Mapping the Bend Loci on the Human Papillomavirus 16 Genome

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Abstract

Physical mapping of the bend loci in HPV 16 may lead to an improved understanding of the transcriptional regulation of this genome. Subcloning small fragments of HPV 16 into pUC18 and pCY7, followed by electrophoretic analysis of the clones, provided a way of determining the location of bend loci. Of the 35.7% of the genome analyzed for bending, three bends were located. One was localized to a region containing an E2 open reading frame. The bend's center was graphically determined to lie between 2850 and 2910 bp.

Introduction

It is apparent that the structure of double stranded DNA is not quite as uniform as Watson and Crick's early model of the double helix demonstrates. Many unique structural characteristics have been identified in the DNA of all organisms (1). One type of observed conformational alteration is referred to as "bent" DNA. Bent DNA is characterized by a positionally distinct curve along the DNA backbone (2). Identification of DNA fragments containing bends can be made on polyacrylamide gels under certain electrophoretic conditions. Fragments containing a bend travel through polyacrylamide at a rate slower than fragments lacking bends, apparently because bent fragments have increased difficulty traveling through gel pores (3).

The position of a bend in a DNA fragment can be determined quantitatively using the circular permutation assay developed by Wu and Crothers (2). This assay involves the cleavage of cloned tandem dimers of a fragment (or the cleavage of a fragment cloned into a tandem dimer), with different restriction enzymes which cut only once in each copy of the tandem sequence. The result is a series of fragments of identical length and nucleotide composition, but whose sequences are circularly permuted. Although these circularly permuted fragments are equal in length, they will not migrate identically if a bend is present. This is because digesting with different enzymes causes the bends to appear at different points along the fragment. When a bend is near an end of the fragment, it has a higher mobility and moves through the gel faster. After

digestion, if the bend lies in the center of the fragment, the fragment is most severely contorted and travels through the gel at a slower rate. A plot of the ratio of the apparent to actual mobilities versus the position of the restriction site in base pairs produces a sinusoidal curve from which the location of the bend can be extrapolated.

Bends are found in both prokaryotic and eukaryotic organisms (1). Because of their universality, it is important to understand their function. It is suspected that bend loci may play a role in the regulation of gene expression, initiation of DNA replication, DNA packaging, and the recognition of specific DNA sequences by proteins (4,1). For example, bends have frequently been localized to regions containing promotors and enhancers. In addition, bacteriophage λ, SV40, adenovirus, and several other viruses all are known to contain bends located near origins of replication (4). The deletion of bends from these regions reduces or eliminates the transcriptional activity of the element, suggesting a functional role in transcription. Bending apparently facilitates SV40 DNA replication by enhancing binding of the SV40 large T antigen to region 1 (5). Similarly, yeast autonomously replicating sequences (ARS1) also contain a bend determined to be a binding site for the protein factor, ABF1 (6). Others have proposed that one function of bends may be to help the DNA package itself into small spaces such as virus heads (3). Until more research is done on DNA bending, the number and type of different functions to which they contribute will remain unclear.

The papillomaviruses are members of the papova DNA virus family. These viruses infect only mucosal and epidermal epithelial

cells resulting in papillomas (warts). The genome of the human papillomavirus 16 (HPV 16) has been sequenced and determined to be 7904 base pairs in length (7). Humans are the only known animal hosts which support HPV infections. Different types of HPVs infect different anatomical locations (9), with HPV 16 being associated with cervical warts (10). In addition, there appears to be some correlation between cervical warts and cervical cancers. HPV 16 and the related strain HPV 18 have been found in 80% of all cervical cancers (7). The large percentage of these tumors containing HPV 16 or HPV 18 DNA, in contrast to the more common cervical viruses such as HPV 6 and HPV 11, suggests that the oncogenic potential varies among genital papilloma viruses.

Although HPVs have not been proven as a direct cause of anogenital cancers, understanding how HPV 16 transforms tumors may provide us with an enhanced understanding of the manifestations of cervical cancers. Normal genital epithelial cells utilize an intracellular control mechanism directed against HPV transcription (8). However, in genital carcinoma cells, cellular functions which control gene expression are absent. Changes of papillomavirus transcription may induce the formation of cancer.

