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Epstein–Barr Virus with Transforming and Early Antigen-inducing Ability Originating from Nasopharyngeal Carcinoma: Mapping of the Viral Genome

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SUMMARY

Epstein–Barr virus (EBV) from a human hybrid epithelial cell line (NPC-KT), derived from the fusion of human adenoidal cells and EBV genome-containing primary nasopharyngeal carcinoma cells (NPC) has both transforming and early antigen (EA)-inducing abilities. EBV DNA from NPC-KT cells was partially digested with *Taq*I and ligated to the cloning vector pJB8 at the *Cla*I site. This cosmid library encompassed the whole genomic DNA of the virus except for several kb of the terminal fragments. The identification and location of each of the cloned DNA fragments have been defined by hybridization to blots prepared with B95-8 and NPC-KT virion DNA. Defective heterogeneous restriction enzyme fragments of the viral DNA were not identified in any of the cosmid clones nor detected in hybridizations to virion DNA, which indicates that a single virus population derived from the NPC tissue has both transforming and EA-inducing activities.

INTRODUCTION

The association of Epstein–Barr virus (EBV) with nasopharyngeal carcinoma (NPC) is consistent and universal. However, the study of the aetiological role of EBV in NPC has been impeded by the lack of any EBV-infected epithelial cell lines, the limited susceptibility of epithelial cells to EBV infection *in vitro* and the lack of detailed biological and molecular characterization of EBV derived from NPC (Raab-Traub *et al.*, 1987; Sixbey *et al.*, 1983).

A hybrid epithelial cell line, NPC-KT, derived from the fusion of a human adenoidal epithelial cell line, Ad-Ah, and primary EBV-containing NPC, produces biologically active virus (Takimoto *et al.*, 1984, 1985). The virus from NPC-KT cells has both transforming and early antigen (EA)-inducing abilities (Sato *et al.*, 1986; Takimoto *et al.*, 1985). Efficient induction of EA and viral DNA synthesis are properties of the non-transforming strain of EBV, HR-1, and have been linked to an unusual rearranged EBV DNA fragment found in the heterogeneous (het) defective DNA molecules present in this strain of EBV (Countryman & Miller, 1985; Rabson *et al.*, 1983). However, at least two strains of EBV without defective viral populations can also induce EA, but not viral DNA synthesis, upon infection of Raji cells (Lin & Raab-Traub, 1987).

This study was undertaken to characterize the viral genome and to determine whether its dual activities could be ascribed to a single virus population. In addition, the virus found in NPC-KT provides an opportunity to characterize EBV of NPC origin in a strain never propagated in lymphocytes. Therefore we report here the molecular cloning and detailed mapping of EBV DNA from NPC-KT cells.

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METHODS

Viral DNA. NPC-KT cells were cultured in Dulbecco's modified Eagle's medium supplemented with 7% foetal calf serum. The EBV producer lymphoid cell line, B95-8, was cultured in RPMI 1640 with 10% foetal calf serum (Miller & Lipman, 1973). Virus was purified from NPC-KT by the method of Dolyniuk *et al.* (1976). Viral DNA was extracted with phenol and purified by equilibrium centrifugation in neutral CsCl. The DNA concentration was determined by optical density and comparison with known amounts of bacteriophage λ DNA cleaved with *Hin*dIII, in agarose gels containing ethidium bromide. About 8 mg of viral DNA was obtained from 3 l of culture supernatant fluid.

Recombinant DNA. The cosmid cloning method of Ish-Horowicz & Burke (1981) was applied with some modifications. Briefly, by digestion of the cloning vector, pJB8, with either *Pvu*II or *Ava*I followed by dephosphorylation and *Cla*I digestion, left-hand and right-hand vector ends were produced which accepted inserted DNA fragments generated by partial digestion with *Taq*I. One mg of NPC-KT viral DNA was partially digested with 1 unit of *Taq*I at 65 °C for 1 h in a total volume of 100 µl. The extent of the reaction was monitored by electrophoresis. The DNA was precipitated with ethanol and redissolved in 2 ml H_2O. Viral DNA was ligated with 0·5 µg of the vector mixture in 5 ml for 24 h at 15 °C. *In vitro* packaging was done using strain BHB2688, and the packaged cosmids were used to infect *Escherichia coli* 490A, which is *recA*$^-$. Single colonies grown on LB broth agar containing 100 mg/ml ampicillin were randomly picked and cultured in 5 ml L broth containing ampicillin. Subclones specific for the right- and left-terminal sequences were obtained from the cosmid clones 35 and 1. Digestion of clone 35 with *Bam*HI resulted in the removal of part of the insert except for an 880 base fragment specific for the left end of the *Bam*HI heterogeneous N fragment (Nhet). The plasmid was religated. A 1500 bp *Bam*HI fragment specific for the left terminus was subcloned from cosmid clone 1 into a plasmid in the same way.

