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Cytoplasmic RNA from normal and malignant human cells shows homology to the DNAs of Epstein-Barr virus and human adenoviruses

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Cytoplasmic RNA prepared from several human cell lines and tissues was hybridised to DNA from Epstein-Barr virus, human adenovirus types 2, 3 and 12 and human papovaviruses BK and JC. RNA from all the cells, regardless of whether or not they were virally infected, hybridised to specific regions of the Epstein-Barr virus or adenovirus genomes but not to papovavirus DNA. The cellular cross-hybridising species appear to be repetitive sequences which are conserved in higher eukaryotes. Mismatch estimations indicate a high degree of homology between the viral and host sequences. Detailed analysis of selected regions of viral DNA failed to reveal any primary-structural peculiarities.

Key words: adenovirus/Epstein-Barr virus/human cells/hybridisation/repetitive sequences

Introduction

The Epstein-Barr virus (EBV) and the human adenoviruses (Ad) readily establish latent infections in human lymphoid tissues (Henle and Henle, 1980; Sambrook *et al.*, 1980). It is generally recognised that EBV is associated with, and probably is a contributing factor to the development of two human malignancies, African Burkitt's lymphoma and poorly differentiated nasopharyngeal carcinoma (Epstein, 1978). On the contrary, extensive studies (Green and Mackey, 1977; Green *et al.*, 1979; Mackey *et al.*, 1976, 1979; Wold *et al.*, 1979) have failed to show an association between adenoviruses and any type of human cancer with the tentative exception of some neurogenic tumours (Ibelgaufts *et al.*, 1982). However, lymphoid neoplasms, in particular leukaemias, have not been well represented in previous investigations of adenovirus-associated tumour diseases.

One of the central themes of virus-mediated oncogenesis is that viral genetic information is expressed in the transformed cell. Here we examine EBV- and adenovirus-related RNA in cells and cell-lines derived from: (i) EBV-associated tumours; (ii) EBV-negative lymphomas and leukaemias; (iii) apparently normal cells.

Previous work along these lines on EBV-related RNA by Kieff and colleagues (Thomas-Powell *et al.*, 1979; Dambaugh *et al.*, 1979; King *et al.*, 1980, 1981; Van Santen *et al.*, 1981) and ourselves (Rymo, 1979; Arrand and Rymo, 1982) has defined the regions of the EBV genome which are expressed in Burkitt lymphoma-related cells. In general, the results obtained using several different approaches were reasonably consistent. However, one major discrepancy was the fact that the Kieff group did not detect any hybridisation to the parts of the EBV genome defined by the fragments *Eco*RI-G1 and *Bam*HI-F which we found to be strongly hybridising regions. The present work examines this hybridisation in more detail and shows that RNA from all human cells examined, whether EBV-infected or not, contains sequences related to EBV DNA.

In common with the EBV results, cytoplasmic RNA from all human cells tested showed complementarity to regions of the genomes of adenovirus types 2, 3 and 12, although we were not able to detect any adenovirus-specific discrete mRNAs. The results agree with a report by Jones *et al.* (1979) who found RNA sequences homologous to Ad2 DNA in human placenta and liver. However, in a recent study Green *et al.* (1979) did not detect Ad2-related sequences in RNA from several different normal human tissues including placenta, or in several tumour RNAs.

Our results suggest that the sequences found in RNA from normal, apparently uninfected cells, which hybridise to certain regions of EBV DNA and adenovirus DNA, are not perfectly homologous to the viral DNAs. Furthermore, they seem to consist of repetitious sequences which are not necessarily related to each other but which seem to be conserved in higher eukaryotes. In contrast to the observations with EBV and adenovirus DNA, cytoplasmic RNA from apparently uninfected cells showed no homology to DNA from the human papovaviruses BK and JC.

Results

Hybridisation of cellular RNA to EBV DNA: a comparison between RNA from EBV-positive and EBV-negative cells

Cytoplasmic RNA was prepared from four EBNA-negative human B-lymphoid cell lines, Ramos, DG75, Riva and BALL-1; two T-lymphoid lines, Molt-4 and HPB-ALL; a human fibroblast cell line Bu, and secondary human fibroblasts; five EBV genome-positive lymphoid lines, Raji, Namalwa, Daudi, Putko and AW-Ramos; and human tonsillar tissue. The RNA was labelled *in vitro* and hybridised to nitrocellulose membranes containing individual cloned restriction endonuclease fragments of B95-8 EBV DNA. A restriction map of EBV DNA is shown in Figure 1 which also includes a graphical summary of our results. RNA from all the cell types hybridised to *Eco*RI fragments A, G1 and D_{end} (D_{end} is a fragment cloned from circular EBV DNA and shown to contain terminal sequences), whereas RNA from the EBV-positive lines showed additional hybridisation to fragments B and J. To map more precisely the hybridising regions within the *Eco*RI-A fragment, a similar experiment was performed using individually cloned *Bam*HI fragments derived from this region of the EBV genome. The results of such experiments with RNA isolated from one EBV-positive cell line (AW-Ramos) and one EBV-negative line (BALL-1) are shown in Figure 2. RNA from all cell lines and the tonsillar tissue hybridised to *Bam*HI fragments F and Q whereas the EBV-positive lines exhibited additional hybridisation to *Bam*HI fragments H, W and (occasionally) Y, and the part of fragment C defined by the *Eco*RI J fragment.

A general pattern emerged from all the RNAs investigated

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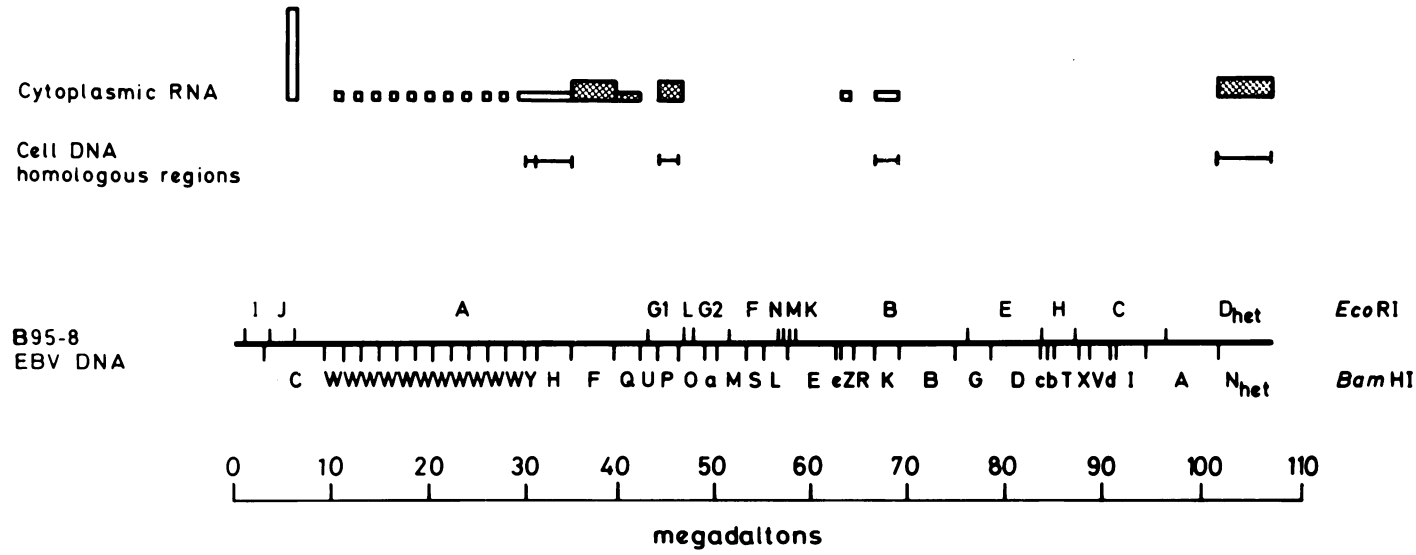