Currently there is little known about the transcriptional regulation or replication of HPV 16. Research on bends in the bovine papillomavirus 1 (BPV 1) has shown that at least some bends are located near cis active elements (1). Since the genomes of HPV 16 and BPV 1 have very similar genetic organizations, identification of bend loci in HPV16 will help to pinpoint potentially important cis elements (see figure 1). Preliminary studies by two dimensional gel

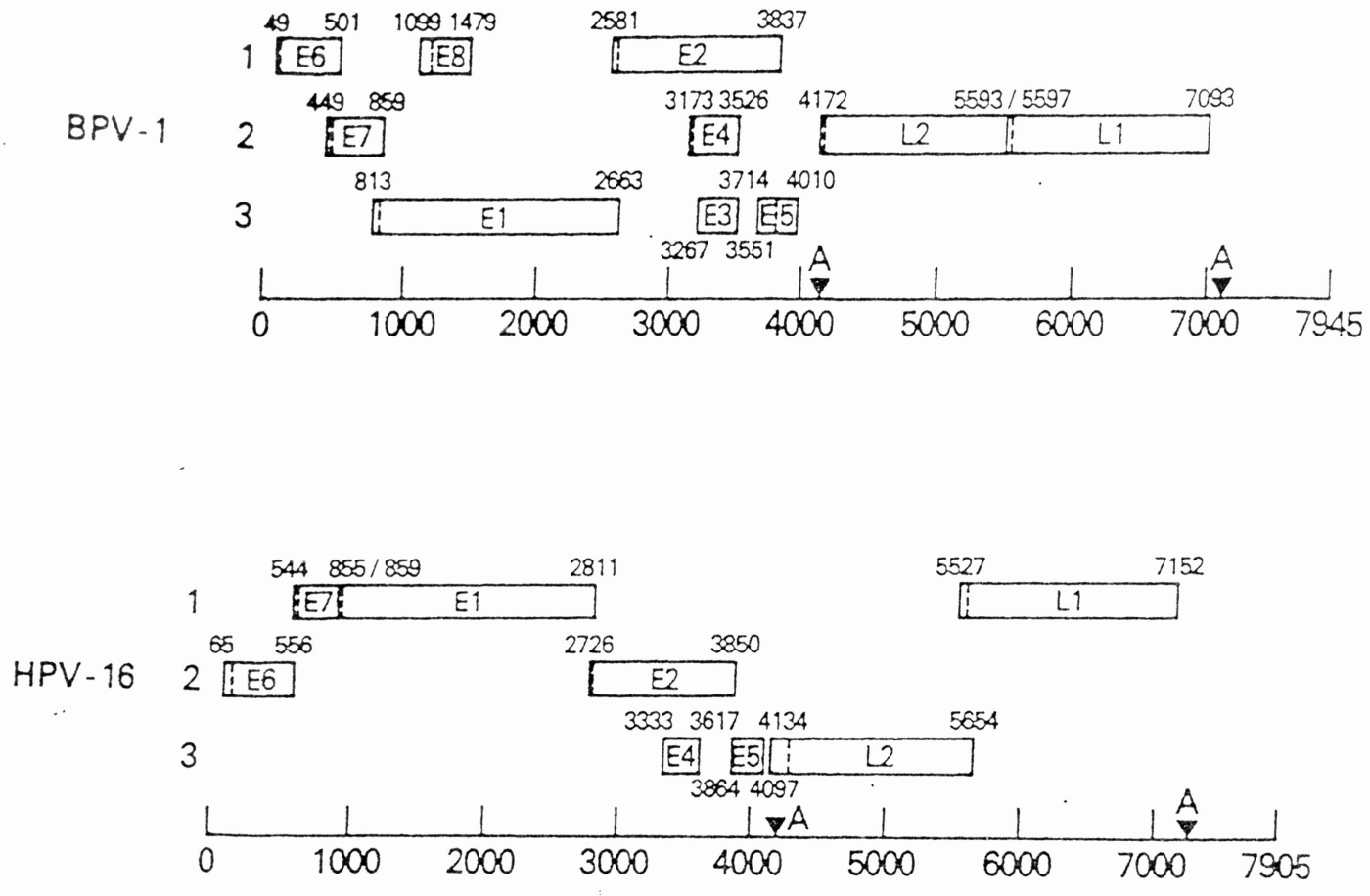


Figure 1. Comparison of genomic organization between BPV 1 and HPV 16

electrophoresis have revealed the presence of multiple bend loci in both BPV1 and HPV 16 genomes (5). Specific mapping of the location of the individual bend sites is required so that their role in transcription or replication can be studied.

The function of bends in cis active regions can be studied by making mutations directly on the bends and the sequences around them. However, determining the function of bends must be preceeded by determining their location. The experiments presented here have yielded a partial map of the bend loci in the HPV 16 genome. Knowledge of the number and location of bend sites will facilitate future attempts at understanding their functional significance.

Methods

Plasmids

Three plasmids were used during different stages of the project, pHPV16, pUC18, and pCY7. pHPV16 is the complete HPV 16 genome cloned into pBR322. DNA fragments from HPV 16 were subcloned initially into the vector pUC18. The pUC18 plasmid contains the *lacZ* gene which, when functional, causes colonies to appear blue in the presence of the sugar x-gal (5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside). Disruption of the *lacZ* gene by the presence of cloned inserts results in white colonies which facilitates identification of recombinants.

The vector selected for the circular permutation assays was pCY7. This vector contains a tandem dimer repeated by a polylinker sequence for insertion of the fragment to be studied. The tandems contain multiple restriction sites used to generate the circularly permuted fragments.

DNA Preparation

