LENGTH HETEROGENEITY IN THE DNA OF VACCINIA VIRUS IS ELIMINATED ON CLONING THE VIRUS

Riccardo WITTEK, Heinz K. MÜLLER, Antonio MENNA and Robert WYLER
Institut für Virologie der Universität Zürich, Winterthurerstrasse 266a, CH-8057, Zürich

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1. Introduction

The genome of vaccinia virus is a linear double-stranded DNA molecule with mol. wt $122 \pm 2.2 \times 10^6$ [1] which is naturally crosslinked at or near the termini [1,2]. Despite its large size, it has recently been possible to establish physical maps of the Elstree strain of vaccinia virus for the bacterial restriction endonucleases *Hin*dIII and *Sst*I [3]. Their results showed that the terminal restriction fragments did not migrate as distinct bands in gel electrophoresis and it was suggested that this was due to length heterogeneity in the DNA population.

In this communication we show that on cloning the virus these heterogeneous fragments form distinct bands in agarose gels. Cleavage of the DNA with *Sst*I, *Eco*RI, *Xho*I and *Kpn*I produces end-fragments of identical length of mol. wt 7.55, 7.0, 5.3 and 4.3×10^6, respectively, from both ends of the genome. This finding suggests a terminal location of the repeated sequences found by the analysis of DNA reassociation kinetics [4,5].

2. Materials and methods

2.1. *Virus growth*

Vaccinia virus (strain Elstree) from a commercially available smallpox vaccine was titrated on the chorioallantoic membrane of developing chick embryos. After 3 days incubation at 37°C the membranes containing 1–5 pocks were harvested. Single pocks were cut out, disrupted by ultrasonic treatment and the resulting suspension again titrated on chorioallantoic membranes. This procedure was repeated 3 times. After this, 5 eggs were inoculated with the homogenate of a single pock lesion to give a stock for further virus multiplication. The virus clone used in this study thus is a 6th passage on chick embryos.

The uncloned virus, grown from the same smallpox vaccine, was the third passage on the chorioallantoic membrane.

2.2. *Virus purification and isolation of DNA*

Virions were purified as in [6]. The DNA was isolated by treatment of the virus suspension with proteinase K in the presence of 0.5% SDS and 27% sucrose followed by extraction with phenol–chloroform (1:1).

2.3. *Restriction enzymes*

*Eco*RI was purchased from Boehringer, Mannheim, *Kpn*I and *Xho*I from New England Bio Labs, Beverly, MA. *Hin*dIII was isolated from *Haemophilus influenzae* R_d by conventional procedures and finally purified by chromatography on heparin–agarose [7]. *Sst*I was isolated from *Streptomyces* stanford from a high-speed supernatant of sonicated cells which was treated with streptomycin sulfate. The supernatant was passed through a DEAE-cellulose column and the enzyme finally purified by chromatography on heparin–agarose [7].

2.4. *Restriction conditions*

Digestions of DNA with restriction enzymes were performed in the buffers listed below (final conc.). *Sst*I reaction mixtures were incubated at 30°C, all others at 37°C.

*Hin*dIII: 50 mM Tris–HCl, pH 7.8, 7 mM $MgCl_2$

*Sst*I: 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 10 mM 2-mercaptoethanol

*Eco*RI: 100 mM Tris–HCl, pH 7.3, 10 mM $MgCl_2$

*Kpn*I: 6 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, 6 mM NaCl, 6 mM 2-mercaptoethanol, 100 μg/ml bovine serum albumin

*Xho*I: 6 mM Tris–HCl, pH 7.4, 6 mM $MgCl_2$, 150 mM NaCl, 6 mM 2-mercaptoethanol, 100 μg/ml bovine serum albumin

2.5. *Gel electrophoresis*

Electrophoresis in agarose slab gels was as in [3].

2.6. *Purification of DNA fragments*

DNA bands were excised from the gels and the agarose slices squeezed through a 22 gauge needle into at least 10 vol. 50 mM Tris–HCl, pH 8, 0.15 M NaCl, 10 mM EDTA and the tubes shaken gently for about 72 h at room temp. The agarose was then pelleted at 48 000 × *g* for 60 min and the supernatant was loaded on a small (approx. 0.5 ml bed vol.) DE 52 column equilibrated with the buffer described above, washed and the DNA eluted with 1 ml 2 M NaCl. The solution was diluted and the DNA precipitated with ethanol.

