CLEAVAGE OF VACCINIA VIRUS DNA BY RESTRICTION ENDONUCLEASE *Bal* I, *Eco* R1, *Bam* HI

Isolation of the natural cross-links

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1. Introduction

Although vaccinia virus was one of the first animal viruses to be purified [1] the anatomy of its double-stranded DNA is not very well defined, partially because of its large size ($122 \pm 2 \times 10^6$ daltons).

The utilisation of restriction endonucleases for the mapping of small DNA molecules such as Simian virus 40, polyoma, lambda and adenovirus [2–6] to determine the initiation and termination sites of DNA replication [7,8] and the location of missing segments in defective DNA molecules [9] is now very common. Similar studies with larger DNA molecules were attempted with Herpes viruses [10] but so far very little has been done with pox viruses.

Previous work has demonstrated that the genome of vaccinia virus corresponds to a single, linear, duplex DNA molecule [11–13] whose complementary strands are naturally crosslinked at or near the two ends [14]. Its molecular weight, determined by contour length measurements from electron micrographs, is $122 \pm 2 \times 10^6$ daltons [15].

Berns and Geshelin [15] have reported the results of studies on the crosslinks contained in vaccinia virus DNA, which indicate that there are two such cross-links per molecule such that the complementary strands are joined by short segments of single-stranded DNA.

Using a different experimental procedure, DeFilippes [16] has recently shown that after shearing of vaccinia virus DNA, denaturation neutralization and digestion by *Hpa* I or *Hpa* II, fragments can be isolated corresponding to rapidly reformed duplex chains.

It appeared interesting to try to identify the fragments at the ends of the DNA molecule. We present data showing that vaccinia virus DNA is specifically cleaved into a limited number of fragments by the restriction endonucleases *Eco* R1, *Bam* HI or *Bal* I, and we also describe the isolation of the fragments which rapidly reform 'snap-backs' after denaturation and neutralization, and removal of single-strand fragments by the nuclease S_1.

2. Materials and methods

2.1. *Vaccinia virus growth and purification*

Two strains of vaccinia viruses were used: the WR strain and the Lister strain.

Kindly supplied by J. Kates (Stony-Brook), strain WR was prepared as follows: HeLa cells were grown as suspension cultures in Eagle's minimal essential medium with 5% calf serum. Vaccinia virus was grown in these HeLa cells in a medium containing 5% calf serum [17], this medium contained in addition 0.2 μCi [^{14}C]thymidine/ml (New England Nuclear Corp.) when labelled DNA was required. The virus was purified by the method of Joklik [18].

The Lister strain was grown on monolayer cultures of KB cells in conditions described previously [19].

2.2. *DNA purification*

Vaccinia DNA was purified using a new procedure developed by Parkhust et al. [20] for the isolation of intact DNA genomes from purified vaccinia virions. The 280 nm to 260 nm ratio was 0.50 to 0.55 for all the DNA preparations used.

The [^{14}C]DNA had spec. act. 5×10^4 cpm/μg. Radioactivity was counted in an Intertechnique liquid scintillation counter in Bray's scintillation fluid with the addition of soluene when the samples contained agarose.

2.3. *Cleavage by restriction endonucleases*

Purified preparations of endonucleases such as:

Bal I from *Brevibacterium albidum*,
SMA from *Serracia Marcesens*,
Kpn I from *Klebsellia pneumoniae* 0K8,
Blg I from *Bacillus globiggi*,
Sal I from *Streptomyces albus* G,
Hpa I from *Haemophilus parainfluenzae*,
Hind III from *Haemophilus influenzae* RD, were a generous gift of R. Robert (Cold Spring Harbor).
Sac I from *Streptomyces achromogenes* was generously supplied by G. Roizes (Montpellier).
Eco R1 from *Escherichia coli* RY 13 and
Bam HI from *Bacillus amyloloquefaciens* H were purchased from New England Biolabs.