After harvesting the DNA from TB1 cells (using large-scale plasmid DNA preparations described in Maniatis), the DNA was purified by CsCl gradient centrifugation. The DNA was then dialyzed in TE buffer (10mM Tris-HCl and .1mM EDTA, pH 7.4), and quantified by UV fluorescence. The restriction enzyme BamHI separated HPV 16 from pBR322. After agarose gel purification, HPV 16 was electroeluted using the Schleicher and Schuell electrophoresis

chamber (Elutrap) and was ready for digestion with restriction enzymes.

Isolation of fragments

The appropriate enzymes required to generate fragments approximately 200 to 600 base pairs long were determined using sequencing data (refer to table 1). After digestion of the HPV16 genome with these enzymes, the desired fragments were isolated by agarose gel electrophoresis. The DNA was eluted from the agarose using Prep A Gene (Bio-Rad Laboratories). In some instances, low melting temperature Nusieve agarose (FMC Bioproducts) was used to avoid having to elute the DNA.

Cloning

Initially, the purified DNA fragments were cloned into pUC18 and transformed into TB1 cells. Only white colonies were suspected to contain inserts. Further screening was done by using the quickscreen method (using the procedure of Sekar). This method distinguishes potential recombinants from parental recombinants based on size differences observed on agarose gels. Clones were confirmed by restriction enzyme analysis of alkaline lysis miniprep DNA (as described in Maniatis).

Upon confirmation of recombination into pUC18, the DNA was then digested with two different restriction enzymes, SacI and BamHI. The liberated fragments were again gel purified by agarose gel electrophoresis and Prep A Gene. Purified fragments were cloned into the vector pCY7 which was linearized by digestion with

SacI and BglII. Ligations were transformed into HB101 cells and screened using quickscreen and miniprep DNA.

Analysis of bending

The fragments cloned into pUC18 were screened for bending by digestion with the enzyme PvuII which cut on both sides of the insert. This digestion liberated the original insert plus a total of 322 extra base pairs. The fragments were loaded on a 6% polyacrylamide gel using TBE sample buffer (90mM Tris-borate, pH 8.2, 2.5 mM EDTA, 10% glycerol, 1% SDS) and electrophoresed at 5-10 V/cm for 36 hrs. The gels were stained with ethidium bromide and photographed under ultraviolet light. Fragments migrating slower than expected were suspected to contain a bend.