2.7. *Determination of end-fragments*

The ends of vaccinia virus DNA are known to be naturally crosslinked [1,2] and to be easily renatured experimentally [8]. Terminal restriction fragments were enriched and identified as follows: Vaccinia virus DNA was digested with *Sst*I, *Eco*RI, *Xho*I and *Kpn*I and the resulting DNA fragments were precipitated with ethanol after phenol–chloroform extraction. DNA fragments were denatured with 0.1 M NaOH for 15 min at 37°C. The solution was neutralized by addition of an equivalent amount of HCl in Tris–HCl, pH 7.6. The samples were analyzed on 0.6% agarose gel in parallel with an untreated corresponding digest as a reference.

3. Results

3.1. *Restriction analysis of the DNA from cloned and uncloned vaccinia virus*

The DNA from cloned and uncloned vaccinia virus was digested with restriction endonucleases *Hin*dIII, *Sst*I, *Eco*RI, *Xho*I and *Kpn*I and the samples run in parallel on a 0.6% agarose gel (fig.1). *Hin*dIII fragments B and G have been shown to be the end-fragments on the restriction map [3]. Fragment G (mol. wt 8.05×10^6 [3]) appears heterogeneous in the DNA of the uncloned virus. This is not obvious for fragment B. However, only relatively large differences in molecular weight can be expected to be resolved in this range (vaccinia *Hin*dIII fragment B, mol. wt 17.1×10^6 [3]). In the DNA of the cloned vaccinia virus, *Hin*dIII fragment G migrates as a distinct band. A similar phenomenon can be observed in the *Sst*I digests. The complex of the fragments *Sst*I G/G′/H (mol. wt $7.68–7.50 \times 10^6$) is known to contain both terminal fragments of the restriction map [3]. Cloning of the virus results in the appearance of one distinct band at the position of the G/G′/H complex. Digestions of vaccinia DNA with the restriction enzymes *Eco*RI, *Xho*I and *Kpn*I also result in the appearance of diffused bands in each restriction pattern of the DNA of the uncloned virus.

The diffused bands are not present in any of the digestions of the DNA from the cloned virus but are seemingly replaced by distinct bands in the same position of the gel. In analogy to digestions with *Hin*dIII and *Sst*I it may, therefore, be assumed that fragments *Eco*RI C (mol. wt 7.0×10^6), *Xho*I F (mol. wt 5.3×10^6) and *Kpn*I F (mol. wt 4.3×10^6) represent end-fragments.

Determination of end-fragments was performed to prove this assumption.

3.2. *Determination of end-fragments*

The genome of vaccinia virus is known to contain a crosslink at both ends [1,2]. This characteristic feature can be used to identify the terminal restriction fragment, as the fragments containing the crosslinks rapidly reform duplexes after denaturation with alkali when the pH is returned to neutrality [3,8]. Figure 2 shows the result of such an analysis.

The complex of the *Sst*I fragments G/G′/H, known to contain both end-fragments in the DNA of the

