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Visualization of an inverted terminal repetition in vaccinia virus DNA

(poxvirus DNA/single-stranded DNA circle/electron microscopy/restriction endonuclease)

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ABSTRACT **An inverted terminal repetition was observed in DNA molecules extracted from vaccinia virus. The repeated sequence was visualized by (*i*) nicking the hairpin loops present at the ends of vaccinia virus DNA, (*ii*) separating the strands of DNA by alkali denaturation, (*iii*) allowing the single strands to self-anneal, and (*iv*) examining the DNA with an electron microscope. Single-stranded circular molecules, each of which contained a duplex projection (3.54 ± 0.12 μm) representing the terminal repetition, readily formed. Similar size projections were also seen in heteroduplex structures formed by crosshybridization of the separated strands of the two terminal *Hin*dIII restriction fragments. Based on contour length measurements and the electrophoretic mobility of the isolated inverted terminal repetition, a molecular weight of approximately 6.9×10^6, equivalent to about 10,500 nucleotide base pairs, was estimated. Evidence was obtained from DNA·RNA hybridization studies that the terminal repetition is transcribed.**

The genome of vaccinia virus appears, in the electron microscope, as a typical linear duplex DNA molecule of approximately 122×10^6 daltons (1). Upon complete denaturation, however, single-stranded circles measuring twice the length of the linear duplex are observed (1). The latter finding, together with the failure of the complementary strands to separate upon sedimentation through an alkaline sucrose gradient (1), indicate that the ends of the DNA duplex form a continuous hairpin loop or are crosslinked in some unknown manner. A similar genome structure has been found in molluscum contagiosum virus (2) and may be characteristic of all poxviruses.

Partial denaturation mapping of vaccinia virus DNA indicated that the base composition at the two ends of the molecule is similar (1, 2). Observations that restriction fragments from opposite ends of rabbitpox virus DNA crosshybridize (3) and that similar size restriction fragments are generated from both ends of the vaccinia virus genome (R. Wittek, personal communication) suggested the presence of a terminal repetition in poxvirus DNA. By using a single-strand-specific endonuclease (4) to cleave the hairpin loops at the two ends of the vaccinia virus genome (1) and then self-annealing the separated strands of DNA, we now provide direct visual evidence for an inverted terminal repetition approximately 10,500 nucleotide base pairs long. Evidence that at least a portion of the repeated sequence is transcribed will also be presented.

MATERIALS AND METHODS

Preparation of Vaccinia Virus DNA. HeLa S-3 cells were infected with cloned vaccinia virus (strain WR) and virions were purified without sonication as described (5). DNA was extracted as described (6) except that digestion with 1 mg of proteinase K (EM Laboratories Inc., Elmsford, NY) per ml for 5 hr was followed by repeated phenol extraction and dialysis against 50 mM NaCl/50 mM Tris·HCl, pH 8.0/1 mM EDTA for 48 hr. The terminal crosslinks of vaccinia virus DNA were removed by digestion with a single-strand-specific DNase isolated from vaccinia virions (1, 4). Reaction mixtures contained approximately 1 μg of DNA, 0.1 M Tris·HCl (pH 7.9), 2.5 mM EDTA, and 10 μl of glycerol-gradient-purified DNase in a final volume of 0.05 ml. Incubations were for 3 hr at 50°. Removal of crosslinks was checked by alkaline sucrose gradient centrifugation and by electron microscopy after partial denaturation. By the latter method we estimated that 85% of the ends of the DNA molecules were nicked without significant cleavage at internal sites.