Each pCY7 clone was digested with a series of restriction enzymes and electrophoresed on a 6% polyacrylamide gel at 4°C. The samples were loaded in TBE sample buffer and electrophoresed at 5-10 V/cm for 24 hrs. The gels were ethidium bromide stained and the DNA was photographed under ultraviolet light.

Apparent sizes of the fragments were determined using a 123bp fragment ladder (Bethesda Research Laboratories) as a marker. The K factors are defined as the ratio of the apparent size to the actual size of each fragment. The bend's center was determined graphically as described by Wu and Crothers (2). The y-axis lists the K-factors and the x-axis plots the relative position of the restriction digests. The minimum corresponded to the location of the bend's center and was determined by extrapolation.

Results

Isolation of fragments

The initial work on this project involved determining which enzymes should be used to generate fragments of DNA from the HPV16 genome. A size restriction was necessary. The goal of this project was to observe the migration of these fragments in polyacrylamide. If the fragments were too large, they would not be able to travel through polyacrylamide gels very effectively. Consequently, fragments were selected which ranged in size from about two hundred to six hundred base pairs (refer to table 1). The fragments selected covered the entire HPV 16 genome. To avoid overlooking any bends that may be localized at the end of a fragment, additional fragments which overlapped each pair of adjacent fragments were selected as well.

Twenty seven fragments were obtained using the enzymes AluI, SspI, and HaeIII. The fragments were separated according to size differences on agarose gels and excised for purification as described in Methods. These fragments accounted for 97% of the genome. The remaining 3% of the genome (218 bp), and four nonoverlapping regions have not yet been obtained.

Cloning into pUC18

The DNA fragments were purified and ligated into pUC18. Cloning into pUC was advantageous for several reasons. First, screening for recombinants was much easier because only white colonies possibly contained an insert. Secondly, a preliminary assay

Size	Location	pUC18	pCY7	Bend
439	1-439	+	-	
673	404-1073	+	-	
473	440-910	-	-	
370	913-1282	-	-	
296	999-1294	-	-	
334	1283-1616	+	+	-
617	1295-1911	+	+	+
396	1912-2307	-	-	
537	1938-2474	+	+	-
458	2475-2932	+	+	+
512	2440-2952	-	-	
264	2933-3196	-	-	
380	2979-3359	-	-	
193	3197-3389	-	-	
212	3390-3601	-	-	
638	3708-4345	+	-	
374	4053-4426	-	-	
299	4346-4644	-	-	
510	4645-5154	-	-	
646	5087-5732	+	+	+
389	5733-6122	-	-	
588	6233-6820	-	-	
577	6285-6861	+	-	
438	6821-7256	-	-	
554	6963-7415	+	+	-
276	7416-7691	-	-	
334	7570-7904	-	-	

Table 1.

could be performed on the pUC recombinants to determine if any bending was apparent. If so, the fragment could be cut out of pUC using enzymes which liberated sticky ended DNA which would then recombine more efficiently with pCY7. The fragments were isolated from pUC using SacI and BamHI. Cloning into pUC also amplified the insert fragment which facilitated subcloning. Nine of the 27 purified fragments have been cloned into pUC and eight were confirmed by restriction digestion. Once cloned into pUC, the plasmid was renamed according to the enzyme it came from and the size of the fragment. pUC clones are followed by an A and pCY7 clones are followed by a B. For instance, pSsp673A was generated by Ssp1, is 673 bp, and is in pUC18.

Bending analysis in pUC18

Five of the pUC clones were screened for bending (see figure 2). Two exhibited migrational patterns suggesting they contain a bend. The apparent size of pAlu617A was 338 bp larger than its actual size. and pSsp646A appeared 159 bp larger. K factors of the clones were 1.36 and1.16 respectively. K factors greater than one indicate possible bending. pHae554A migrated as if it contained no bend, and the other two require additional bending analysis to determine if a bend is present.