The digestion of the DNA (1 μg/50 μl assay) was performed at 37°C in the specific assay condition relative to each enzyme *Bal* I, *Bam* HI and *Eco* R1 the most frequently used enzymes in the work reported here.

Bal I: vaccinia virus DNA was cleaved by incubating overnight in a buffer containing 6 mM Tris–HCl, pH 7.9, 6 mM $MgCl_2$ and 6 mM 2-mercaptoethanol and 15 μl *Bal* I.

Bam HI: the DNA was incubated for 4 h with 5 μl *Bam* HI in the same buffer as used for digestion with *Bal* I except that the pH was 7.4 and that 100 μg/ml gelatin (Difco, autoclaved) were included.

Eco RI: cleavage of the DNA was performed for 4 h in a mixture containing 5 μl enzyme, 100 mM Tris–HCl, pH 7.5, 50 mM NaCl and 5 mM $MgCl_2$.

In one instance, a vaccinia DNA fragment (band A, fig.2: about 1 μg) eluted from an agarose gel was digested by 5 μl of *Sac* I in the same buffer conditions as used for cleavage by *Eco* R1. Incubation was for 6 h.

In all cases the reaction was stopped by addition to an aliquot of 1/5 vol. solution containing 50% glycerol, 0.2 M EDTA, pH 7.5 and 0.12% bromophenol blue and the samples was analyzed by slab-gel electrophoresis. The remainder of the incubation mixture was

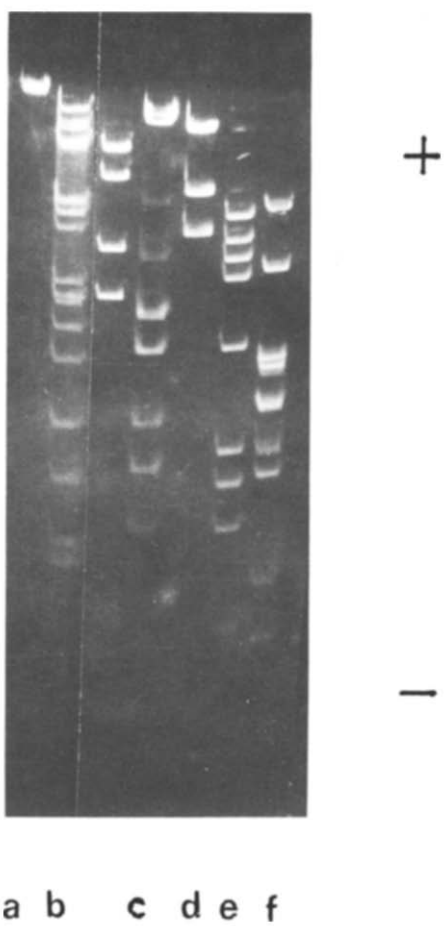

Fig.1. Electrophoresis of vaccinia DNA *Bal* I fragment and molecular weight markers on an 0.7% agarose gel. (a) Ad-2 DNA control; (b) vaccinia *Bal* I DNA digest; (c,d,e f) Ad-2 DNA digested by *Eco* R1, *Sal* I, *Kpn* I and *Hind* III, respectively.

brought to 0°C and further treated as indicated below.

The DNA fragments generated by the digestion of either adenovirus-2 (Ad-2) DNA (with *Eco* R1, *Sal* I, *Hind* III or *Kpn* I) or in certain cases bacteriophage γ DNA (with *Eco* R1) were used as molecular weight markers in the gel electrophoresis. The molecular weights of the Adenovirus-2 DNA fragments were obtained from M. Mathews (personal communication). The molecular weight of each fragment of vaccinia virus DNA digested by *Bal* I, *Bam* HI or *Eco* R1 was determined by comparing the migration profiles with the previously characterized Ad-2 or λ DNA fragments, respectively (fig.1). Ad-2 DNA was a gift of M. Mathews and λ DNA of P. Yot.