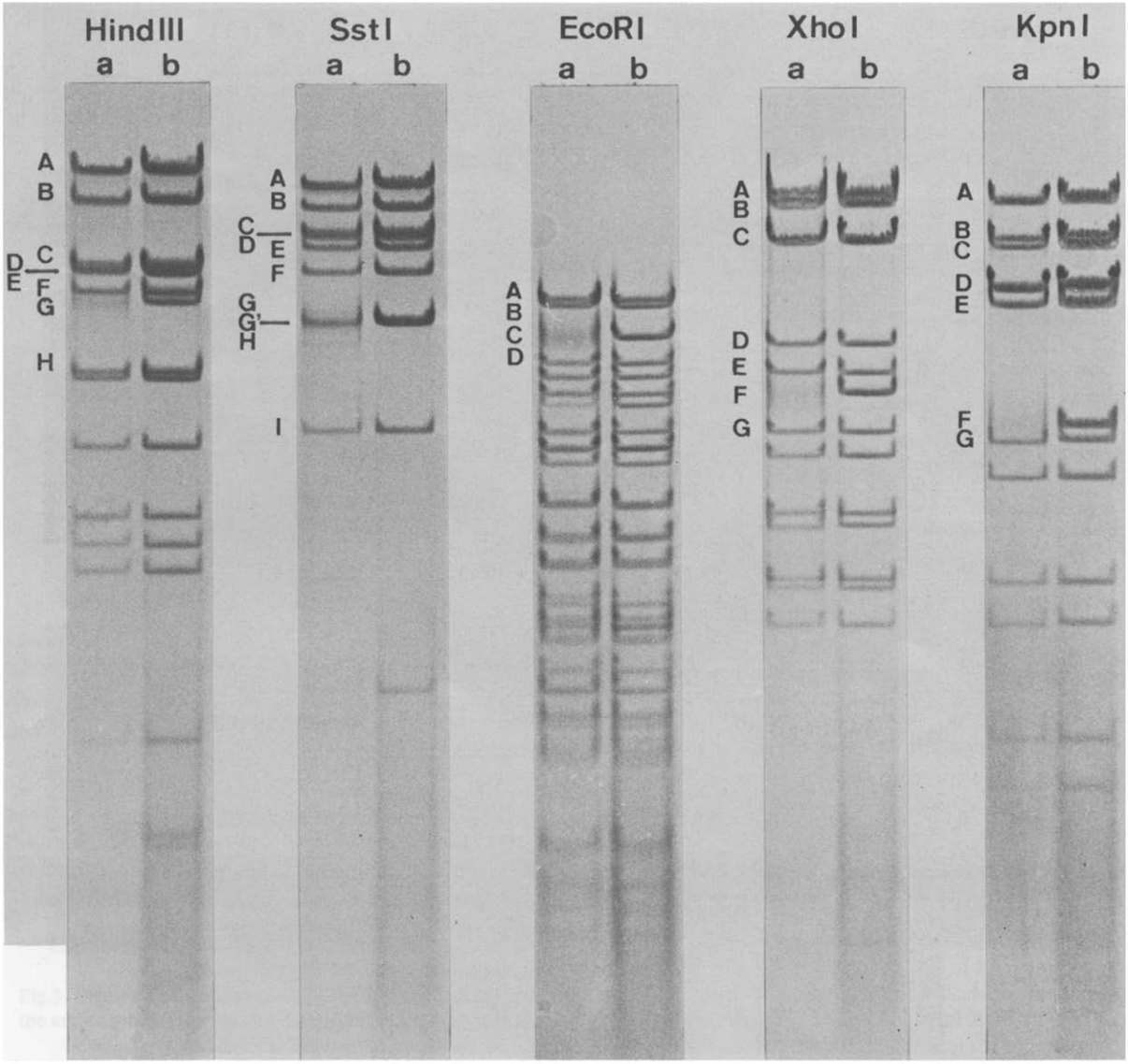

Fig.1. DNA cleavage patterns of uncloned (a) and cloned (b) vaccinia virus. Electrophoresis was in 17 cm, 0.6% agarose gels at 30 V for 17 h.

uncloned virus, also contains the crosslinked fragments in the DNA of the cloned virus as these are clearly enriched by the denaturation–renaturation procedure. The same holds true for the fragments *Eco*RI C, *Xho*I F and *Kpn*I F. Therefore, these fragments also contain the crosslink and represent end-fragments as hypothesized above.

Interestingly, the distinct bands (fig.1) replacing the diffused ones upon cloning of the virus appear to occur in more than one molar amount in the *Sst*I, *Eco*RI, *Xho*I and *Kpn*I restriction patterns as judged by their higher fluorescence after staining with ethidium bromide. This view is supported by the fact that only these fragments are clearly enriched by the denaturation–renaturation experiment. If *Sst*I, *Eco*RI, *Xho*I and *Kpn*I cleave identical fragments