Purification of Restriction Fragments. Approximately 2 μg of unlabeled vaccinia virus DNA was mixed with tracer amounts of [^{3}H]thymidine-labeled DNA and digested with 6 units of *Hin*dIII restriction endonuclease (Bethesda Research Laboratories, Bethesda, MD) in 60 mM NaCl/7 mM Tris·HCl, pH 7.4/7 mM $MgCl_2$ at 37° for 4 hr. The digest was layered directly on a 5–30% linear sucrose gradient in 100 mM NaCl/50 mM Tris·HCl, pH 7.6/1 mM EDTA and centrifuged for 18 hr at 23,000 rev per min in an SW41 rotor at 10°. Fractions (0.24 ml) were collected by piercing the bottom of the tube, and a small portion of each was used for determination of radioactivity in a scintillation spectrophotometer. The peak fractions containing fragments B and C were dialyzed for 6 hr as described above and then concentrated 5-fold by application of dry Sephadex G-200 to the outside of the dialysis membrane. The purity of the DNA fragments was checked by agarose gel electrophoresis as described below.

Electron Microscopy. Terminally nicked duplex DNA molecules or purified terminal restriction fragments were denatured with 0.1 M NaOH at room temperature for 10 min. After neutralization, the separated DNA strands were reannealed in 50% (vol/vol) formamide/0.1 M Tris·HCl, pH 8.5/1 mM EDTA for 2 hr at 34°. The DNA was mounted for microscopy by the formamide procedure essentially as described by Davis *et al.* (7). Grids were examined in a Siemens Elmiskop 101 at 40 kV accelerating voltage. Electron micrographs were taken on Kodak Electron Image Plates at magnifications of 4000–8000. Magnification was calibrated with a grating replica (E. F. Fullam, cat. no. 1000), and contour lengths were measured with a Numonics Graphic calculator interfaced to a Wang 2200 computer.

Hybridization of RNA to DNA Fragments. The heteroduplex mixture obtained by annealing the separated strands of *Hin*dIII fragments B and C was dialyzed to remove formamide and digested with single-strand-specific DNase as described above. After phenol extraction and ethanol precipitation, the duplex fragments were analyzed by electrophoresis in a 0.6% agarose slab gel. The DNA was transferred to a nitrocellulose sheet by the method of Southern (8) and hybridized at 68° for 48 hr in 0.9 M NaCl/0.09 M sodium citrate to cytoplasmic RNAs labeled with [^{3}H]uridine from 0 to 2 hr and 5 to

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7 hr after vaccinia virus infection. The RNA had been purified by CsCl centrifugation to eliminate DNA (9). The nonhybridized RNA was removed by digestion with RNase A (50 μg/ml) and RNase T1 (2.5 μg/ml) at 37° for 30 min and extensive washing. After drying, the nitrocellulose sheet was impregnated with 20% (wt/vol) 2,5-diphenyloxazole in toluene and fluorographed with Kodak XR-2 x-ray film.

RESULTS

Formation of Single-Stranded Circular DNA. The strategy used to visualize the inverted terminal repetition in vaccinia virus DNA is outlined in Fig. 1. Essentially, the procedure consisted of (*i*) selectively denaturing and nicking the hairpin loops at the ends of the DNA molecule with a single-strand-specific DNase isolated from purified vaccinia virions, (*ii*) separating the strands of DNA by alkali denaturation, and (*iii*) allowing the single strands to self-anneal at a relatively low DNA concentration in 50% formamide. By this procedure, single-stranded circular molecules readily formed (Fig. 2). A projection, which appeared double-stranded, was clearly visible on every circle observed in these preparations. The duplex nature of the projection was demonstrated by partial denaturation, which separated the ends of the duplex to reveal two strands (not shown). In addition, when the aqueous mounting technique (7) was used to visualize the DNA molecules, the single-stranded circles collapsed into "bush-like" structures, whereas the contour of the double-stranded projections remained unaltered. After long hybridization times, some complex structures clearly formed by annealing of two single-stranded circles were also seen, providing evidence that both strands of DNA contain an inverted terminal repetition.

Accurate quantitation of the percentage of circular structures was difficult because the large DNA molecules were subject to tangling upon mounting for microscopy. In addition, shear damage to the DNA, failure to nick some terminal hairpins, nuclease damage, and reannealing to form intermolecular duplexes would all serve to reduce the circle-forming potential of the DNA. Nevertheless, up to 40% circular forms could be observed in some preparations, leading us to believe that most if not all viral genomes contain a terminal repetition.