2.4. *DNA denaturation and neutralization*

The vaccinia virus DNA fragments were denatured at room temperature for 15 min in a 0.2 N NaOH solution, then neutralized by the addition of an equal vol. solution containing 0.2 N HCl in Tris–HCl, pH 7.5. An aliquot was removed, the reaction stopped by addition of bromophenol blue mixture (see above) and analyzed by gel electrophoresis. The remainder of the incubation mixture was further digested by nuclease S_1.

2.5. *Nuclease S_1 digestion*

Nuclease S_1 isolated from *Aspergillus oryzae* and purified on sulfo-Sephadex according to Vogt [21]

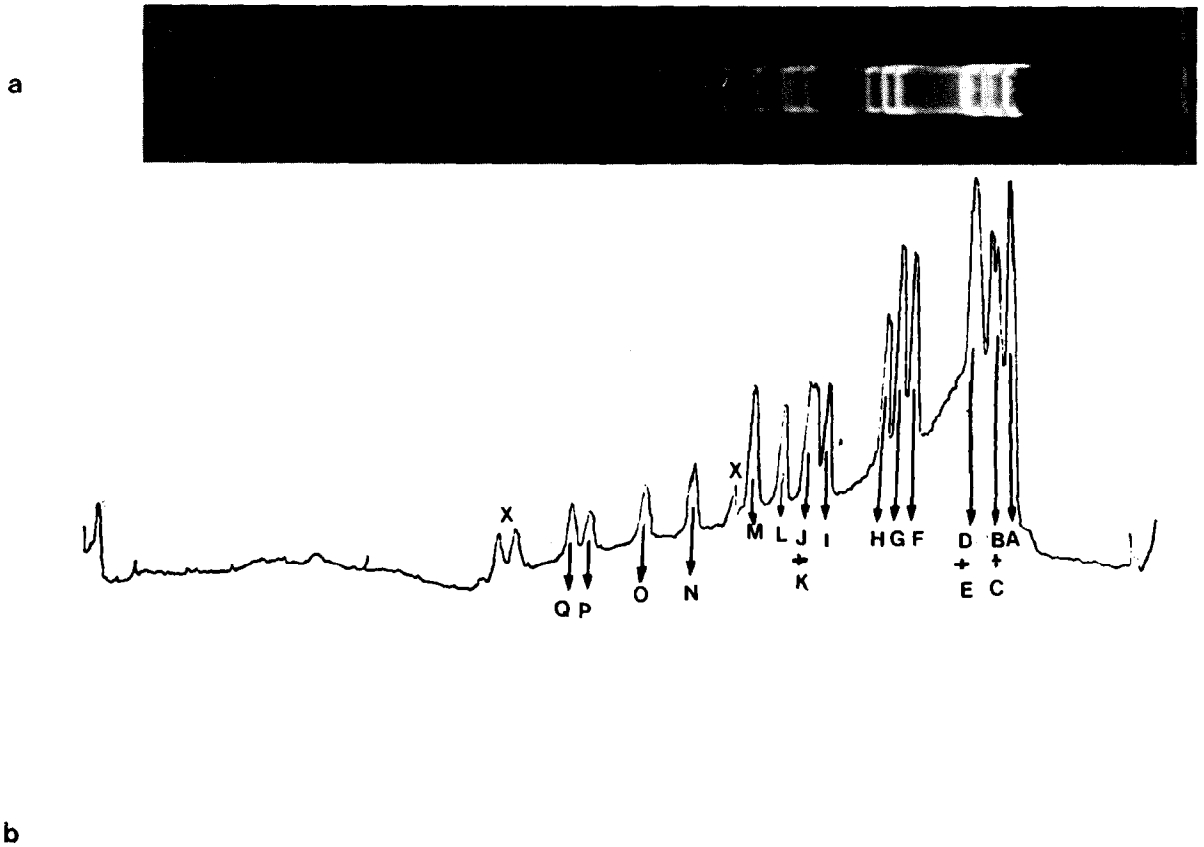

Fig.2. Electrophoresis of vaccinia DNA fragments produced after digestion by *Bal* I. (a) Electrophoresis pattern of 1 μg *Bal* I DNA digest obtained as described in Materials and methods. The agarose gel (0.7%) was run at 60 V for 3.5 h at room temperature. The gel was stained and photographed. (b) Corresponding scan. Peaks indicated by 'X' are artefacts of scanning which were not reproduced in other scans of the same gel.

was kindly provided by P. Nardeu (Villejuif).

The DNA (10 μg) digested by *Eco* R1 or *Bam* HI denaturated and neutralized was incubated at 45°C for 30 min in 30 mM Na acetate, pH 4.6, 250 mM NaCl, 5 mM $ZnSO_4$ with 20 units enzyme. The reaction was stopped by addition of the bromophenol blue mixture and analyzed by gel electrophoresis.

2.6. *Gel electrophoresis*

The fragments of digested DNA were fractionated by slab-gel electrophoresis using two kinds of gel. The first consisted in a composite of 1.8% acrylamide and 0.5% agarose with a pH 8 buffer, containing 0.04 M Tris-base, 0.02 M Na-acetate and 2 mM EDTA; on such a gel (16 X 40 X 0.18 cm) the DNA fragments were electrophoresed for 12 h at 80 V. The other kind used with the smaller (16 X 14 X 0.5 cm) slabs consisted in 0.7% or 0.4% agarose in the above buffer containing in addition 0.18 M NaCl; electrophoresis was at 60 V for 3.5 h. The digests were detected by soaking the gel in ethidium bromide (5 μg/ml), visualizing with a long-wavelength ultraviolet lamp, and then photographing with a polaroid camera. The negative photograph of the gels were scanned with a Joyce-Loeb apparatus.

3. Results and discussion