Cloning into pCY7 and bending analysis

The construction of the tandem dimer in pCY7 allows circular permutation assays to be performed on the HPV 16 inserts to localize the centers of any bend loci (see figure 3). pCY7 was cut

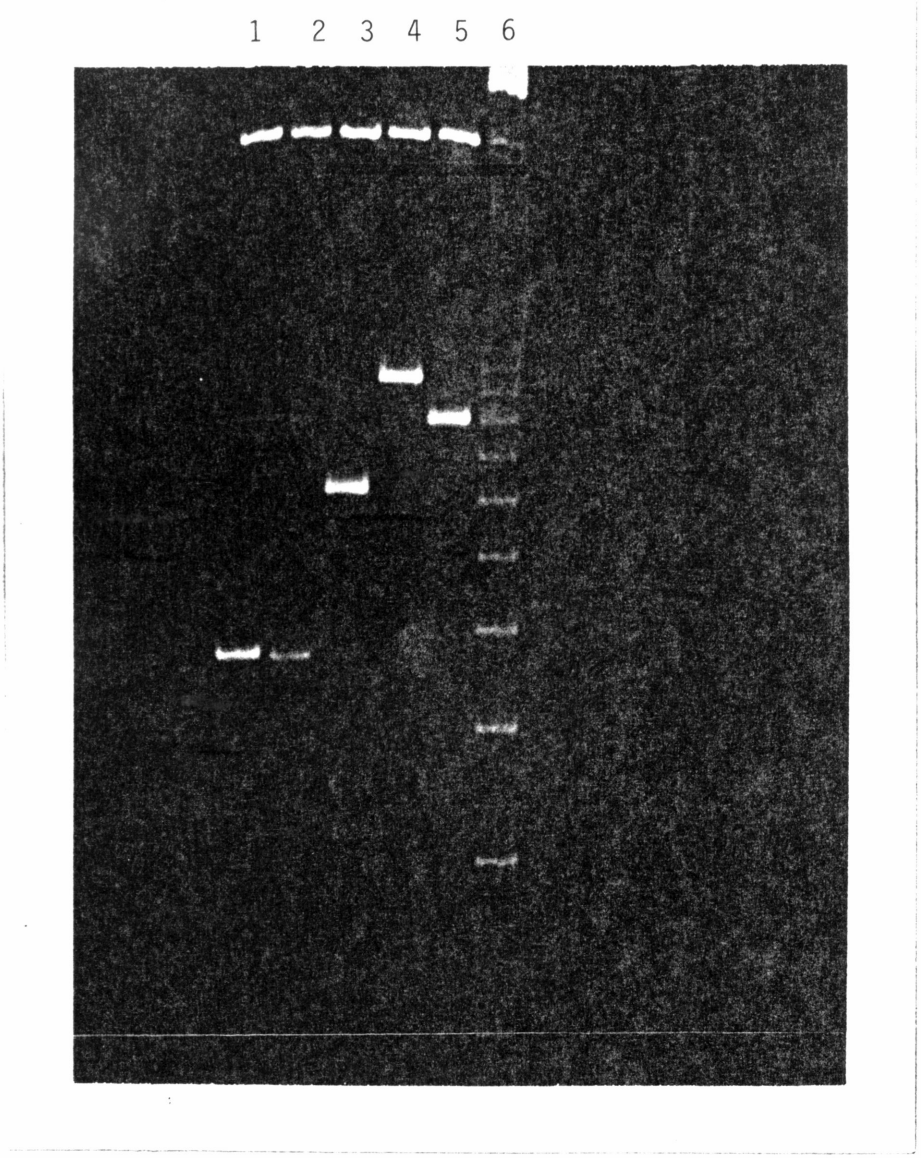


Figure 2. Results of bending analysis using PvuII restriction enzyme electrophoresed on 6% polyacrylamide.