Analysis of inserted DNA. The DNA from each recombinant bacterial clone was digested with *Eco*RI, *Hin*dIII, *Sal*I or *Bam*HI, electrophoresed in agarose gels, and the mobilities of each fragment of the EBV inserts were compared with total digests of B95-8 and NPC-KT EBV DNA. For the analysis of small fragments, 2·5 mg of DNA was digested and separated on 4% acrylamide gel using *Hae*III-digested colicin E1 DNA fragments as size markers. Blot hybridization was employed to map the insert. Viral DNAs from B95-8 and NPC-KT digested to completion with each enzyme were separated on agarose gels and transferred to nitrocellulose filters (Southern, 1975). Identical filter strips of each restriction endonuclease digest were made by slicing preparative Southern blots into strips 5 mm wide. DNA from the cosmid clones or from virus was labelled with [^{32}P]dCTP by nick translation. Hybridization was carried out at 65 °C for 16 h in 50 mM-Tris–HCl pH 7·5, containing 0·02% polyvinylpyrrolidone, 0·02% bovine serum albumin, 0·02% Ficoll, 10 mM-EDTA, 1 M-NaCl, 0·1% SDS and 20 mg/ml heat-denatured salmon sperm DNA. After hybridization, the nitrocellulose strips were washed with 2 × SSC (1 × SSC is 150 mM-NaCl, 15 mM-trisodium citrate, pH 7·0) at room temperature and then with 0·1 × SSC containing 0·1% SDS at 50 °C. The filters were covered with Saran Wrap and exposed to X-ray film with an intensifying screen for autoradiography. The nomenclature and the sizes of each fragment of B95-8 DNA derived from the complete sequence of EBV DNA were utilized (Baer *et al.*, 1984). For the fragments of NPC-KT DNA, the nomenclature was assigned corresponding to the M_r values of the fragments (Fig. 1).

RESULTS

DNA from each clone was analysed by electrophoresis in agarose gels containing ethidium bromide after digestion with *Eco*RI, *Hin*dIII, *Sal*I or *Bam*HI and visualized with u.v. light (Fig. 2*a* to *d*). Small DNA fragments were separated on 4% acrylamide gels (Fig. 2*e*). These data enabled the accurate determination of the M_r values of the *Bam*HI, *Sal*I, *Eco*RI and *Hin*dIII fragments within each cosmid clone. The faint unlabelled bands in *Sal*I digests represent partial digestion products. It should be noted that the fragments located at both ends of the insert DNA are ligated to vector DNA at the *Taq*I site and therefore have altered M_r values and are designated by arrowheads. The DNAs from 10 clones were analysed further by blot hybridization using these DNAs as probes against NPC-KT or B95-8 viral DNA (Fig. 3*a* to *d*). It was assumed that NPC-KT DNA fragments that comigrated with B95-8 DNA and hybridized to the same cosmid DNA were identical or closely related and had the same map positions on the genome (Dambaugh *et al.*, 1980).

Comparison of these digestions indicated that the NPC-KT viral DNA structure was similar to that of B95-8 viral DNA except for the deletion characteristic of the B95-8 strain in the *Eco*RI C fragment (Raab-Traub *et al.*, 1980). The NPC-KT *Eco*RI A fragment, identified by cosmid clones 1, 6, 9 and 20, was 6 kb shorter than that of B95-8, suggesting that it had fewer copies of the large internal repeat sequence, IR1 (Fig. 3*a*). The NPC-KT viral B fragment, identified by