3.1. *Cleavage of vaccinia virus DNA by restriction endonucleases*

3.1.1. *Bal* I

Vaccinia virus DNA (WR strain) was incubated with endonuclease *Bal* I and the digested DNA fractionated by electrophoresis through a 0.7% agarose slab gel. The bands were located by ethidium bromide staining (fig.2). At least, 17 distinct bands appeared (A,B,C. . . Q). No fragment smaller than the last visible fragment (Q) of fig.2 could be detected in more concentrated (1%) agarose gels and no change in the pattern of the bands was observed with increasing enzyme concentration or as a result of longer incubation times.

Estimates of the molecular weights of the DNA fragments were determined by comparing the migration distances of the fragments with those appearing in parallel Ad-2 DNA cleaved by *Eco* R1 and *Kpn* I (table 1).

Table 1
Molecular weights and relative amounts of the vaccinia DNA *Bal* I fragments separated in a 0.7% agrose gel

Electrophoresis band	mol. wt (× 10^6)	Relative amounts (%)
A	15.4	25.3
B	11.5	23.5
C	11.3	
D	9.4	22.8
E	9.2	
F	5.4	4.4
G	5.1	5.3
H	4.5	3.7
I	3.0	3.1
J	2.8	2.8
K	2.6	
L	2.4	1.6
M	2.1	3.3
N	1.6	1.1
O	1.3	0.8
P	1.0	1.1
Q	0.92	1.1
	Total 116.4	

The lettering of the electrophoresis bands is the one adopted in fig.2. The molecular weights were determined by comparing the migration profiles of Ad-2 DNA cleaved *Eco* R1 or *Kpn* I (fig.1). The relative amounts were obtained by surface measurements of the corresponding scan

To determine the relative amounts of the fragments, vaccinia virus DNA fragments resulting from a cleavage by *Bal* I were separated on a 0.7% agarose gel (fig.2). After scanning of the stained gel the surface of each peak was measured with a planimeter (table 1). Band A apparently consists of two fragments of the same molecular weights (see below) as do also bands D or E, and M and Q, whereas the other bands represent unique fragments. To define the composition of band A, it was eluted from the gel essentially as described by Thuring et al. [22] and digested by *Sac* I. The analysis of the digest by gel electrophoresis revealed four fragments (fig.3). The summing of these pieces gives a total mol. wt 15.2 X 10^6 suggesting that band A is a single fragment present two times in the genome.

