX174 DNA**—The accuracy of the map of barriers in Fig. 5 was limited by the fact that the alkaline agarose gel system did not have resolution sufficient to determine the exact size of the products. To establish the exact location of the major barrier (Barrier C), and to learn whether the polymerase halted at a unique nucleotide or at one of several nucleotides in the region of the barrier, we analyzed the products of polymerization on a gel of much higher resolution. The primer used was Hha I-3, the 3' terminus of which is very close to Barrier C (see map in Fig. 5 and sequence in Fig. 6). As shown by alkaline agarose gel electrophoresis (Fig. 7A), the major product of synthesis, even after a 60-min incubation, was a fragment only slightly longer than the primer. To characterize this product more fully, it was cleaved with EndoR-Hha I, liberating a small radioactive fragment corresponding to the sequence between the primer terminus and Barrier C. This small fragment was then electrophoresed together with standards, on a 20% polyacrylamide gel in the presence of 7 M urea (Fig. 7B). This gel system, which separates DNA species which differ in length by only a single nucleotide (5), revealed that the major component appears to be 16 nucleotides long. A minor component, containing less than 10% of the radioactivity, appears to be 17 nucleotides long. Therefore, the vaccinia polymerase polymerizes about 16 nucleotides onto the Hha I-3 primer until its progress is impeded by Barrier C. Most of the polymerase molecules stop at this unique site, but a few may proceed one nucleotide further. There is no evidence for a gradual increase in product size as the enzyme proceeds further into the barrier region. Inspection of the φX174 sequence shows that the barrier to polymerization is located within the hairpin region (see Fig. 6).

**Discussion**

To study the action of vaccinia DNA polymerase on a long single-stranded DNA template, we have used as a substrate φX174 DNA primed with a strand of unique restriction enzyme fragments. Alkaline agarose gel electrophoresis of the products of synthesis revealed that they consist of a small number of species of discrete size. This finding, which is similar to Sherman and Gefter's observation (2) on E. coli DNA polymerase II, indicates that the polymerase is hindered by barriers at specific sites on the template. The enzyme polymerizes nucleotides very rapidly between the barriers, but pauses at the barriers. The barriers are not absolute, but only serve to delay the progress of the enzyme. Some barriers are more effective than others. In spite of a number of barriers on the template (about 15 are detected at 37°C; see map in Fig. 5), some polymerase molecules are able to traverse the entire template sequence. The barriers are a unique property of the template sequence, since the same barriers are detected in experiments with different primers (Fig. 5) and also in experiments using on a gel of much higher resolution. The primer used was Hha I-3, the 3' terminus of which is very close to Barrier C (see map in Fig. 5 and sequence in Fig. 6). As shown by alkaline agarose gel electrophoresis (Fig. 7A), the major product of synthesis, even after a 60-min incubation, was a fragment only slightly longer than the primer. To characterize this product more fully, it was cleaved with EndoR-Hha I, liberating a small radioactive fragment corresponding to the sequence between the primer terminus and Barrier C. This small fragment was then electrophoresed together with standards, on a 20% polyacrylamide gel in the presence of 7 M urea (Fig. 7B). This gel system, which separates DNA species which differ in length by only a single nucleotide (5), revealed that the major component appears to be 16 nucleotides long. A minor component, containing less than 10% of the radioactivity, appears to be 17 nucleotides long. Therefore, the vaccinia polymerase polymerizes about 16 nucleotides onto the Hha I-3 primer until its progress is impeded by Barrier C. Most of the polymerase molecules stop at this unique site, but a few may proceed one nucleotide further. There is no evidence for a gradual increase in product size as the enzyme proceeds further into the barrier region. Inspection of the φX174 sequence shows that the barrier to polymerization is located within the hairpin region (see Fig. 6).

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ments with either vaccinia polymerase or T4 polymerase (Fig. 3, Tracks I and II).

Several observations indicate that some, if not all, barriers are regions of template secondary structure. First, the strength of some barriers which are present at 27°C increases at 15°C, and other barriers not present at 27°C appear at 15°C. Second, E. coli DNA polymerase I, an enzyme which is able to utilize a duplex template by strand-displacement (18), is not affected by the barriers (Fig. 3, Track III). Finally, the major barriers (designated A, B, and C in Figs. 5 and 6) are in regions which contain hairpin structures of relatively high stability. In the case of Barrier C, the major barrier in φX174 DNA, the stopping point for the polymerase is located within the stem of the hairpin (Fig. 7, see below for further discussion).