Fig. 1. *Eco*RI and *Bam*HI restriction endonuclease maps of B95-8 EBV DNA and the relative positions of the hybridising cytoplasmic RNA species. The RNAs are shown as bars, the height of which represents the relative abundance of each species. Open bars indicate regions of the genome which hybridise cytoplasmic RNA exclusively from EBNA-positive cells. The hatched bars indicate regions to which poly(A)$^+$ RNA from all cell types studied will hybridise. 'Cell DNA homologous regions' refers to fragments of EBV DNA which hybridise to cellular DNA in the spot hybridisation test (Figure 4). The results of hybridisation of cytoplasmic RNA from EBV-positive cells to EBV DNA are in general agreement with those of Kieff and colleagues (Thomas-Powell *et al.*, 1979; Dambaugh *et al.*, 1979; King *et al.*, 1980, 1981; Van Santen *et al.*, 1981). However, there is one major discrepancy between the results of Kieff *et al.* and those obtained in this work and in earlier studies (Rymo 1979; Arrand and Rymo, 1982) in that the Kieff group did not detect any RNA complementary to *Eco*RI-G1 or *Bam*HI-F. This can, however, very likely be explained as being due to the fact that eukaryotic DNA was used as carrier in their hybridisations. We show in Figure 4 that calf thymus DNA competes out the hybridisation of these fragments of EBV DNA to uninfected cell DNA. Additional experiments (not shown) have demonstrated that inclusion of calf thymus DNA instead of yeast RNA as carrier in our standard RNA-DNA hybridisations also greatly diminishes or eliminates the observed hybridisation to these fragments.

(Figure 1). In both EBV-negative and -positive cells EBV-related cytoplasmic RNA sequences were found which were complementary to regions of the genome defined by the restriction enzyme fragments *Eco*RI-D$_{end}$ and -G1 and *Bam*HI-F and -Q. In EBV-positive cells, additional hybridisation was observed to *Bam*HI fragments W, H, K and (inconsistently) Y and very strong hybridisation to *Eco*RI-J. This has been discussed in detail previously (Arrand and Rymo, 1982; Jat and Arrand, 1982).

To examine whether the EBV DNA-related RNA sequences found in uninfected cells are polyadenylated, cytoplasmic RNA was fractionated on oligo(dT)-cellulose columns, poly(A)$^+$ RNA was collected, labelled *in vitro* and hybridised to blots containing EBV DNA fragments. The results showed that RNA sequences complementary to the *Bam*HI-F and *Eco*RI-G1 fragments had been enriched in the poly(A)$^+$ fraction as compared with non-fractionated cytoplasmic RNA. To find out if the EBV DNA-related RNAs behave as discrete species with defined sizes, total cytoplasmic RNA and oligo(dT)-selected RNA were denatured, size fractionated by electrophoresis through agarose gels, transferred to nitrocellulose membranes and hybridised with ^{32}P-labelled cloned *Bam*HI-F and *Eco*RI-G1 fragments. No discrete bands were, however, detected, either with total cytoplasmic or with poly(A)$^+$ RNA (data not shown).

Fine structure mapping of some regions of EBV DNA which are complementary to RNA found in all cells examined

To define more precisely some of the regions of the EBV genome which are complementary to cytoplasmic RNA from both EBNA-positive and -negative cells, *Eco*RI fragment G1 and *Bam*HI fragment F have been mapped in more detail using several restriction endonucleases (Figure 3b). Labelled cytoplasmic RNA was hybridised to blots of the subfragments and the result of one such experiment is shown in Figure 3a. RNA hybridises exclusively to an ~1.3-kb segment at the right end of this fragment. Similarly, cellular RNA was found to be complementary to two regions within *Eco*RI-G1 (data not shown; see Figure 3b).

Hybridisation of EBV DNA to cellular DNA and competition by eukaryotic DNA

The presence in uninfected human cell DNA of sequences homologous to EBV DNA was demonstrated by hybridising selected ^{125}I-labelled cloned EBV DNA fragments to nitrocellulose filters containing DNA from EBNA-negative lymphoid cell lines (Ramos, DG75, HPB-ALL, BALL-1, Molt-4), from secondary human fibroblasts and a human liver biopsy, and from EBV genome-positive cell lines (Raji, Namalwa). As seen in Figure 4, both EBV-positive and EBV-negative cells contain sequences which hybridise to regions of the EBV genome defined by the restriction enzyme fragments *Bam*HI-Y, -H, -P, -K and *Eco*RI-D$_{end}$. It is notable that the hybridisation can be competed out to a large extent by the presence of calf thymus DNA in the hybridisation mixture (Figure 4B). Addition of poly(C) to the mixture at a concentration of 20 μg/ml did not affect the hybridisation pattern (data not shown).

EBV-related cellular DNA sequences were characterised further by cleaving high mol. wt. DNA from DG75 cells with *Eco*RI in the presence or absence of two genome equivalents of B95-8 EBV DNA. The fragments were separated by agarose gel electrophoresis and blotted onto nitrocellulose membranes. Strips cut from the membranes were hybridised to ^{32}P-labelled cloned B95-8 EBV DNA fragments *Bam*HI-W, -Y, -H, -F, -P, -K and *Eco*RI-D$_{end}$. The results are shown