Furthermore, the relative amounts of the bands were also determined by using [^{14}C]DNA cleaved by *Bal* I; the DNA fragments were separated by agarose (0.4%) gel electrophoresis, the bands excised from the gel and the radioactivity in each band determined.

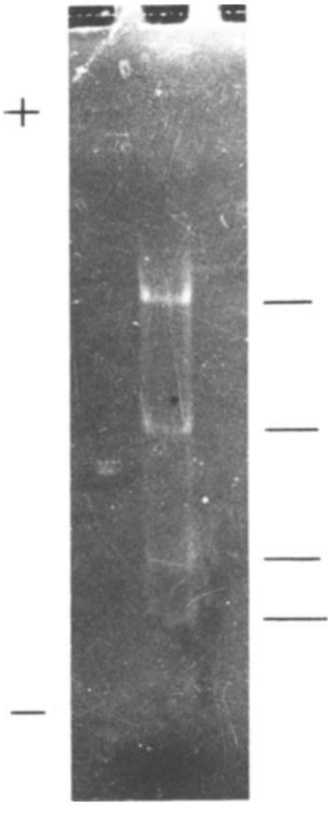

Fig.3. Electrophoresis of band A fragments after digestion by *Sac* I.

The results obtained by this method were in good agreement with those presented above (using stained DNA) with respect to fragments A through L, but concerning the bands of lower mol. wt (M–Q) a certain discrepency was denoted; the labelled DNA method suggests that band M represents one fragment, whereas band O represents two and band Q three fragments. In these conditions the molecular weights of the fragments add up to 117.4×10^6 daltons; however, the molecular weight obtained by surface measuring was 116.4×10^6 daltons which is quite in accordance with the value determined by Geshelin and Berns [15] by electron microscopy.

The fact that fragment A appears to be homogenous with respect to the *Sac* I restriction sites it contains, and that it is present in twice the relative amount expected (table 1) suggests that the vaccinia virus genome may be redundant for a region of genetic sequences at least, as large as fragment A (15.4×10^6 daltons). This possibility is currently under investigation.

3.1.2. *Eco* R1

Vaccinia virus DNA extracted in the same conditions from either the Lister of the WR strain was digested by *Eco* R1, and was analyzed by electrophoresis through a 1.8% polyacrylamide 0.5% agarose slab gel (fig.4). Twenty-five fragments can be visualized after ethidium bromide staining, the largest having est. mol. wt 12×10^6.

If one compares the pattern of the *Eco* R1 digests

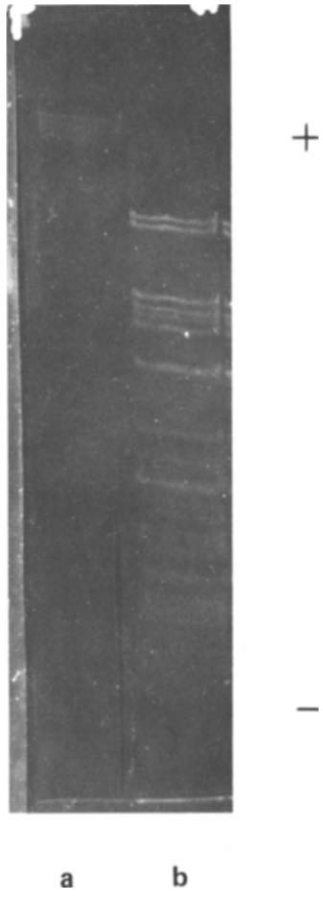

Fig.4. Electrophoresis of vaccinia DNA fragments produced after digestion by *Eco* R1. (a) Vaccinia DNA control (0.5 μg); (b) electrophoresis pattern of 1 μg *Eco* R1 digest in a 1.8% acrylamide and 0.5% agarose gel run at 80 V for 12 h at room temperature. Gels were stained and photographed as described in Materials and methods.