There are four regions of the φX174 DNA sequence which contain extensive hairpin structures (19), and three of these appeared as the major barriers in our experiments (Fig. 6). Barrier A, near nucleotide 4000, is between genes H and A. Barrier B, near nucleotide 3000, is within gene H, and Barrier C, near nucleotide 2300, is between genes F and G. There are two hairpins in the region of Barrier C. The remaining hairpin, near nucleotide 1000 and between genes J and F, was not unambiguously detected as a major barrier to vaccinia polymerase, but that most of the polymerase molecules had synthesized past this position after 10 minutes of incubation, our earliest time sample. The hairpins between genes A and H and between genes F and G are sufficiently stable to resist digestion by single strand-specific nucleases (19, 20).

We determined the precise stopping point of the polymerase at Barrier C by using as primer a strand of Hha I-3. The 3' terminus of this primer is within the small hairpin in Barrier C and is within 20 nucleotides of the first base pair of the stem of the larger hairpin (see location of primer terminus in Fig. 6C). Analysis of the products of synthesis by alkaline agarose gel electrophoresis indicated that even after 60 min only a few nucleotides had been added to most of the primer molecules (Fig. 7A). To determine the exact stopping point of the polymerase, the newly synthesized DNA was cleaved from the primer by digestion with EndoR-Hha I, and its size was determined by electrophoresis on a 20% polyacrylamide gel. Most of these fragments appeared to be 16 nucleotides in length, but a small fraction of them were one nucleotide longer (Fig. 7B). Therefore, the major stopping point for the polymerase, indicated by the horizontal arrow in Fig. 6C, is unexpected, because it resides within the stem of the hairpin. Three base pairs must have been disrupted to reach this point, and yet only two base pairs ahead of the stopping point must have prevented the enzyme from proceeding further. By similar arguments, the minor stopping point, 17 nucleotides from the primer terminus, is even more puzzling.

We cannot explain the apparent stopping points for the polymerase within Barrier C. It is possible that the DNA does not exist in a simple hairpin secondary structure as shown in Fig. 6, but that instead it assumes a more complicated tertiary structure analogous to that found in tRNA. Only when this tertiary structure is known would it be possible to interpret its effect on the polymerase. It is interesting to note that the QB replicase was recently reported to pause at hairpin sequences in MDV-1 RNA, and in this case also the precise stopping points could not be explained by a simple inspection of the nucleotide sequence (21). However, there are several possible reasons why our data on the precise location of the stopping point could be misleading. First, the standards used to establish the exact size of the small fragments were the dyes bromphenol blue and Xylene cyanol FF (5), with the spacing between fragments differing by one nucleotide determined by a partial DNase I digest of [32P]T7 DNA. These indirect standards could result in an incorrect size determination. Second, it is possible that due to mutation our phage DNA contained a sequence in the region of Barrier C which was different from that of the DNA used by Sanger and co-workers for sequence determination (3). Examination of the corresponding sequence in G4 DNA reveals a hairpin of completely different sequence, indicating that this region of the DNA evolves rapidly (22). Furthermore, since our φX174 virus had not been cloned recently, the 16- and 17-nucleotide fragments may conceivably derive from templates of different sequence. At present we are investigating these possibilities. Nevertheless, these experimental results indicate that the vaccinia polymerase stops near or within the stem of a hairpin structure, at least in the case of Barrier C.

It is of interest to determine how the polymerase molecules penetrate the barriers. The most likely explanation is that the barriers occasionally melt and their sequences then are quickly copied by the polymerase. However, it is also possible that a polymerase molecule occasionally gets through a barrier by bypassing the sequence in the hairpin. This bypass reaction could be related to "strand-switching" during displacement synthesis by E. coli DNA polymerase I (23) or by T7 DNA polymerase (24). We are currently investigating this possibility. We are also studying the precise location of stopping points at other barriers in the φX174 DNA template. This investigation should not only reveal new information about the mechanism of action of DNA of polymerases, but should also provide an effective way of studying the secondary structure of φX174 DNA.

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