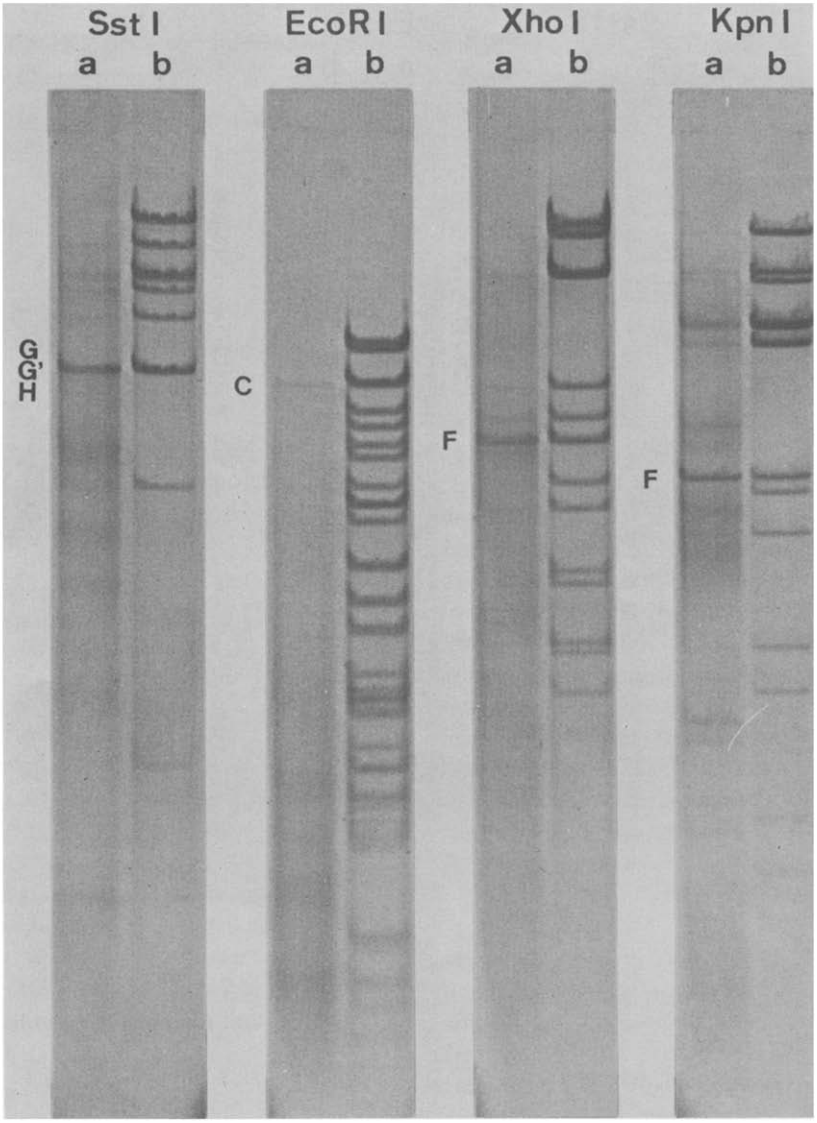

Fig.2. End-fragment determination in the DNA of cloned vaccinia virus. The lettered bands indicate the positions of the cross-linked fragments enriched by the denaturation-renaturation procedure. (a) Denatured-renatured digests; (b) untreated total digests. Electrophoresis was in 17 cm, 0.6% agarose gels at 30 V for 17 h.

from both ends of the genome, one expects digestion of each *Hin*dIII end-fragment (B and G) with these enzymes to yield a cleavage product comigrating with the end-fragments in the total digest. This is confirmed by the experiment presented in fig.3. Length heterogeneity in the DNA molecules of the uncloned virus, therefore, results from length variation of both terminal regions.

4. Discussion

In this communication we have analyzed the DNA of cloned and uncloned vaccinia virus (strain Elstree) with the restriction endonucleases *Hin*dIII, *Sst*I, *Eco*RI, *Xho*I and *Kpn*I. This study has shown that the terminal regions of the genome are subject to considerable length variation. The biological implica-