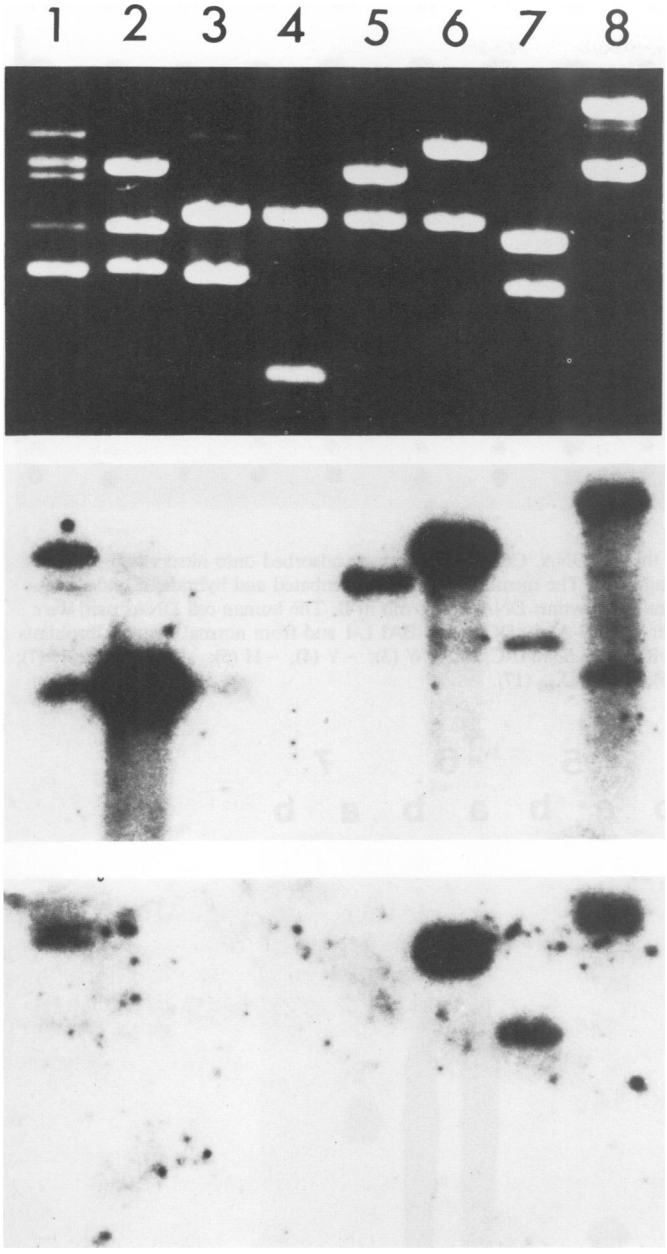

Fig. 2. Hybridisation of cellular cytoplasmic RNA to individual cloned *Bam*HI fragments of EBV DNA and a cloned *Eco*RI fragment which contains sequences from both ends of the linear virion DNA. The *Bam*HI fragments in this experiment are all subfragments of *Eco*RI-A. Plasmid DNA was cleaved with *Eco*RI plus *Bam*HI (**lanes 1** and **2**), *Bam*HI (**lanes 3–7**) or *Eco*RI (**lane 8**). **Lane 1** contains *Eco*RI-A, **lanes 2–6** contain the single *Bam*HI fragments C, W, Y, H and F, **lane 7** contains *Bam*HI fragments Q and U and **lane 8** contains *Eco*RI $-D_{end}$. DNA was fractionated on 0.8% agarose gels, transferred to nitrocellulose membranes and hybridised with ^{32}P-labelled RNA. The **upper panel** shows the pattern of fragments after ethidium bromide staining and visualisation under u.v. light. The **middle** and **lower panels** show, respectively, the patterns of hybridisation obtained with total cytoplasmic RNA from AW-Ramos cells and polyadenylated RNA from BALL-1 cells. The origin of the band visible in **lane 8** of the **middle panel** but not present in the upper and lower panels is not clear. It may represent a submolar fragment of EBV DNA which is released by a contaminant in the restriction endonuclease which hybridises to EBV-specified RNA from this region (Van Santen *et al.*, 1981).

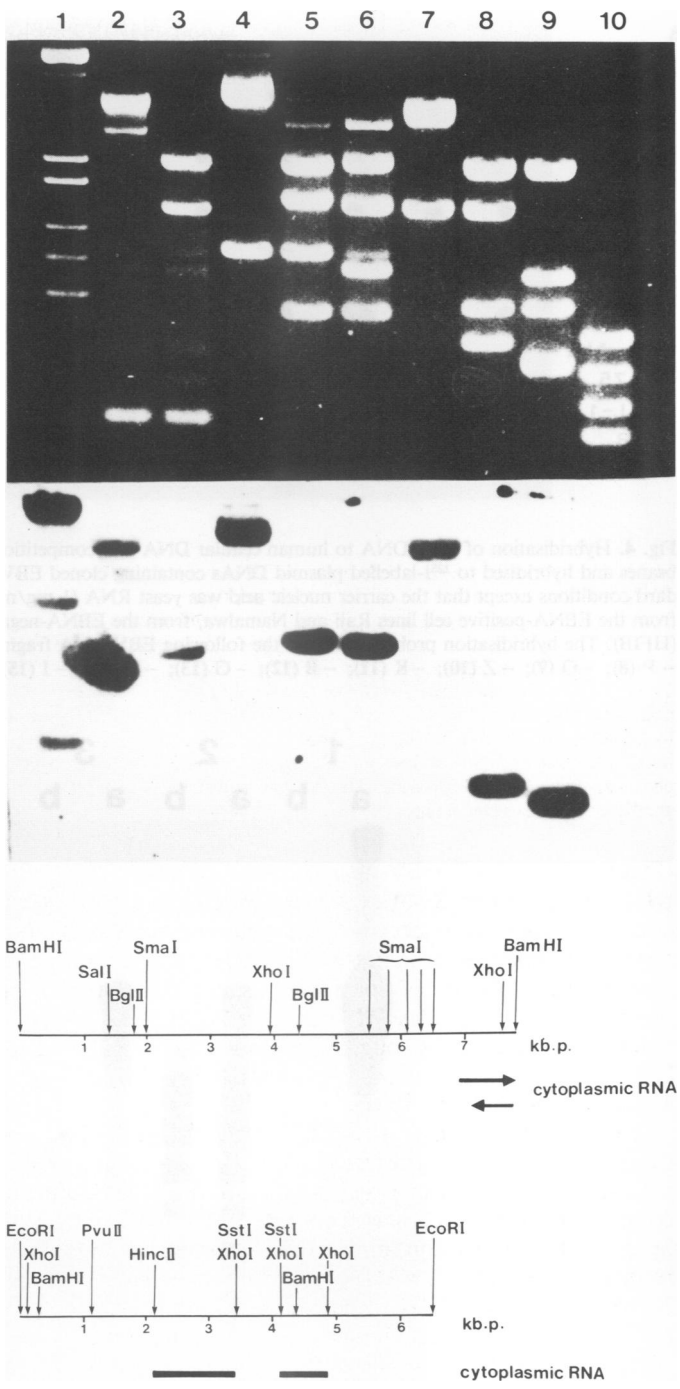

Fig. 3. (a) Hybridisation of cellular cytoplasmic RNA to Ad2 DNA and subfragments of cloned EBV DNA fragments *Eco*RI-G1 and *Bam*HI-F. Conditions were as in Figure 2 except that the fragments were separated on a 1% agarose gel. The **upper panel** shows the fragment pattern and the **lower panel** shows the pattern of hybridisation obtained using ^{32}P-labelled polyadenylated cytoplasmic RNA from Namalwa cells. Ad2 DNA was cleaved with *Eco*RI (**lane 1**); plasmid pBR322 containing the EBV *Bam*HI-P fragment was cleaved with *Sst*I (**lane 2**) or *Sst*I plus *Bam*HI (**lane 3**); plasmid pBR322 containing the EBV *Bam*HI-F fragment was cleaved with *Bgl*II (**lane 4**), *Bgl*II plus *Bam*HI (**lane 5**), *Bgl*II, *Bam*HI and *Xho*I (**lane 6**), *Sma*I (**lane 7**), *Sma*I plus *Bam*HI (**lane 8**), *Sma*I, *Bam*HI and *Xho*I (**lane 9**). **Lane 10** contains polyoma virus DNA cleaved with *Hpa*II. In common with its human counterparts BK and JC (see Figure 6), DNA from the murine papovavirus polyoma does not hybridise to human cellular RNA. **(b)** Restriction endonuclease maps orientated as in Figure 1 of *Bam*HI-F (**top**) and *Eco*RI-G1 (**bottom**). The positions of cytoplasmic RNA from all human cells tested, which hybridises to these fragments are indicated as solid lines below the corresponding map.