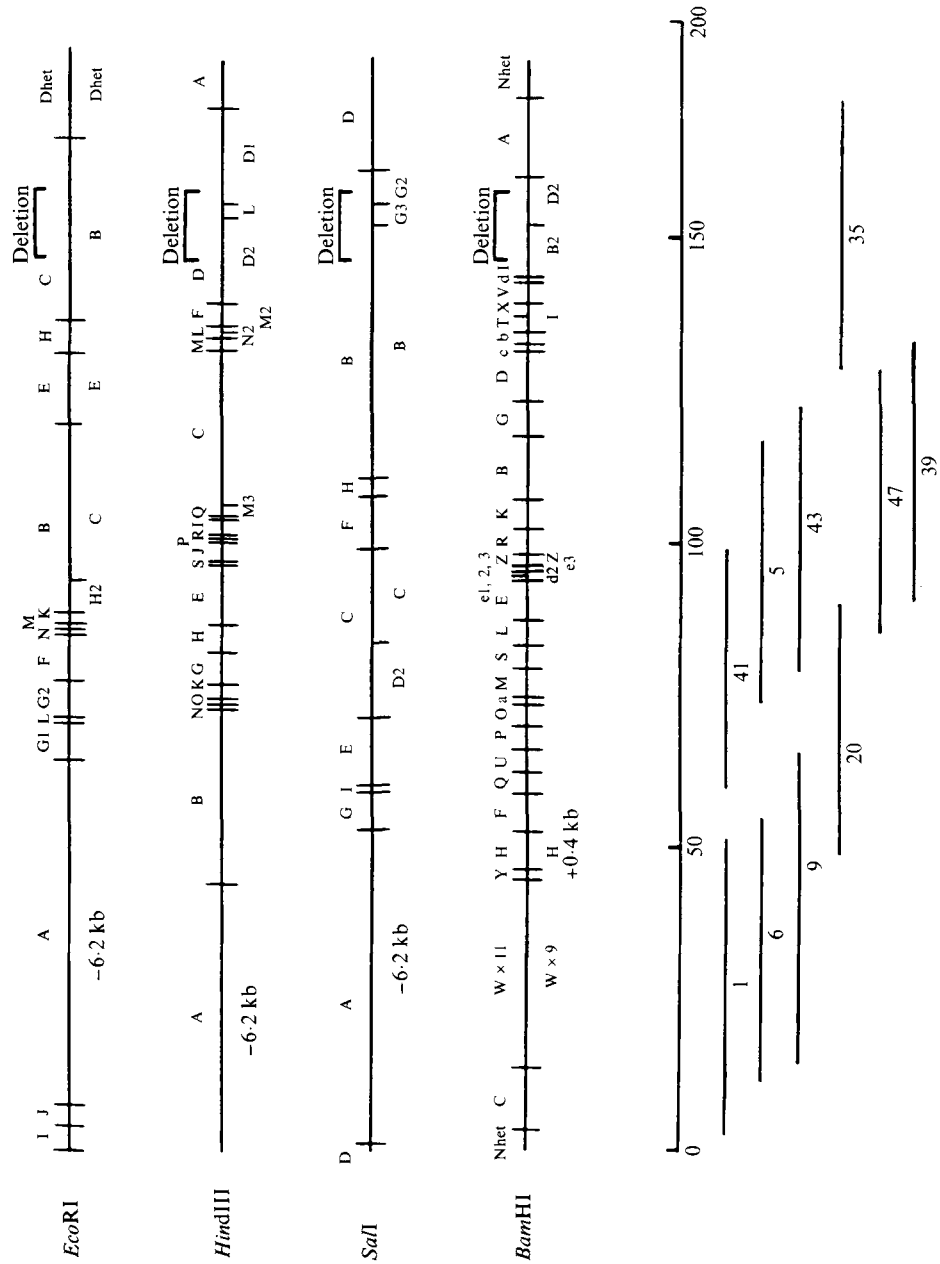
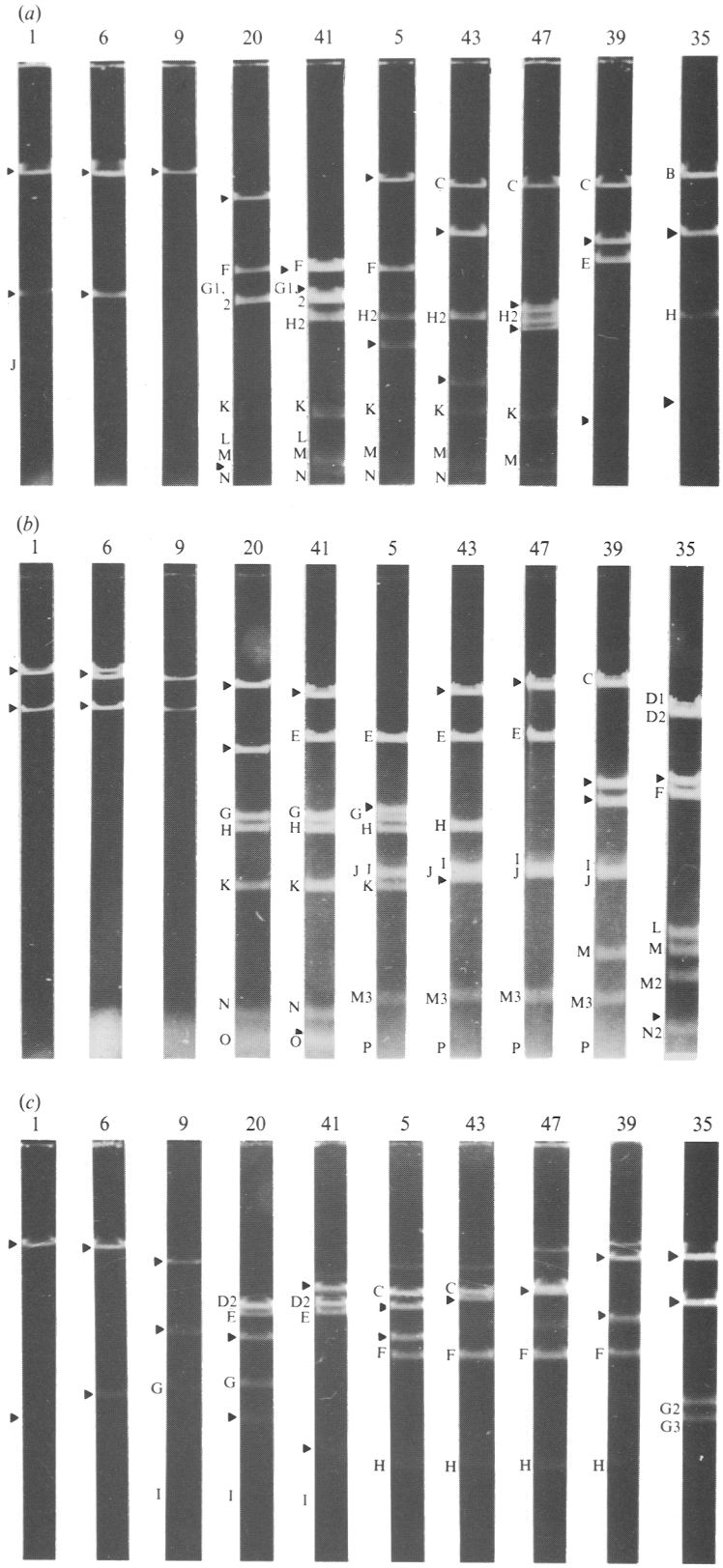


Fig. 1. Restriction enzyme linkage map of NPC-KT virus compared with B95-8. The *Eco*RI, *Hin*dIII, *Sal*I and *Bam*HI restriction enzyme fragments of NPC-KT (shown below each line) were mapped in comparison with the B95-8 strain of EBV (shown above each line). NPC-KT restriction enzyme polymorphisms are indicated. The scale is shown below (kb). The map positions of the NPC-KT cosmid clones which were used in determining the restriction enzyme maps are indicated at the base of the figure.