lane 1. pHae334A

lane 2. pSsp537A

lane 3. pHae554A

lane 4. pAlu617A K>1

lane 5. pSsp646A K>1

lane 6. 123 bp ladder

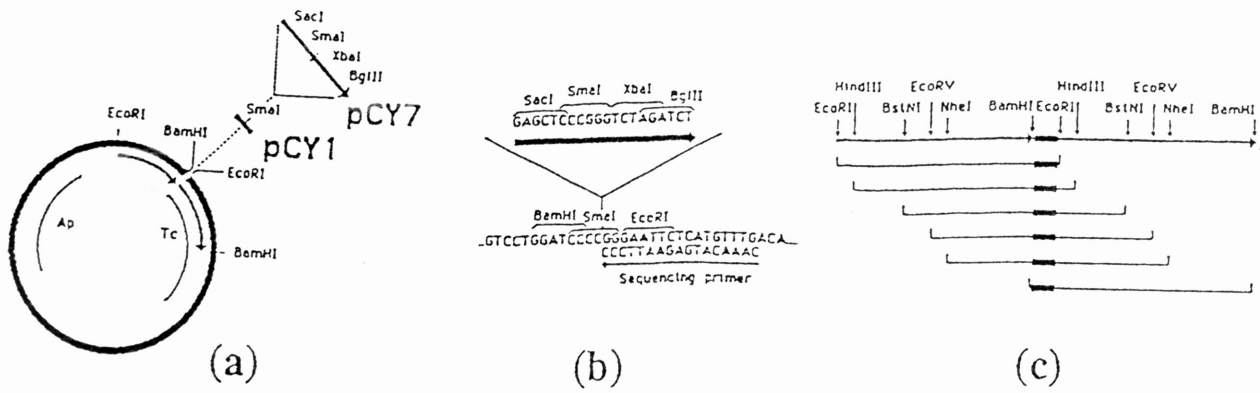


Figure 3. a. The plasmid pCY7

b. The polylinker between the repeats

c. The multiple restriction sites in the tandem repeat

with two different enzymes (SacI and BglII). After restriction of the pUC clones with SacI and BamHI and gel purification, the fragments were ligated into pCY7.

The pCY7 clones were digested with five different enzymes in separate reactions. EcoRI, HindIII, EcoRV, NheI, and BamHI, cut only once within each of the tandem repeats. Each digestion was analyzed by electrophoresis on low cross-link polyacrylamide gels at 4°C. The presence of a bend in a DNA fragment causes retarded migration of the DNA. Bending is best observed when the acrylamide gels are electrophoresed at very low temperatures. At temperatures greater than 50-55°C, bends in DNA are not visible.

Five fragments have been cloned into pCY7. Only one has been assayed by circular permutation. Clone pSsp458B contained a bend (see Fig 4). The bending assay clearly demonstrated the varying mobilities of the fragment dependent on the location of the bend in the fragments. The apparent sizes were determined relative to the 123 bp ladder. The K factors were determined and the resulting graph produced the expected sinusoidal curve (see Graph 1). From the graph, the center of the bend was extrapolated to 430 bp from the EcoRI site. The 458 bp insert begins 351 bp from the EcoRI site. Accordingly, the center of the bend is about 50 bp from the end of the insert. The insert fragment contained HPV 16 sequences from 2475-2932 oriented with the 2932 end adjacent to the SacI site in pCY7 (see figure 3). Therefore, the center of a bend has been localized to an area between 2850 to 2910 bp.