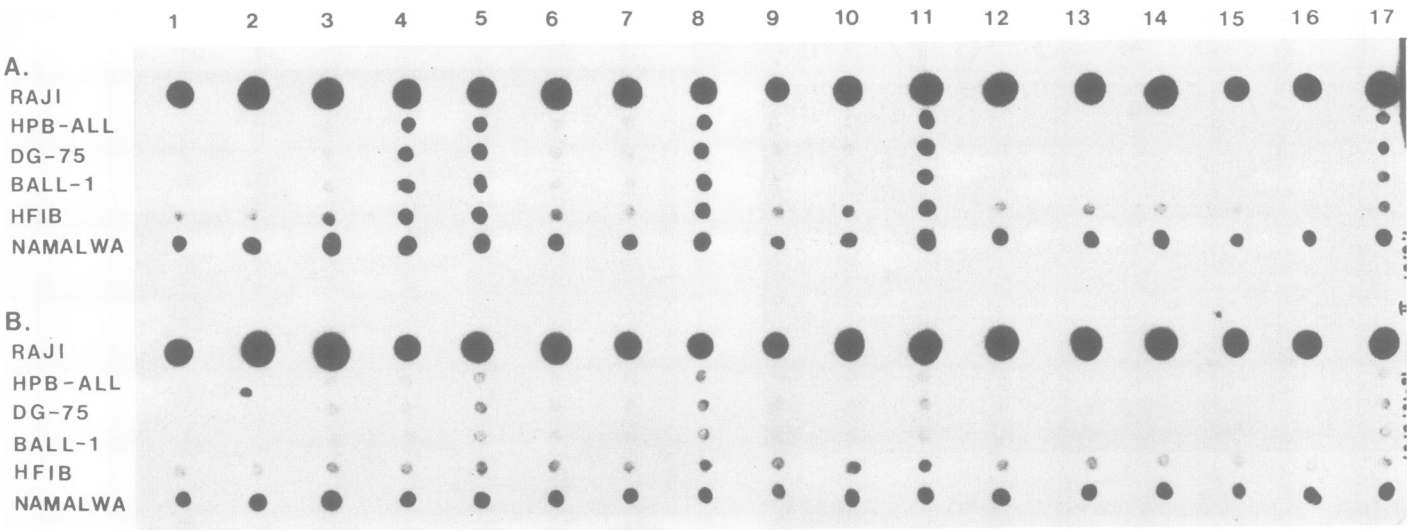

Fig. 4. Hybridisation of EBV DNA to human cellular DNA and competition by calf thymus DNA. Cellular DNAs were adsorbed onto nitrocellulose membranes and hybridised to ^{125}I-labelled plasmid DNAs containing cloned EBV DNA fragments. The membranes were preincubated and hybridised under standard conditions except that the carrier nucleic acid was yeast RNA (1 mg/ml) in **A** and calf thymus DNA (1 mg/ml) in **B**. The human cell DNAs used were from the EBNA-positive cell lines Raji and Namalwa, from the EBNA-negative cell lines HPB-ALL, DG75 and BALL-1 and from normal human fibroblasts (HFIB). The hybridisation probes contained the following EBV DNA fragments: *Eco*RI-J (1); *Bam*HI-C (2); −W (3); −Y (4); −H (5); −F (6); −Q+U (7); −P (8); −O (9); −Z (10); −K (11); −B (12); −G (13); −D (14); −I (15); −A (16); *Eco*RI-D_{end} (17).

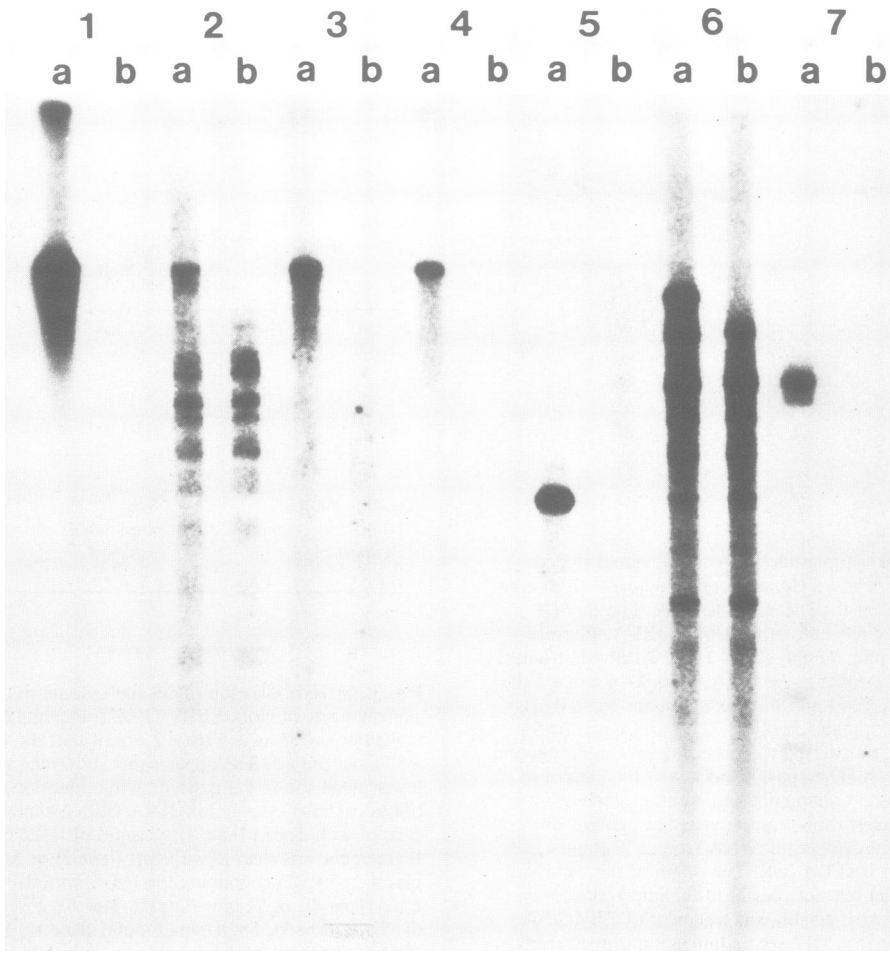

Fig. 5. Hybridisation of EBV DNA to *Eco*RI cleavage fragments of human cellular DNA. DNA from the cell line DG75 was cleaved by *Eco*RI with (**a**) or without (**b**) the addition of two genome equivalents of B95-8 EBV DNA. The fragments were fractionated on a 0.4% agarose gel and transferred to a nitrocellulose membrane. Strips were cut from the membrane and hybridised with ^{32}P-labelled B95-8 EBV DNA fragments *Bam*HI-W (1); −Y (2); −H (3); −F (4); −P (5); −K (6) and *Eco*RI-D_{end} (7). All the strips are from the same gel. Yeast RNA was used as carrier.