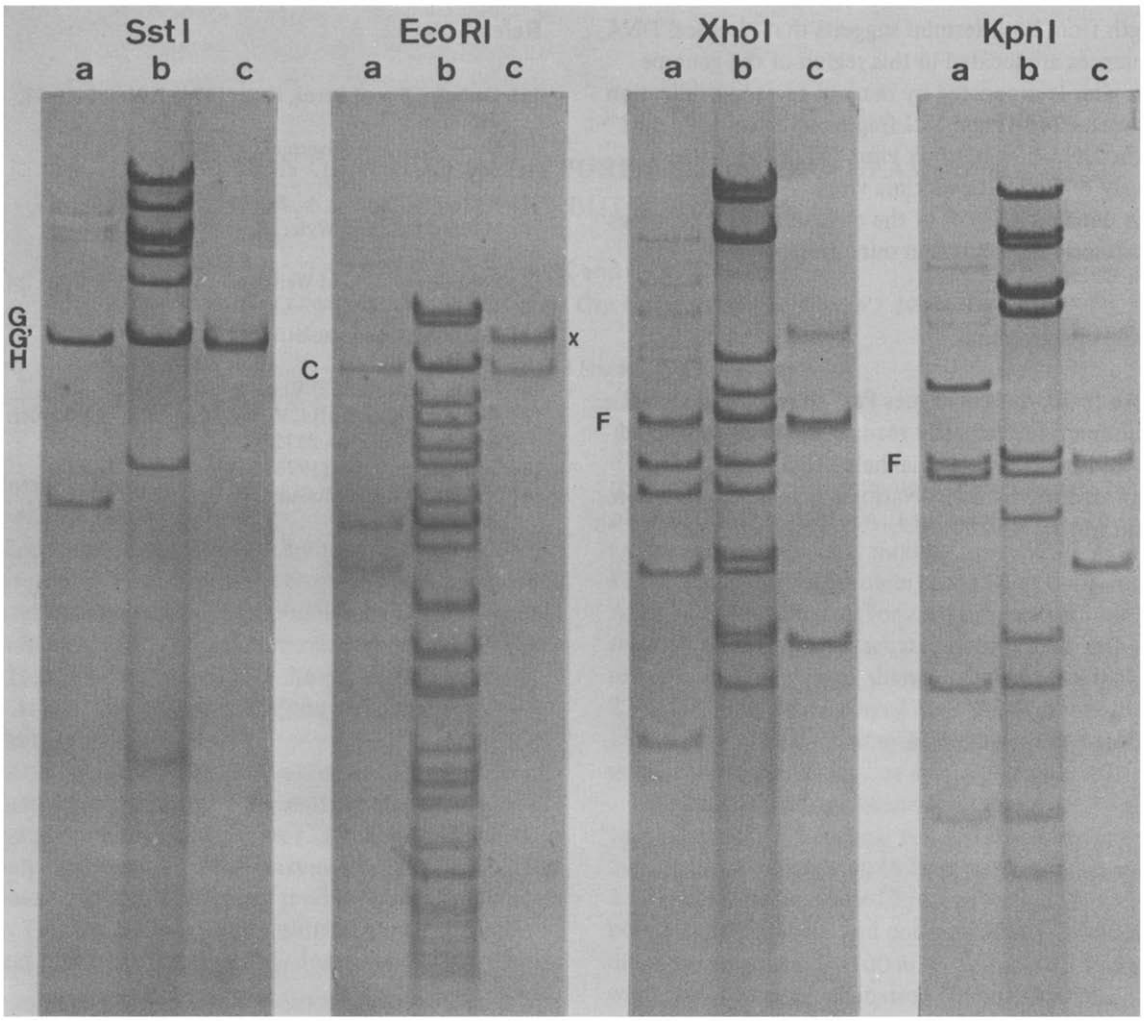

Fig.3. Digestion of isolated *Hin*dIII end-fragments B (a) and G (c). (b) Total digests. The lettered bands indicate the positions of the end-fragments. (x) Undigested *Hin*dIII end-fragment G. Electrophoresis was in a 17 cm, 0.6% agarose gel at 30 V for 17 h.

tion of these findings is not clear, but since physical properties such as sedimentation velocity and buoyant density can vary considerably within a whole population of vaccinia virus particles [9] it is not surprising that also heterogeneity at the level of the genetic material is found. Comparison of the physical maps of vaccinia virus and rabbitpox virus DNA [3] as well as cleavage patterns of the DNAs of several vaccinia virus strains (L. Archard, personal communication; H. K. M., unpublished) show that an internal genome section of approx. 60% is highly conserved whereas the differences found are due to sequence divergence in the terminal regions. Moreover, it was found that the genomes of vaccinia virus and rabbitpox virus differ in length by about 6×10^6 daltons [3]. This difference can also be located at the ends. The length heterogeneity in the DNA of the uncloned vaccinia virus may, therefore, be the result of a high tendency of these regions to evolve.

Repeated sequences in vaccinia virus DNA have been reported [4,5]. The fact that *Sst*I, *Eco*RI, *Xho*I and *Kpn*I produce end-fragments of identical

length from both termini suggests that identical DNA sequences are located in this region of the genome. This view is supported by marked cross-hybridization between *Hin*dIII and *Sst*I fragments from both ends of the DNA of rabbitpox virus [3], a species very closely related with vaccinia virus.

A detailed analysis of the ends of these 2 genomes is currently in progress in our laboratory.

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