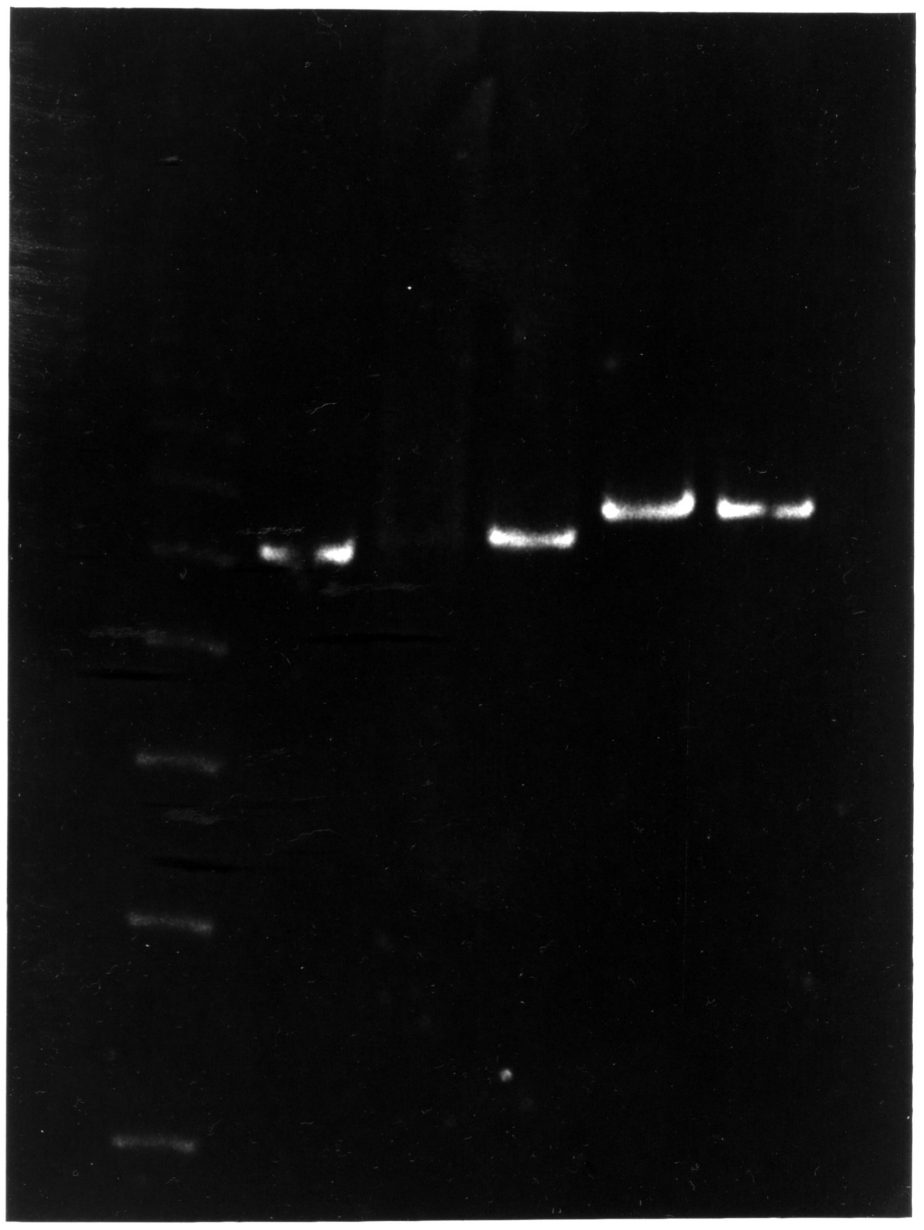


Figure 4. Circular permutation of pSsp458B.

lane 1. 123 bp ladder.