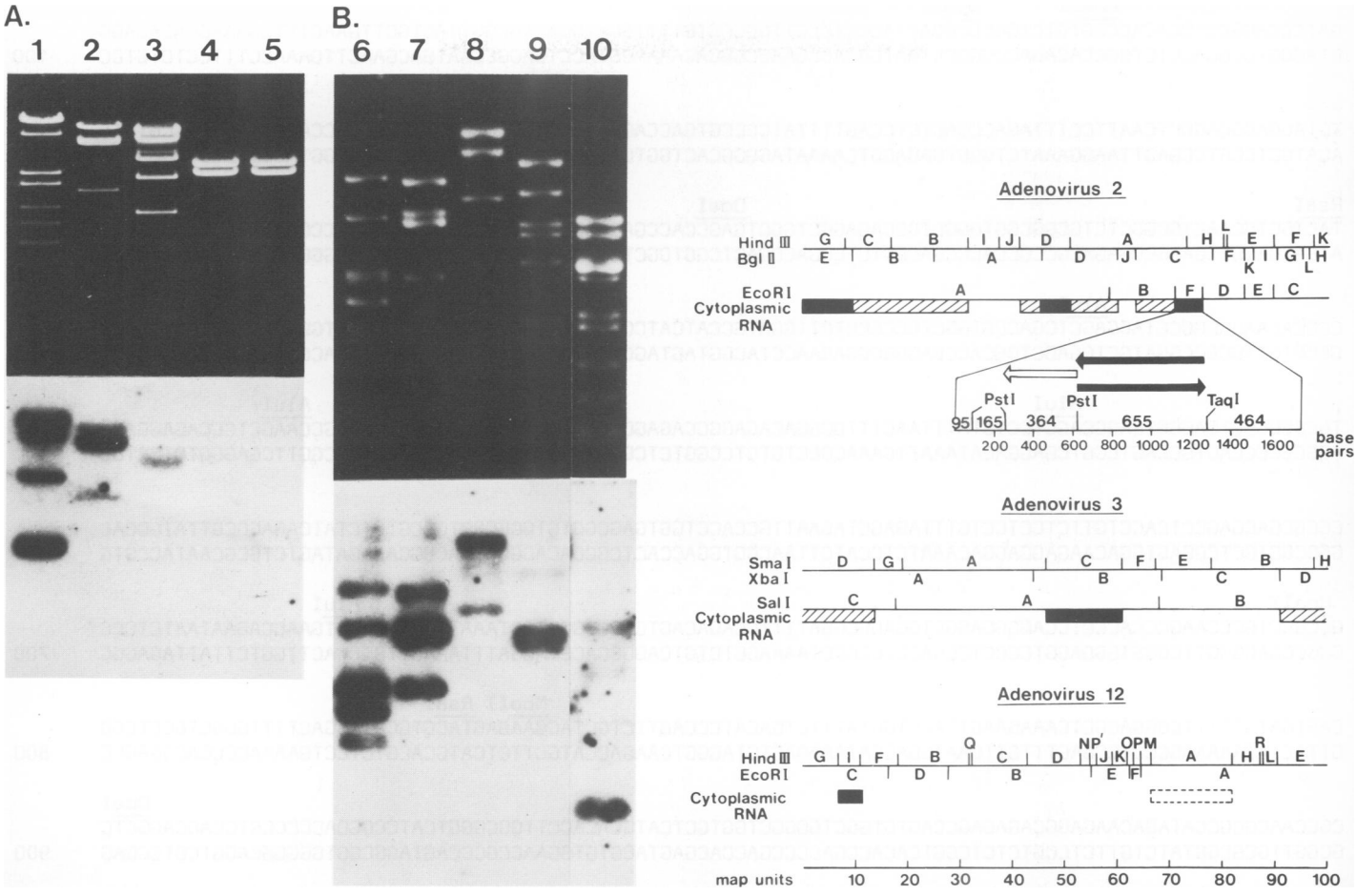

Fig. 6. Hybridisation of cytoplasmic RNA to DNA from human adenoviruses and papovaviruses. DNA was cleaved with restriction endonucleases, fractionated on a 0.8% agarose gel, transferred to a nitrocellulose membrane and hybridised with ^{32}P-labelled RNA from BALL-1 cells. The **upper panels** show the DNA fragment pattern and the **lower panels** show the hybridisation pattern. **(a) Lane 1**, Ad2 DNA cleaved with *Eco*RI; **lane 2**, Ad3 DNA cleaved with *Xba*I; **lane 3**, Ad12 DNA cleaved with *Eco*RI; **lane 4**, plasmid pBR322 containing *Bam*HI-linearised BK virus DNA cloned in the *Bam*HI site and cleaved with *Bam*HI; **lane 5**, plasmid pBR322 containing *Bam*HI-linearised JC virus DNA cleaved with *Bam*HI. This particular preparation of Ad2 DNA contains a small amount of DNA which originates from Ad2-defective species. This gives rise to the extraneous submolar bands which run between *Eco*RI -A and -B and between -E and -F. **(b) Lane 6**, Ad2 DNA cleaved with *Hind*III; **lane 7**, Ad2 DNA cleaved with *Bgl*II; **lane 8**, Ad3 DNA cleaved with *Sal*I; **lane 9**, Ad3 DNA cleaved with *Sma*I; **lane 10**, Ad12 DNA cleaved with *Hind*III. **(c)** Restriction endonuclease maps (Tooze, 1981) of Ad2, 3 and 12 and the relative positions of the hybridising cytoplasmic RNA species. The regions which show major hybridisation are indicated by solid bars whilst the hatched bars represent areas of lesser hybridisation. The dashed bar under Ad12 represents a region of very weak hybridisation which is detectable only after prolonged exposure of the autoradiographs. The *Eco*RI -F fragment of Ad2 is shown on an expanded scale to indicate the subfragments which were cloned in phage m13. The regions of this fragment to which hybridisation was observed are indicated by the horizontal arrows which represent the 5′-3′ polarity of the hybridising RNA species. Green *et al.* (1979) were unable to find Ad2-related sequences in RNA from different human tissues. However, the method for detection used by them, DNA-RNA hybridisation in liquid using adenovirus DNA as labelled probe and cell RNA in excess, is not very sensitive for the short, repetitious RNA sequences homologous to only very limited regions of the adenovirus genome, that seem to be responsible for our hybridisation results.

in Figure 5. On the strips with the added EBV DNA (lanes a) the expected homologous hybridisation is observed but, in addition, *Bam* Y and *Bam* K reveal extra multiple, discrete bands of hybridisation which are also present in the samples without exogenously added EBV DNA (lanes b). *Bam*HI-H similarly displays an extra heterogeneous, diffuse hybridisation. These results indicate that portions of the EBV genome are related to human repetitive DNA sequences.

A large proportion of the human genome consists of repetitive sequences of which the *Alu* family is the single most abundant one (Schmid and Jelinek, 1982). These sequences also seem to be transcribed. To test the hypothesis that the virus has incorporated *Alu* sequences during evolution, and that this partly or wholly explains the findings reported here, a cloned *Alu* sequence purified from the plasmid BLUR-8 (Rubin *et al.*, 1980) was isotope labelled and hybridised to blots containing *Eco*RI and *Bam*HI cleaved B95-8 EBV DNA. The results showed that although the *Alu* probe hybridised to the *Eco*RI-A and -J, and the *Bam*HI-C and -W fragments, no hybridisation was found to the fragments shown above to have partial homologies with sequences found in EBV genome-negative cells (data not shown).

Hybridisation of cellular RNA to human adenovirus and papovavirus DNA

It was observed (Figure 3a) that cytoplasmic RNA from various lymphoid and fibroblast cell lines hybridised to specific fragments of Ad2 DNA. Strong hybridisation was observed to Ad2 *Eco*RI fragments A and F whilst fragment B hybridised less well and fragments C, D and E were negative. This result agreed with that of Jones *et al.* (1979) who showed that human placental cell RNA hybridised to Ad2 *Eco*RI fragments A and F. They did not detect the weaker hybridisation to *Eco*RI-B.