of the DNA of the two vaccinia virus strains, differences appear in the migration of certain fragments in the range 4.6–8 × 10^6 daltons although the total number of visible fragments is the same.

Using the WR strain (ATCC) of vaccinia virus, Gangemi and Sharp [23] obtained no specific fragmentation with *Eco* R1; it might be that the vaccinia virus DNA preparation used by these authors contained an inhibitor of the endonuclease; a less likely interpretation would be a difference in the strains used. Our results obtained with two different strains of vaccinia virus do reveal differences in the cleavage patterns of their DNAs but in both cases a large number of specific fragments were obtained.

3.1.3. *Bam* HI

When vaccinia virus DNA (WR strain) was cleaved by the endonuclease *Bam* HI and the fragments separated on a 0.7% agarose gel ethidium bromide staining revealed 25 fragments (fig.5A) whose molecular weights were determined using *Eco* R1 digested λ DNA fragments as marker.

3.1.4. Other restriction endonucleases

Cleavage of vaccinia virus DNA with the restriction

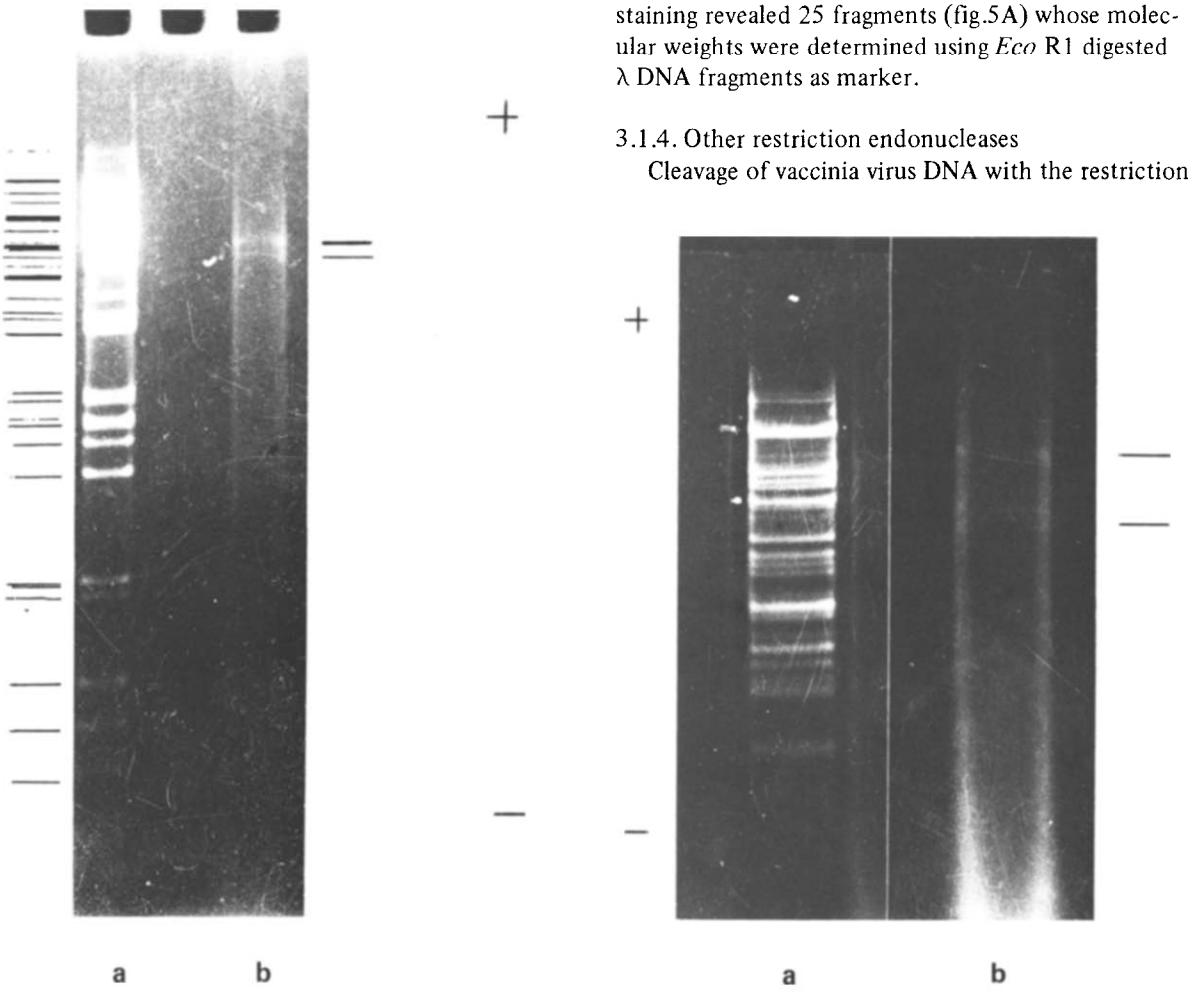

Fig.5A. Electrophoresis of vaccinia DNA fragments produced after digestion by *Bam* HI. (a) 0.7% agarose gel was run at 60 V for 3.5 h at room temperature. Electrophoresis pattern of 1 μg *Bam* HI DNA digest. (b) Electrophoresis of a vaccinia *Bam* HI DNA digest after denaturation, neutralization and treatment by nuclease S_1. The gel was stained and photographed as described in Materials and methods. Fig.5B. Electrophoresis of vaccinia DNA fragments produced after digestion by *Eco* R1. (a) 0.7% agarose gel was run at 60 V for 3.5 h at room temperature. (a) Electrophoresis of 1 μg *Eco* R1 DNA digest; (b) electrophoresis of a vaccinia *Eco* R1 DNA digest after denaturation, neutralization and treatment by nuclease S_1. The gel was stained and photographed as described in Materials and methods.