(a)
1 6 9 20 41 5 43 47 39 35
J
F G1, 2 K L M N
F G1 2 H2 K L M N
F H2 K M N
C H2 K M N
C H2 K M
C E
B H
(b)
1 6 9 20 41 5 43 47 39 35
E G H K N O
E G H K N O
E G H J I K M3 P
E H J I M3 P
E I J M3 P
C I J M M3 P
D1 D2 F L M M2 N2
(c)
1 6 9 20 41 5 43 47 39 35
D2 E G I
D2 E I
C F H
C F H
F H
F H
G2 G3

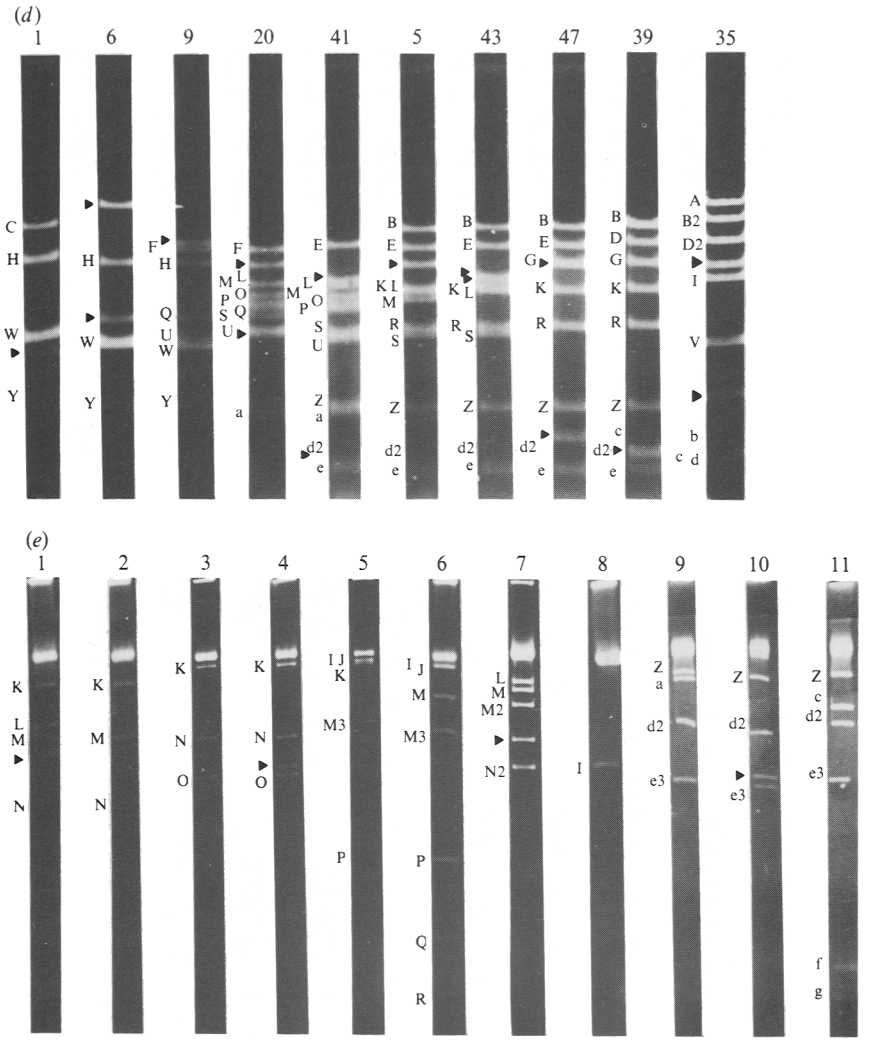
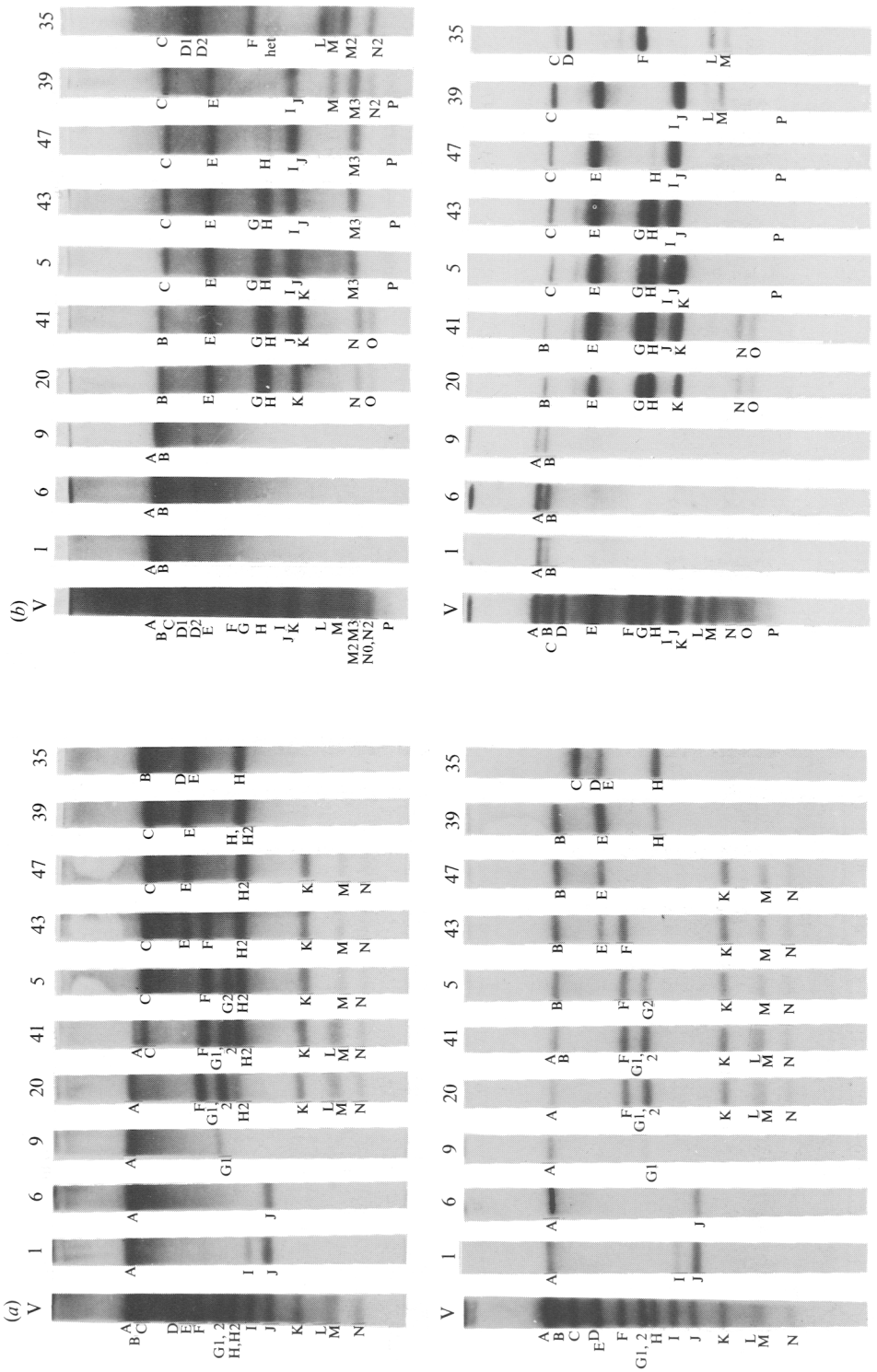