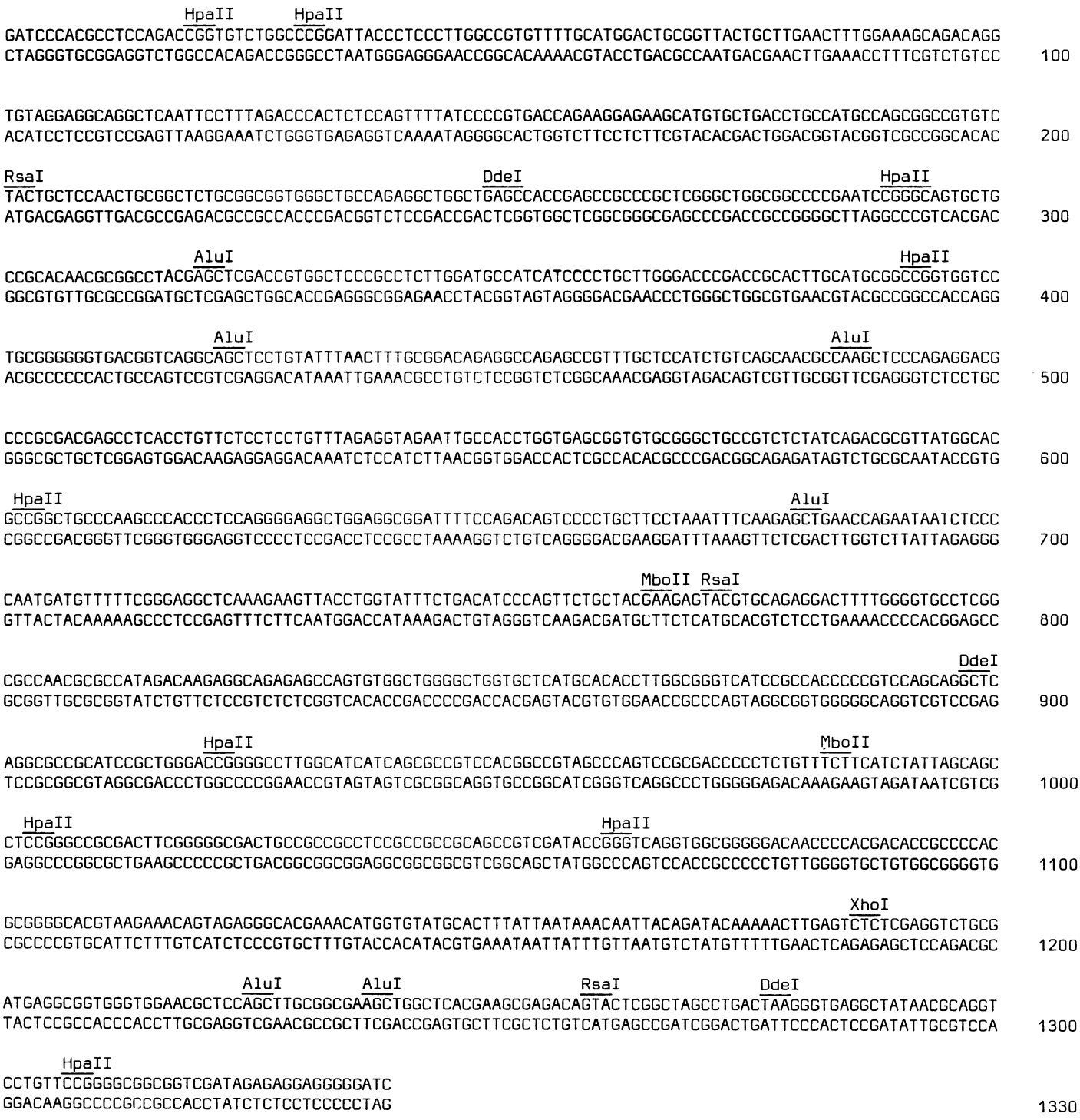

Fig. 7. The sequence of EBV *Bam*HI -F from the vicinity of the rightmost *Sma*I site (see Figure 3b) to the right terminus. The recognition sites of restriction endonucleases referred to in the text are indicated. The sequence shown includes the *Bam*HI linker at the left end. The linking/cloning process appears to have resulted in the loss of a few nucleotides since a *Bam* linker coupled to a 5′ end derived from *Sma*I cleavage should give the sequence 5′GATCCGGGG------3′. The residue at position 12 is the leftmost residue obtained from sequencing subclones derived from authentic (i.e., not *Bam* linked) fragments.

Since RNA from apparently uninfected human cells hybridised to DNA from a human herpes-family virus (EBV) and a human adenovirus (Ad2), it was of interest to see if the same phenomenon was exhibited by DNA from human papovaviruses. Figure 6a shows the results of hybridising polyadenylated cytoplasmic RNA from BALL-1 cells to a nitrocellulose membrane containing DNA from the human papovaviruses BK and JC and the human adenoviruses, type 2 [subgroup C, (Sambrook *et al.*, 1980], type 3 (subgroup B) and type 12 (subgroup A). As before, regions of Ad2 show strong hybridisation. Ad3 shows strong hybridisation to the *Xba*I-B and/or -C fragment and weak hybridisation to the -A and -D fragments whilst Ad12 exhibits hybridisation to the *Eco*RI-C fragment only. The two papovaviruses show no hybridisation.

The regions of hybridisation within the various adenoviral genomes were defined more precisely by similar experiments using different restriction endonucleases to fragment the