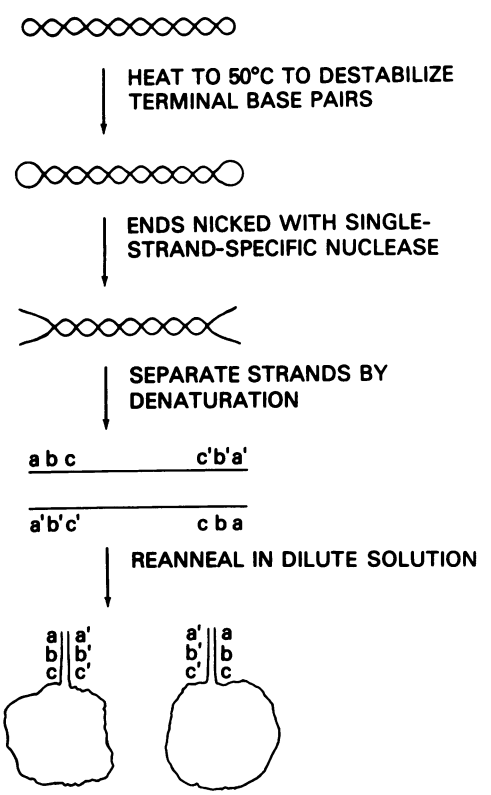

FIG. 1. Formation of single-stranded circular molecules from vaccinia virus DNA and the possible arrangement of terminal nucleotide sequences. Complementary sequences are designated by primed letters.

Terminal Location of Repeated Sequence. If the duplex projections observed on every single-stranded circle formed as a result of hybridization of an inverted terminal repetition, then a similar interaction would be expected to occur upon crosshybridization of separated strands obtained from the two terminal restriction fragments. *Hin*dIII restriction endonuclease cleaves vaccinia virus (strain WR) DNA into 15 fragments (10, 11). The second and third largest fragments are derived from the two ends of the DNA (F. DeFilippes, personal communication; and ref. 11). We also found that fragments B and C, with molecular weights determined by contour length of approximately 17.8×10^6 and 13.4×10^6, respectively, contain the crosslinked ends of vaccinia virus DNA by their ability to rapidly renature and chromatograph as duplex structures on benzoylnapthoyl-DEAE cellulose (12).

In order to form heteroduplex molecules, purified *Hin*dIII fragments B and C were first treated with single-strand-specific DNase to remove the crosslinks, then mixed together in equimolar ratios and denatured with alkali to separate the strands. After reannealing, three types of duplex structures were identified by electron microscopy. Two of these were simply the reformed homoduplex fragments B and C. The third type of molecule, accounting for up to 30% of the total, were Y-shaped heteroduplex structures with single-stranded arms of dissimilar length (Fig. 3). The end of the molecule, opposite the single-stranded fork, was shown to be double stranded by partial denaturation.

Contour Length of Terminal Repetition. Contour lengths of *Hin*dIII terminal fragment heteroduplex projections (e.g., Fig. 3) were measured and compared with duplex projections seen on single-stranded circular molecules (e.g., Fig. 2). Histograms of the results are presented in Fig. 4. Contour length measurements gave a mean value (±SEM) of 3.54 ± 0.12 μm for duplex projections on intact single-stranded circles and a similar mean value of 3.49 ± 0.12 μm for the duplex projections associated with branched heteroduplex molecules. These values represent molecular weights of 6.94×10^6 and 6.84×10^6, respectively.

Transcription of Inverted Terminal Repetition. The inverted terminal repetition was isolated by agarose gel electrophoresis after single-strand-specific DNase digestion of the mixture containing the *Hin*dIII B-C heteroduplex as well as the reannealed homoduplex molecules. Three bands with molecular weights corresponding to fragment B, fragment C, and the inverted terminal repetition (6.8×10^6) were visualized by ethidium bromide staining. The latter molecular weight, determined by comparison with the electrophoretic mobilities of *Eco*RI digests of λ DNA, agreed with the value determined from contour length measurements. After ethidium bromide staining, the DNA fragments were transferred from the gel to a nitrocellulose sheet by the blotting procedure of Southern (8) and then allowed to hybridize with a mixture of ^{3}H-labeled cytoplasmic RNAs obtained at early and late times after infection. The fluorograph (Fig. 5) indicated that the inverted terminal repetition as well as fragments B and C are transcribed.