endonucleases *Blg* II, *Xba, Kpn* or *Hpa* I resulted in a large number of fragments that were difficult to resolve by gel electrophoresis (not shown here). On the other hand, cleavage by the endonuclease SMA did not lead to any visible fragmentation under conditions wherein Ad-2 DNA was cleaved.

3.2. *Isolation of cross-linked fragments*

The complementary strands of vaccinia virus DNA do not separate under denaturing alkaline conditions, indicating that covalent bonds link the two strands [14]. If the DNA is cleaved by a restriction endonuclease, the strands of most of the duplex fragments will separate after denaturation, and it should be possible to isolate the fragments which rapidly reform a 'snap-back' after neutralization of the denatured fragments.

To check this point the *Eco* R1 or the *Bam* HI digests were denatured by alkali and neutralized. The mixtures were treated by nuclease S_1 and analyzed by 0.7% agarose gel electrophoresis and stained.

Under these conditions only two reproducible crosslinked fragments appeared representing 4.3% and 4% of the genome, respectively, when the DNA had been cleaved by *Bam* HI (fig.5A). The position of these crosslinked fragments correspond to the position of two DNA fragments obtained by *Bam* HI digestion of native DNA. The recovery of the crosslinked end was 16.4% of the original material concerning the biggest band and 6.2% for the little one. The relative amounts were obtained by surface measurements of the corresponding scan.

In the case of cleavage by *Eco* R1 the crosslinked fragments represented 5.9% and 4% of the genome, respectively (fig.5B).

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