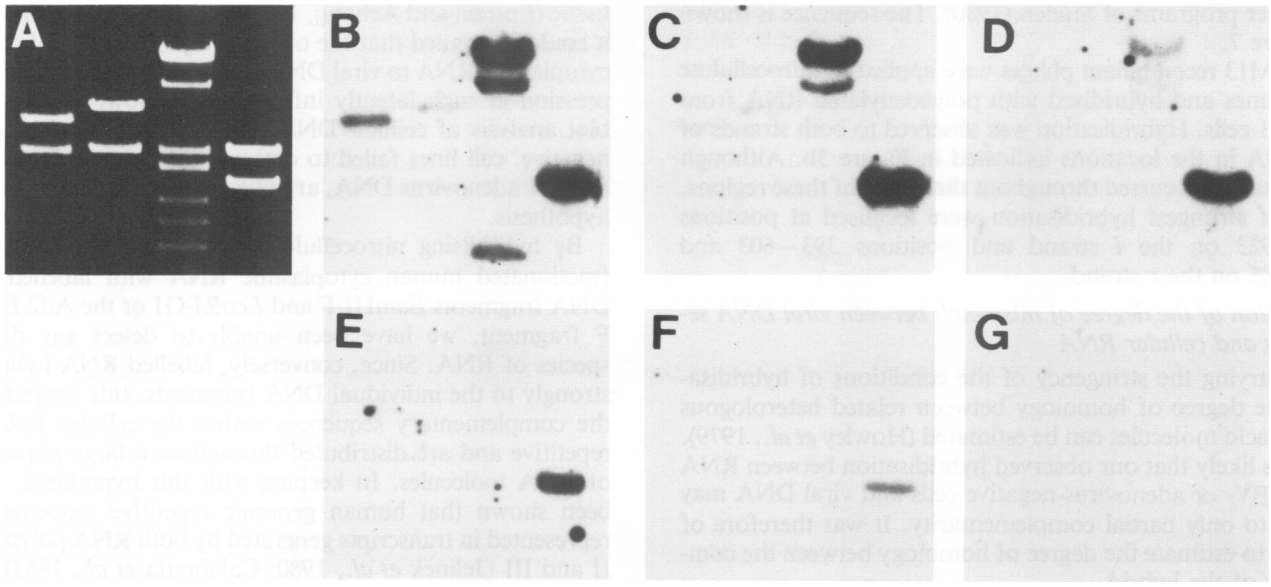

Fig. 8. Estimation of the degree of mismatch in hybrids between cellular cytoplasmic RNA and regions of the EBV or Ad2 genomes. **Panel A** shows the ethidium bromide stained, gel fractionated DNA fragments following (from left to right) *Eco*RI cleavage of plasmid pBR322 containing EBV *Eco*RI -G1, *Bam*HI cleavage of plasmid pBR322 containing EBV *Bam*HI -F, *Eco*RI cleavage of Ad2 DNA, *Eco*RI cleavage of plasmid pBR322 containing EBV *Eco*RI-J. Duplicate nitrocellulose membranes containing these fragments were hybridised with ^{32}P-labelled cytoplasmic RNA from AW-Ramos cells and washed under conditions of **(B)** $T_m - 20°C$, **(C)** $T_m - 15°C$, **(D)** $T_m - 10°C$, **(E)** $T_m - 5°C$, **(F)** T_m, **(G)** $T_m + 5°C$. T_m is related to the base composition of the DNA, the ionic strength of the solvent and the formamide concentration by the empirical equation: $T_m = 81.5 + 16.6$ (log M) + 0.41 (% G+C) − 0.72 (% formamide) where M is the monovalent salt molarity and (% G+C) is the percentage of G+C residues in the DNA (Schildkraut and Lifson, 1965; McConaughy *et al.*, 1969; Frank-Kamenetskii, 1971). In contrast to the situation with DNA-DNA hybrids, depression of the T_m of a DNA-RNA duplex by increasing concentrations of formamide is non-linear (Casey and Davidson, 1977). However, it was determined experimentally that at a formamide concentration of 50%, the reduction in the T_m of a DNA-DNA duplex was very close to that of a DNA-RNA duplex (Casey and Davidson, 1977). Thus, at this formamide concentration, the above equation should be applicable to DNA-RNA hybrids. Since each 1% base mismatch in a hybrid lowers the T_m by 1.4°C (Hyman *et al.*, 1973) it is possible to use the above information to estimate the degree of homology between cellular RNA and viral DNA. If T is the temperature at which a perfect hybrid is eluted from the filter and t is the corresponding temperature for a mismatched hybrid then, assuming that the melting curves are of comparable width, the degree of mismatch can be estimated from the formula: $\frac{T-t}{1.4}$ (Howley *et al.*, 1979; Hyman *et al.*, 1973).

DNA. Ad2 DNA was cleaved with *Hind*III or *Bgl*II, Ad3 DNA was cleaved with *Sal*I or *Sma*I and Ad12 was cleaved with *Hind*III. The patterns of hybridisation of cytoplasmic RNA to these fragments are shown in Figure 6b. Again, the results for Ad2 were consistent with those of Jones *et al.* (1979). Ad3 showed strong hybridisation to the *Sma*I-C fragment and weaker hybridisation to some other regions, whilst in Ad12 the hybridisation was confined to the region defined by *Hind*III-I. A graphical summary of these results is shown in Figure 6c.

Since hybridisation of cellular RNA to the transforming regions of both Ad2 and Ad3 was observed, the possibility was considered that viral infection may play an oncogenic role in some leukaemic cells. However, we were unable to detect viral genomes or discrete fragments containing viral sequences in the cellular DNA by restriction endonuclease cleavage and Southern blot analysis, indicating that these cells are uninfected by virus.

The nature of the viral sequences complementary to cellular RNA

To determine whether there was anything unusual about the primary structure of the viral DNA which hybridised to RNA from apparently virus-negative cells, we examined in detail the *Eco*RI-F fragment of Ad2, the complete nucleotide sequence of which is known (Galibert *et al.*, 1979) and the rightmost *Sma*I-*Bam*HI fragment of EBV *Bam*HI-F (Figure 3b). The *Eco*RI restriction map of Ad2 is shown in Figure 6c together with a fine structure map of the F fragment. The five subfragments obtained by cleavage of Ad2 *Eco*RI-F with *Pst*I and *Taq*I were individually cloned in coliphage M13, and single-stranded recombinant phages which contained each individual strand of each subfragment were selected by sequence analysis. The *l* strand of the 655 nucleotides long *Pst*I-*Taq*I subfragment was not obtained. The nine recombinants were applied to nitrocellulose membranes and hybridised with *in vitro* labelled polyadenylated cytoplasmic RNA from BALL-1 cells. Strong hybridisation was observed to the *r* strand of the 655 nucleotide subfragment and very weak hybridisation to the *l* strand of the 364 nucleotide subfragment. None of the other subfragments showed any hybridisation.

To test the *l* strand of the 655 fragment, the rightmost *Pst*I-*Eco*RI subfragment was strand separated using two different, successive gel systems. Following transfer of the DNA to nitrocellulose and hybridisation as before, RNA was found to be complementary to both the *l* and *r* strands. The locations and polarity of the hybridising RNA species are indicated in Figure 6c.

The rightmost *Sma*I-*Bam*HI subfragment of EBV *Bam*HI-F was cleaved with *Mbo*II, *Alu*I or *Hpa*II. The mixture of fragments generated by each enzyme was ligated to phage M13 sequencing vector and EBV-positive recombinants were selected by hybridisation. These clones were sequenced using the dideoxy method. *Rsa*I, *Xho*I and *Dde*I subfragments from the same region were sequenced by the method of Maxam and Gilbert (1980). The sequences thus obtained were assembled into a contiguous sequence using the

computer programs of Staden (1980). The sequence is shown in Figure 7.

The M13 recombinant phages were applied to nitrocellulose membranes and hybridised with polyadenylated RNA from BALL-1 cells. Hybridisation was observed to both strands of the DNA in the locations indicated in Figure 3b. Although hybridisation occurred throughout the whole of these regions, areas of strongest hybridisation were localised at positions 1060–923 on the *l* strand and positions 393–603 and 765–985 on the *r* strand.

Estimation of the degree of mismatch between viral DNA sequences and cellular RNA

By varying the stringency of the conditions of hybridisation, the degree of homology between related heterologous nucleic acid molecules can be estimated (Howley *et al.*, 1979). It seems likely that our observed hybridisation between RNA from EBV- or adenovirus-negative cells and viral DNA may be due to only partial complementarity. It was therefore of interest to estimate the degree of homology between the components of the hybrid.

Duplicate nitrocellulose membranes each containing the cloned EBV DNA fragments *Eco*RI-G1 and -J and *Bam*HI-F together with an *Eco*RI digest of Ad2 DNA were hybridised to ^{32}P-labelled total cytoplasmic RNA from AW-Ramos cells. The filters were then washed at 38°, 43°, 48°, 53°, 58° and 63°C as described in Materials and methods. Taking the G+C content of both EBV and Ad2 DNA as 58% (Pritchett *et al.*, 1975; Pina and Green, 1965) these temperatures, under the washing conditions used, correspond to T_m −20°, T_m −15°, T_m −10°, T_m −5°C, T_m and T_m +5°C. The results are shown in Figure 8. The G+C content of the EBV J-RNAs used as the positive control is close to 58% (Arrand and Rymo, 1982). This hybrid is only barely detectable at 63°C (predicted T_m +5°C). A comparable level of hybridisation is attained at 48°C by the EBV fragments *Eco*RI-G1 and *Bam*HI-F and by the Ad2 *Eco*RI fragments B and F. This corresponds to a level of mismatch of ~11%. At this temperature, hybridisation is still evident to the Ad2 *Eco*RI-A fragment, but at 53°C the hybrid is unstable, indicating a mismatch of ~7%.

Discussion

This study has demonstrated that cytoplasmic RNA from apparently uninfected human cells shows complementarity to specific regions within the genomes of EBV and human adenoviruses but not human papovaviruses. Specific cloned segments of the viral genomes will hybridise to human cellular DNA in the presence of yeast tRNA but if calf thymus DNA is substituted then the hybridisation is diminished or abolished. This suggests that EBV and adenoviruses contain sequences which are related to conserved sequences of higher eukaryotes. This conclusion is reinforced by the observation that cytoplasmic RNA from mouse 3T6 cells hybridises to the same regions of EBV DNA as does RNA from EBNA-negative human lymphoid cells (our unpublished results) and that RNA extracted from gorilla organs hybridises to the same regions of Ad2 DNA as does human RNA (Jones *et al.*, 1979). Similarly, normal rat cell DNA contains sequences complementary to Ad5 DNA (Frolova and Georgiev, 1979).

It is well known that both EBV and the group C human adenoviruses readily establish latent infections in lymphoid tissue (Epstein and Achong, 1977; Sambrook *et al.*, 1980) and it could be argued that the observed hybridisation of cellular cytoplasmic RNA to viral DNA is due to limited viral gene expression in such latently infected cells. However, Southern blot analysis of cellular DNA prepared from several 'virus-negative' cell lines failed to detect any discrete fragments of EBV or adenovirus DNA, arguing against the latent infection hypothesis.