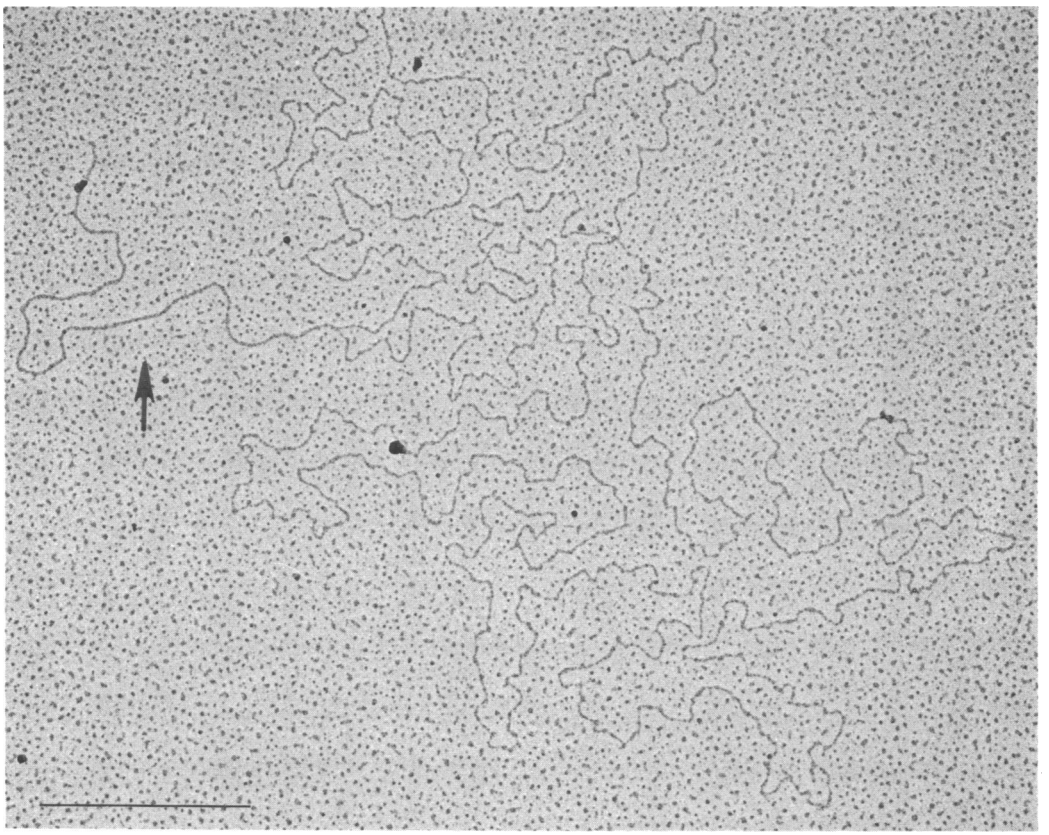

FIG. 2. Electron micrograph of a single-stranded circular vaccinia virus DNA molecule formed after terminally nicked duplex molecules were denatured and renatured at a concentration of less than 1 μg/ml. DNA was mounted for electron microscopy by the formamide technique and was rotary shadowed with platinum/palladium. Bar represents 1 μm. Arrow points to the duplex projection extending from the single-stranded circle.