Fig. 2. Identification of the *Eco*RI (*a*), *Hin*dIII (*b*), *Sal*I (*c*) and *Bam*HI (*d*) fragments within the NPC-KT cosmid clones. The designated cosmid clones (number given above each lane) were digested with the required restriction enzyme and subjected to electrophoresis in a 0·6% agarose gel. The M_r values of each of the fragments were determined by comparison with the *Hin*dIII fragments of phage λ. The arrowheads designate the terminal fragments which are ligated to vector DNA. The individual fragments are designated with the NPC-KT fragment letter determined after hybridization. Small fragments were separated on a 4% acrylamide gel using *Hae*II-digested colicin E1 DNA as a molecular standard (*e*). Lanes 1 and 2, *Eco*RI-digested clones 20 and 5 respectively; lanes 3 to 7, *Hin*dIII-digested clones 20, 41, 5, 39 and 35 respectively; lane 8, *Sal*I-digested clone 9; lanes 10 to 12, *Bam*HI-digested clones 41, 5 and 39 respectively.

clone 35, corresponded to B95-8 *Eco*RI C but was 12 kb larger, indicating that virus from NPC-KT, like other prototype strains of EBV, does not have a deletion in the *Eco*RI C fragment (Raab-Traub *et al.*, 1980). The NPC-KT C fragment, identified by clones 41, 5, 43, 47 and 39, was 5·4 kb smaller than the B95-8 *Eco*RI B fragment. However, these clones also hybridized to a novel *Eco*RI fragment, NPC-KT H2 (5·4 kb). NPC-KT H2 hybridized to the B95-8 B fragment (Fig. 4) and mapped between the NPC-KT K and C fragments. The size of B95-8 B was equal to the sum of NPC-KT C and H2, suggesting that the B95-8 B fragment was split into C and H2