lane 2. digestion with EcoRI

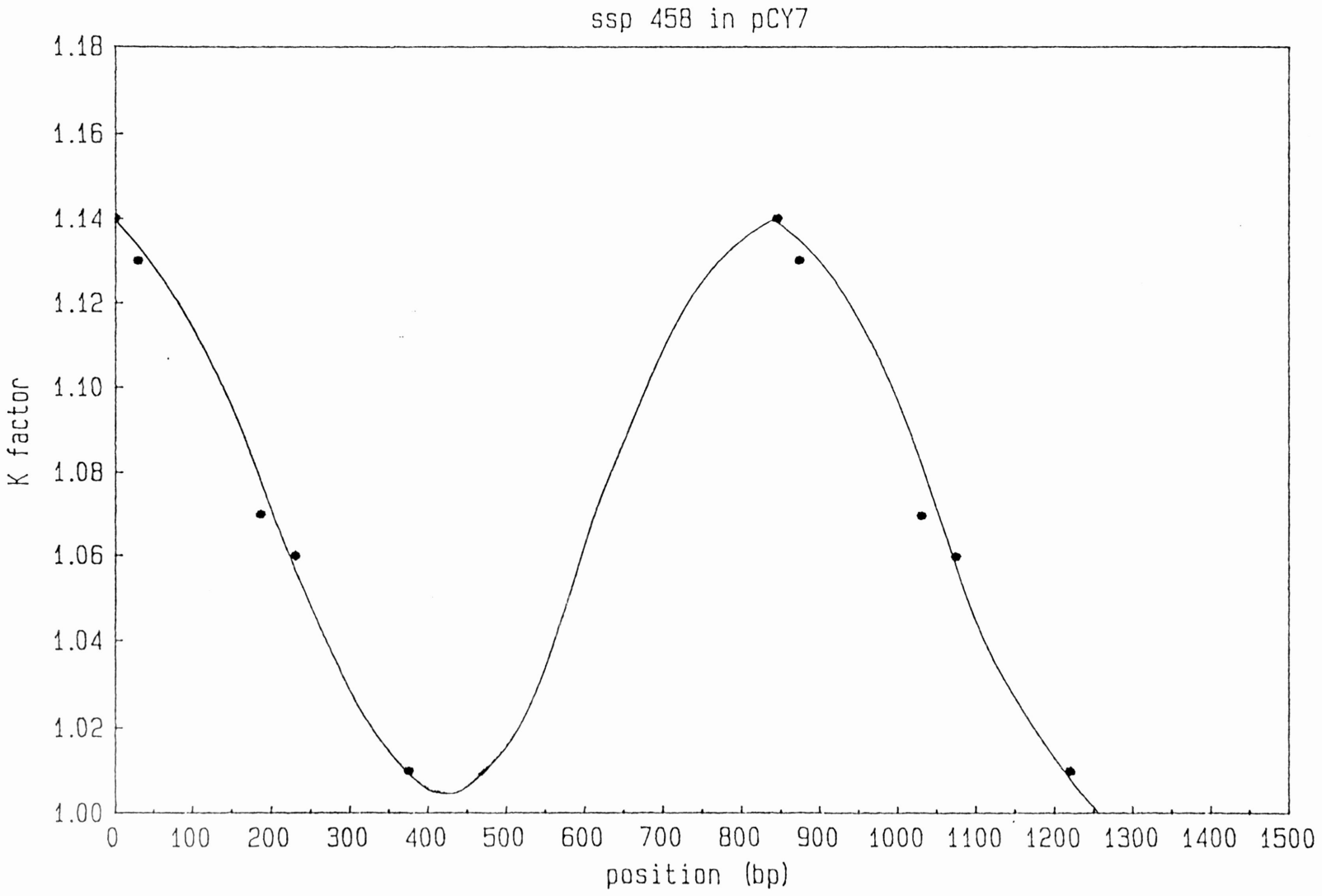
lane 3. with HindIII

lane 4. with EcoRV

lane 5. with NheI

lane 6. with BamHI

Graph 1. pSsp458B extrapolation of bend center using K factors obtained from figure 4.



Discussion

Twenty seven fragments were isolated from HPV 16 using various restriction enzymes. Nine of the fragments were cloned into pUC18 and five were cloned into pCY7. Six fragments were analyzed for bending. Five pUC clones were screened and pSsp646A and pAlu617A showed bending based on K factors obtained from polyacrylamide gel electrophoresis. To date, one pCY7 clone has been analyzed and determined to contain a bend site. In total, 35.8% of the HPV 16 genome has been analyzed for bends.

Of the three clones which contain bends, only one was assayed by circular permutation. pSsp458B was localized to a region which contains a known E2 open reading frame in HPV 16. BPV 1 contains a promotor in that area which suggests that HPV16 may also have a promotor in that region. However, the bend appears to be downstream of the initiation sequence which doesn't provide much insight about its association with the transcriptional regulatory sequences of that region. The HPV fragment also contains sequences which resemble TATA boxes and transcriptional binding sites which may be influenced by bending.

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