DISCUSSION

An inverted terminal repetition in vaccinia virus DNA was visualized by self-annealing of the separated DNA strands. Single-stranded circles containing a duplex projection representing the terminal repetition were seen. The key technical problem involved removing the crosslinks at the ends of the duplex DNA, without otherwise damaging the molecule, so that intact DNA strands could be separated. A single-strand-specific DNase was used for this purpose, as described by Geshelin and Berns (1). Further evidence for the inverted terminal repetition

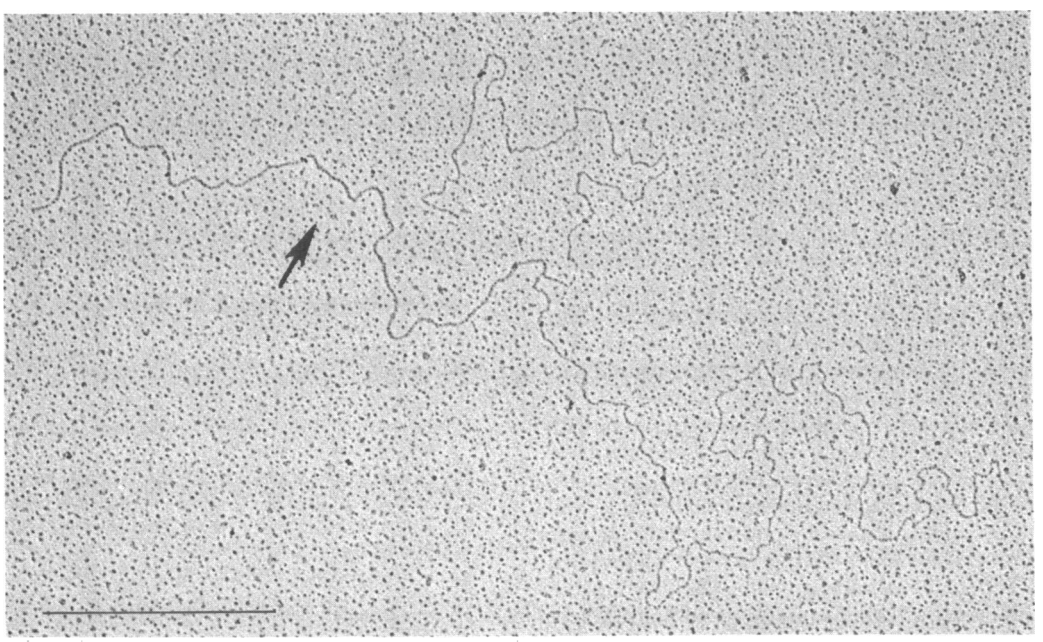

FIG. 3. Heteroduplex molecule formed by mixing equimolar amounts of terminally nicked *Hin*dIII fragments B and C followed by denaturation and then renaturation in 50% (vol/vol) formamide. Bar represents 1 μm. Formamide spread. In this molecule, the duplex projection indicated by the arrow measured 3.59 μm and the single-stranded arms measured 7.03 and 3.64 μm.

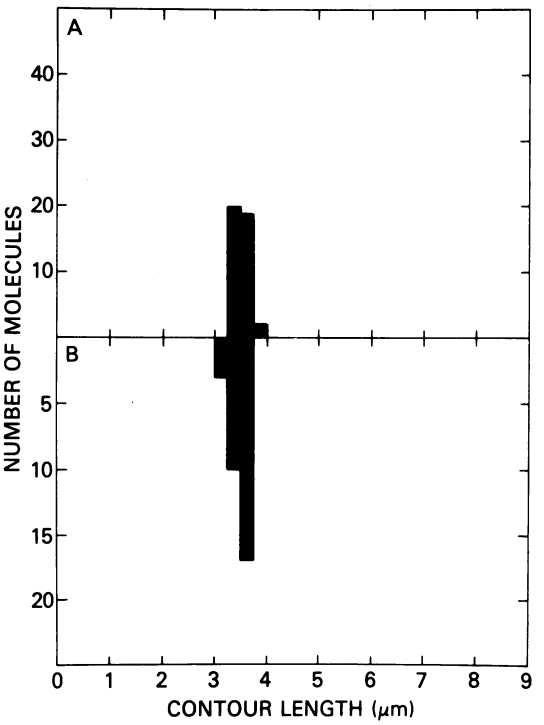

FIG. 4. Contour length measurements of (*A*) duplex projections visible on intact single-stranded circular molecules and (*B*) duplex projections on *Hin*dIII fragment B·C heteroduplex molecules.