(a)
(b)
V
1
6
9
20
41
5
43
47
39
35

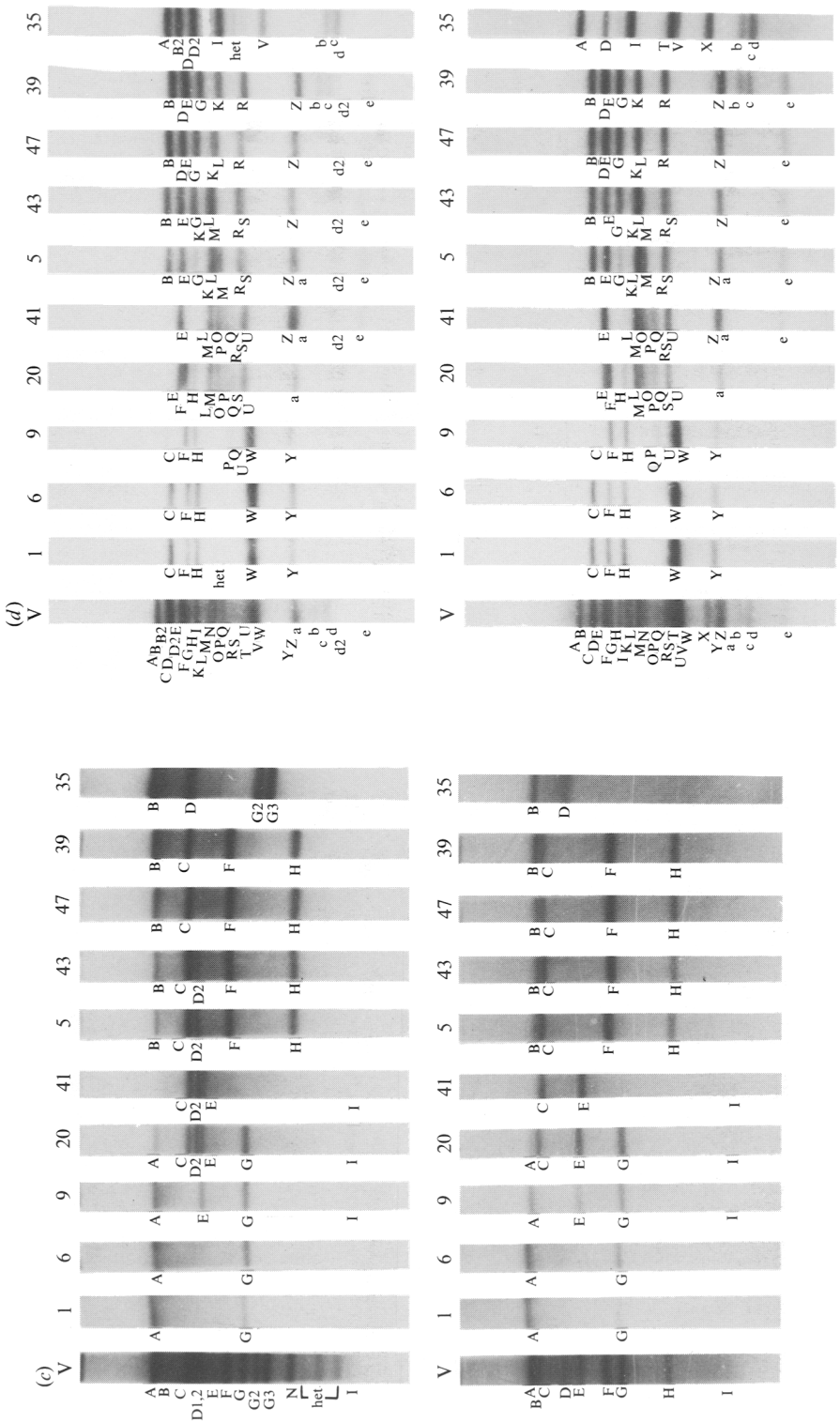


Fig. 3. Hybridization of the NPC-KT cosmid clones to B95-8 and NPC-KT virion DNA. Virion DNA prepared from the supernatant fluids of induced B95-8 or NPC-KT cell lines was digested with *Eco*RI (*a*), *Hin*dIII (*b*), *Sal*I (*c*) or *Bam*HI (*d*), subjected to electrophoresis in a preparative gel and transferred to nitrocellulose. Duplicate strips of B95-8 (lower gel of each pair) and NPC-KT (upper gel of each pair) blots were hybridized to each of the cosmid clones (identified by the number above each line). The fragments are denoted with the restriction enzyme fragment letter as indicated in Fig. 1. Total virion DNA digests are in lanes V.

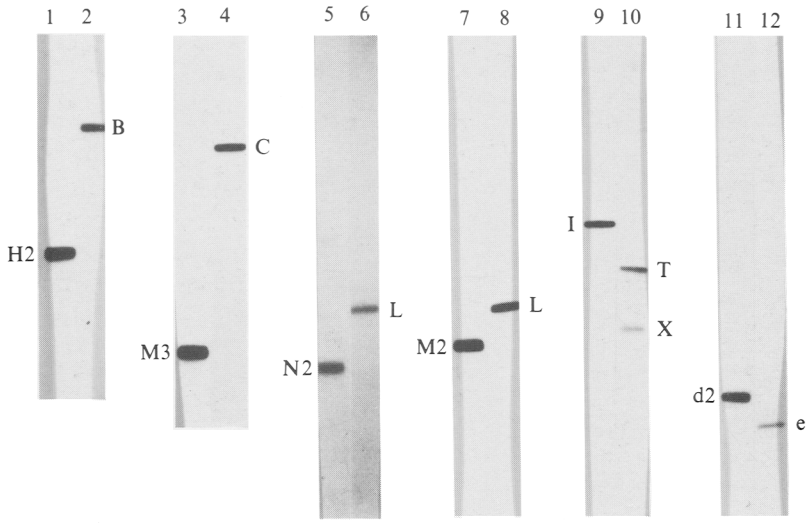


Fig. 4. Mapping of NPC-KT unique fragments. The restriction enzyme fragments that were detected in the NPC-KT clones but which were not present in B95-8 were labelled with ^{32}P and hybridized to duplicate blots of NPC-KT (odd-numbered lanes) or B95-8 (even-numbered lanes) virion DNA to identify which B95-8 fragment contained sequences homologous to the novel NPC-KT fragments. The restriction enzymes used to generate the novel fragments were: *Eco*RI (H2 fragment, lanes 1 and 2), *Hin*dIII (M3 fragment, lanes 3 and 4; N2 fragment, lanes 5 and 6; M2 fragment, lanes 7 and 8) and *Bam*HI (I fragment, lanes 9 and 10; d2 fragment, lanes 11 and 12). They were used to digest the NPC-KT or B95-8 DNA to prepare the duplicate blots.

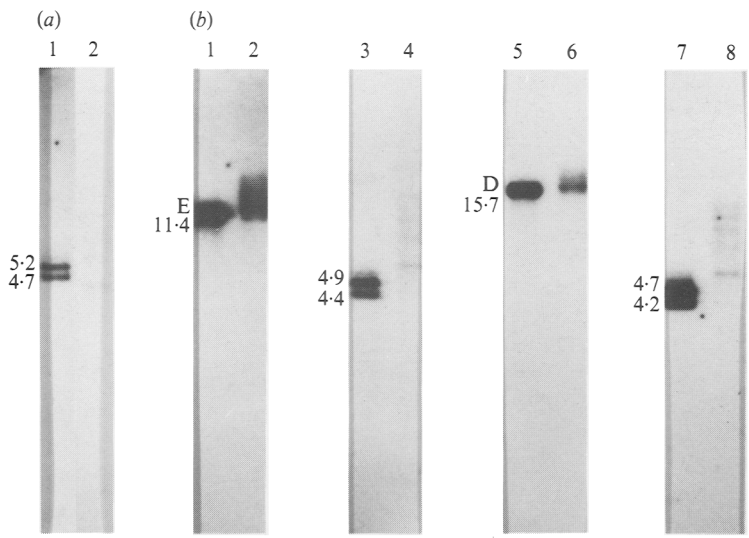


Fig. 5. Identification of the terminal fragments of NPC-KT. To identify these, a probe specific for the left terminus (*a*) was hybridized to NPC-KT (lane 1) and B95-8 (lane 2) virion DNA digested with *Bam*HI. A probe specific for the right terminus (*b*) was hybridized to NPC-KT (odd-numbered lanes) and B95-8 (even-numbered lanes) virion DNA digested with *Eco*RI (lanes 1 and 2), *Hin*dIII (lanes 3 and 4), *Sal*I (lanes 5 and 6) and *Bam*HI (lanes 7 and 8).

fragments in NPC-KT viral DNA. The predominant *Eco*RI fragment of the NPC-KT viral DNA right terminus was 11·4 kb, about 1 kb shorter than the B95-8 right-terminal fragment Dhet, suggesting that the NPC-KT terminal fragment has fewer copies of the terminal repeat sequence, TR (Fig. 5).

On *Hin*dIII digestion, the size of the A fragment, like that of the *Eco*RI A, was smaller than that of B95-8 *Hin*dIII A by 6 kb, suggesting a loss of IR1 sequences (Fig. 3*b*). NPC-KT viral DNA M2 (1·4 kb), M3 (1·1 kb) and N2 (0·9 kb) were additional fragments not found in B95-8 viral DNA digestions. The N2 and M2 fragments of NPC-KT hybridized to B95-8 L, the size of which was equal to the sum of NPC-KT N2 and M2, indicating an additional *Hin*dIII site in NPC-KT (Fig. 4). The NPC-KT M3 fragment hybridized to the C fragment of B95-8, and B95-8 C was equal in size to the sum of the sizes of NPC-KT C and M3, suggesting that the B95-8 *Hin*dIII C fragment was split into C and M3 fragments in NPC-KT DNA (Fig. 4). Two predominant right-terminal fragments of 4·4 and 4·9 kb were detected in NPC-KT DNA, whereas B95-8 DNA was more heterogeneous (Fig. 5). Cosmid clone 35, which hybridized to the *Hin*dIII D fragment of B95-8, contained three *Hin*dIII fragments, D1, D2 and L, similar to other EBV strains (Bornkamm *et al.*, 1980).

On *Sal*I digestion, the NPC-KT A fragment, which spans the IR1 repeats, was also 6 kb shorter than that of B95-8 (Fig. 3*c*). Hybridization of clones 20 and 41 identified unique fragments in NPC-KT, C (16·7 kb) and D2 (13·3 kb), which hybridized to the B95-8 C fragment (30·0 kb) indicating an additional *Sal*I site in NPC-KT. In addition, NPC-KT had two *Sal*I fragments, G2 and G3, identified by clone 35 which spanned the B95-8 deletion.

In the *Bam*HI digestions, the H fragment of NPC-KT was 0·4 kb longer than that of B95-8 (Fig. 3*c*). This fragment encompasses the small repeat sequence, IR2, and varies in size between strains of EBV. The presence of a single homogeneous *Bam*HI H fragment suggests that a single virus strain is present. Similarly, the *Bam*HI K fragment varies in size between strains of EBV and a single *Bam*HI K fragment was detected in NPC-KT viral DNA. The number of IR1 repeat sequences represented by *Bam*HI W was estimated to be nine in NPC-KT, because the *Eco*RI, *Hin*dIII and *Sal*I A fragments were 6 kb larger in B95-8, which has 11 copies of IR1. *Bam*HI Y of NPC-KT was equal in size to that of B95-8, indicating that the sequences that encode EBNA 2 were not deleted in NPC-KT. The NPC-KT d2 fragment (810 bp) mapping between E and Z was unique; e1 (306 bp) and e2 (507 bp) mapping at this position in B95-8 (Baer *et al.*, 1984) were not detected, and only 0·8 kb d2 and e3 (520 bases) were included in cosmid DNAs encompassing this region, indicating a loss of the *Bam*HI site separating e1 and e2 (Fig. 4). NPC-KT I (5·5 kb) hybridized to B95-8 T (3·4 kb) and X (2·1 kb), indicating that the *Bam*HI cleavage site between the T and X fragments of B95-8 DNA was lost in NPC-KT DNA (Fig. 4).