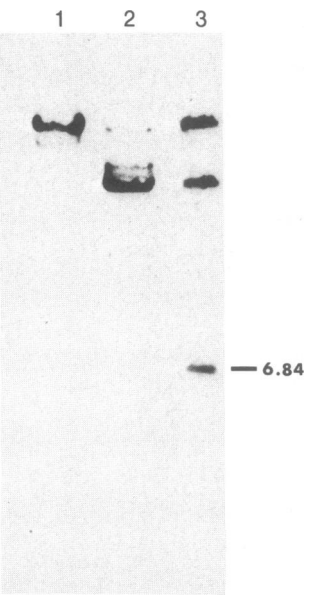

FIG. 5. Fluorogram showing hybridization of ^{3}H-labeled RNA to DNA fragments separated by 0.6% agarose gel electrophoresis and transferred to nitrocellulose sheets. RNA was a mixture obtained by labeling cells from 0 to 2 hr and from 5 to 7 hr after infection. *1*, Purified *Hin*dIII B fragment; *2*, purified *Hin*dIII C fragment; *3*, products formed by reannealing the separated strands of *Hin*dIII B and C fragments and then treating with single-strand-specific DNase. Number on the right is the molecular weight ($\times 10^{-6}$) of the inverted terminal repetition determined by coelectrophoresis of an *Eco*RI digest of λ DNA.

came from the visualization of heteroduplex structures formed by crosshybridization of the separated strands from terminal *Hin*dIII restriction fragments.

Inverted terminal repetitions have previously been found in the DNA of adenovirus (13, 14), adenovirus-associated virus (15, 16), and herpesvirus (17), suggesting an important role, perhaps in DNA replication. The repeated sequences range in size from 140 base pairs in adenovirus serotype 2 (18) to 6000 base pairs in adenovirus type 18 (19). Based on contour length measurements and electrophoretic mobility in agarose gels, we estimated that the terminal repetition in vaccinia virus (strain WR) DNA is approximately 10,500 nucleotide base pairs or 6.9 $\times 10^6$ daltons. Added together, the homologous sequences at the two ends of the DNA comprise more than 11% of the genome. From restriction endonuclease analysis, Wittek and coworkers have obtained evidence for a similar size terminal repetition in DNA from the Lister strain of vaccinia virus (personal communication).

The absence of visible areas of strand mismatch in the vast majority of heteroduplex structures examined and the apparent resistance of the heteroduplex to single-strand-specific endonuclease suggest close sequence homology at the two ends of the DNA molecule. That some sequence variation does occur within the inverted terminal repetition, however, was indicated by the detection of small deletion loops within terminal heteroduplex structures of DNA obtained from uncloned vaccinia virus (our unpublished results). This variation is consistent with the observation that the terminal restriction endonuclease fragments of vaccinia or rabbitpox virus DNA do not give sharp bands on agarose gel electrophoresis unless cloned virus is used (3, 11). In addition, a comparison of restriction endonuclease sites of vaccinia virus DNA and rabbitpox virus DNA revealed differences primarily in the terminal segments (3). The methods used in the present report should be useful for visualization of heteroduplex structures formed from the separated strands of DNA from different poxviruses.

In experiments in which terminally nicked, full-length DNA molecules or *Hin*dIII restriction fragments B or C were separately denatured and briefly reannealed, inverted internal repetitions or long internal palindromic sequences were not observed. More detailed analysis under a variety of conditions is necessary, however, as our experiments do not rule out short palindromes or other repeated sequence rearrangements that could account for the presence of reiterated DNA detected by the analysis of reassociation kinetics (20, 21).

The finding that the inverted terminal repeat sequence is transcribed suggests that some proteins may be encoded in this region.

Note Added in Proof. Evidence that the length heterogeneity of vaccinia virus DNA is eliminated by cloning the virus (22) and that both vaccinia virus and rabbitpox virus contain inverted terminal repeat sequences (23) has been obtained by Wittek and coworkers.

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