To examine the right terminus of NPC-KT DNA, an 880 bp fragment from the rightmost *Bam*HI site was cloned and hybridized to NPC-KT and B95-8 DNA digests (Fig. 5). This fragment identified the right-terminal fragments, *Eco*RI Dhet (11·4 kb) and *Sal*I Dhet (15·7 kb) and predominant *Bam*HI fragments of 4·2 and 4·7 kb and *Hin*dIII fragments of 4·9 and 4·4 kb. The B95-8 DNA right terminus was more heterogeneous. A 1500 bp fragment specific for the left terminus identified *Bam*HI fragments of 4·7 and 5·2 kb in NPC-KT DNA. The B95-8 DNA left terminus was also more heterogeneous. The 1500 bp fragment did not hybridize to the NPC-KT DNA *Sal*I het which is located at the left end of the genome to the left of the 1500 bp probe.

DISCUSSION

Despite its consistent presence in NPC, data on the molecular or biological properties of EBV in this epithelial malignancy are sparse. The NPC-KT virus represents a strain of EBV from NPC that has not been passaged in lymphocytes and therefore has not been selected for non-lytic properties that might result from such passage. The virus is unusual in that it has the capacity not only to transform lymphocytes, but also to superinfect latently infected cell lines and induce the synthesis of viral replicative antigens (Sato *et al.*, 1986). Previous subcloning of the NPC-KT cell line failed to separate the biological properties, suggesting that two viral strains were not responsible for the dual properties.

In this study the genome of NPC-KT has been cloned and compared with the well characterized B95-8 strain of EBV. In summary, the NPC-KT EBV strain has fewer copies of the IR1 sequence and fewer copies of the TR at each terminus. There are no deletions of unique

sequences, and the sequences that encode EBNA 2 have been retained. The sequences that encode the EBNA 2 gene of EBV are deleted in the non-transforming strains of EBV produced by the HR-1 and Daudi cell lines (Jones *et al.*, 1984; King *et al.*, 1982; Rabson *et al.*, 1982). However, NPC-KT is similar to B95-8 and other transforming strains of EBV in that the sequences which encode EBNA 2 have been retained, indicated by non-deleted *Bam*HI H and Y fragments. This suggests that the loss of EBNA 2 sequences is not a factor contributing to the ability of the HR-1 strain to superinfect.

Although cloning of the viral DNA from a single specimen of unpassaged NPC revealed no striking differences when compared with the W91 strain of EBV, derived from a Burkitt lymphoma (Heller *et al.*, 1981; Raab-Traub *et al.*, 1980, 1987), multiple distinctive restriction enzyme polymorphisms do in fact characterize the NPC-KT genome. These restriction enzyme polymorphisms are homogeneous in all preparations and indicate that NPC-KT represents a single virus strain. In addition, restriction enzyme fragments of EBV that contain varying numbers of small repeat sequences, such as the *Bam*HI H and K fragments, differ in M_r between EBV isolates; however, single *Bam*HI H and K fragments are detected in NPC-KT virus. These structural characteristics are distinctive for NPC-KT and indicate that a single virus strain possesses both of the key biological properties of EBV.

The HR-1 strain of virus, which possesses the ability to superinfect Raji cells and activate DNA replication in latently infected cell lines, is also a single strain of virus. However, analysis of subclones of this strain indicated that the ability to induce EA synthesis efficiently appeared to be dependent on the presence of defective EBV genomes, although it is possible to induce EA without a defective subpopulation if the m.o.i. is high enough (Heston *et al.*, 1982; Lin & Raab-Traub, 1987; Rabson *et al.*, 1983). Gene transfer experiments have revealed that the replication activator function is encoded by a defective fragment in which sequences from the *Bam*HI Z fragment are linked to sequences from IR1 resulting in aberrant expression of the BZLF1 gene product (Countryman *et al.*, 1987). However, unlike HR-1, defective fragments could not be detected in NPC-KT viral DNA (Fig. 3) even during long exposures of the strong hybridizations of the cosmids to virion DNA, and were not obtained in any of the recombinant cosmid DNA clones which span the NPC-KT genome. The superinfection process with NPC-KT virus has recently been characterized further (Sato *et al.*, 1988). After superinfection both the endogenous and superinfecting genomes are amplified without evidence of the presence or production of defective DNAs. Defective genomic DNA is preferentially amplified after superinfection with HR-1 virus (Miller *et al.*, 1985).

The basis for the unusual property of NPC-KT to induce efficiently both EA and viral DNA synthesis is as yet unknown because it cannot be attributed to rearranged fragments as in HR-1. Analysis of the DNA structure of the *Bam*HI Z fragment, which encodes the EBV replication activator protein, does not reveal any major structural differences in NPC-KT virion DNA or in DNA replicated after superinfection (Sato *et al.*, 1988). However, NPC-KT virus is unique in that its ability to activate EA synthesis and viral replication is enhanced by treatment with DMSO (Sato *et al.*, 1988). It is possible that small sequence changes in the promoter of the *Bam*HI Z activating function could result in enhanced expression after treatment with DMSO. Alternatively some other structural feature of the NPC-KT virus could contribute to its ability to replicate after superinfection. NPC-KT virus circularizes or forms concatemers very efficiently after superinfection, which could contribute to establishment of the superinfecting viral genome and promote viral expression (Sato *et al.*, 1988).

NPC-KT is the first virus strain obtained from NPC that has not been passaged in lymphocytes. Therefore it is possible that the superinfecting capacity of NPC-KT is characteristic of wild-type epithelial strains or any wild-type strain not selected by transformation of lymphocytes. This property could be responsible for the elevated serological titres to EA and other replicative antigens which are characteristic of patients with NPC.

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