By hybridising nitrocellulose membranes containing gel-fractionated human cytoplasmic RNA with labelled EBV DNA fragments *Bam*HI-F and *Eco*RI-G1 or the Ad2 *Eco*RI-F fragment, we have been unable to detect any discrete species of RNA. Since, conversely, labelled RNA hybridises strongly to the individual DNA fragments, this suggests that the complementary sequences within the cellular RNA are repetitive and are distributed throughout a large population of RNA molecules. In keeping with this hypothesis, it has been shown that human genomic repetitive sequences are represented in transcripts generated by both RNA polymerase II and III (Jelinek *et al.*, 1980; Calabretta *et al.*, 1981).

DNA-DNA hybridisation experiments (Figure 4) revealed several regions of homology between EBV DNA and virus genome-negative human cellular DNA. Some of these regions (defined by *Bam*HI fragment P and *Eco*RI fragment D_{end}) corresponded to those portions of the genome which were partially complementary to cytoplasmic RNA sequences, whilst others (*Bam*HI fragments Y, H and K) did not appear to be significantly represented in cellular RNA. However, although human cytoplasmic RNA hybridises strongly to *Bam*HI-F (Figures 2 and 3), this fragment does not itself hybridise significantly to cellular DNA. We interpret this to mean that *Bam*HI-F complementary sequences are amplified in the cellular RNA population relative to the DNA. This could arise, for example, by the splicing of a common leader sequence onto the bodies of many different mRNAs.

Heller *et al.* (1982a) have also observed that human DNA contains multiple sequences with homology to *Bam*HI-K. This fragment of EBV DNA itself contains extensive repeated sequences (Heller *et al.*, 1982b; R. Baer, personal communication) as do fragments *Bam*HI-H (Hayward *et al.*, 1982), *Eco*RI-D (Given and Kieff, 1978; Kintner and Sugden, 1979) and *Bam*HI-Y (our unpublished results) which we have shown also hybridise to host sequences (Figures 4 and 5). Recently, Peden *et al.* (1982) also observed homology between *Bam*HI-Y and human DNA.

The finding of viral DNA sequences which have close homology to sequences within the host cell may have implications in virus evolution. Many retroviruses appear to have acquired host genetic information (Weiss *et al.*, 1982) and it is possible that DNA viruses could, in the course of their evolution, have done likewise. Precedents for this hypothesis can be found in studies on papovaviruses. Ding *et al.* (1982) characterised a polyoma virus variant which had incorporated into its genome a 95 nucleotide pair segment of mouse DNA, and Dhruva *et al.* (1980) isolated an SV40 variant which contained an insertion of monkey DNA. Furthermore, three segments of DNA isolated from a monkey genomic library have been shown to hybridise to SV40 DNA (Queen *et al.*, 1981). In both instances with SV40, the homologous cellular segments of DNA were members of a family of repetitious host sequences; a situation analogous to that proposed in this work for EBV and adenoviruses. Several families of human repetitive DNA have been defined

(Rubin *et al.*, 1980; Miesfield *et al.*, 1981; Shafit-Zagardo *et al.*, 1982; Manuelidis, 1982) and some of these show significant evolutionary conservation (Miesfield *et al.*, 1981; Schmid and Jelinek, 1982; Shafit-Zagardo *et al.*, 1982). Such sequence conservation is in keeping with our observation of hybridisation competition by bovine DNA (Figure 4).

EBV DNA has a G+C content of ~58% (Pritchett *et al.*, 1975) whilst Ad2, Ad3 and Ad12 contain, respectively, ~57%, 53% and 40% (Pina and Green, 1965). Since the extent of hybridisation of cytoplasmic RNA from apparently uninfected cells to these DNAs seems to follow the G+C content of the DNA, one trivial explanation for the results could be that the hybridisation is due to 'sticky' G+C-rich regions within the DNA. However, we do not support this hypothesis since: (a) BK virus DNA, which has a G+C content of 40% (Seif *et al.*, 1979) does not hybridise to cellular RNA whereas Ad12 *Hind*III-I with a similar G+C content (42.5%) does (Figure 6); (b) the addition of poly(C) to the hybridisation mix does not affect the level of hybridisation (data not shown); (c) regions of EBV DNA with higher than average G+C content, e.g., the internal tandem reiteration (G+C content 65%, Hayward *et al.*, 1980) or the *Not*I repeat (G+C content 83%, Hayward *et al.*, 1982) do not hybridise any cytoplasmic RNA from EBV-negative cells (Figure 2); (d) the Ad2 *Eco*RI-F fragment has a G+C content of 58% (Galibert *et al.*, 1979), similar to whole Ad2 DNA, whilst the hybridising region of EBV *Bam*HI-F contains ~61% G+C. Allowing for these base compositions, our estimates of the degree of mismatch within these fragments suggest that only 3–4 bases in every 30 are imperfectly paired.

Examination of the sequences of the EBV DNA fragments *Bam*HI-F (Figure 7) and *Eco*RI-G1 (T. Gibson, personal communication), Ad2 *Eco*RI-F (Galibert *et al.*, 1979) and Ad12 *Hind*III-I (Bos *et al.*, 1981) within the limits defined by our RNA mapping data (Figures 3b and 6c) failed to reveal any obvious or unusual structural features which may account for the observed hybridisation of RNA from virus-negative cells. The sequences showed no substantial homology to each other, neither were any extensive symmetric, self-complementary or internally repetitive sequences apparent, as compared with, e.g., the right hand terminal 134 nucleotides of Ad2 DNA (Arrand and Roberts, 1979), a sequence which does not exhibit complementarity with cellular RNA.

The finding that normal host cells contain endogenous seqences with a high degree of homology to potential tumour virus RNA and DNA introduces a note of caution into the interpretation of hybridisation data obtained when either the probe or target is of high complexity. For example, the *in situ* hybridisation experiments of Maitland *et al.* (1981) and Ibelgaufts *et al.* (1982) which indicated that cells from some cervical or neurogenic tumours contained Ad2-related RNA sequences, were performed under conditions corresponding to $\sim T_m - 25°C$. We show here that, under such conditions, virus-negative cell RNA hybridises efficiently to Ad2 DNA (Figure 8).

Cellular transformation by adenoviruses involves integration of viral DNA into the host genome. It is clear that the integration site is not unique in either the cellular or viral DNA and that, in some cases, host repetitious sequences are involved (Sambrook *et al.*, 1979; Stabel and Doerfler, 1982). The DNA sequences of several virus-host junctions (Sambrook *et al.*, 1979; Deuring *et al.*, 1981; Westin *et al.*, 1982; Gahlmann *et al.*, 1982; Stabel and Doerfler, 1982) have revealed the existence of extensive 'patch homologies', i.e., short, interspersed, homologous sequences between viral DNA and host DNA. These homologies are often distant from the point of integration and it was postulated that these partially homologous sequences could align the viral DNA with the host as part of the integration process. In one instance (Stabel and Doerfler, 1982), the patch homologies were shown to involve middle repetitive cellular DNA. These results are consistent with the observations discussed in the present work. It may be significant that adenoviruses from the three major groups all show regions of homology close to the transforming region at the left end of the viral genome. On the basis of the results presented in this paper, a similar 'patch homology' directed process could be involved in the integration of EBV DNA.

Two recent reports (Peden *et al.*, 1982; Puga *et al.*, 1982) described homology between human or murine DNA and herpes simplex virus DNA (and also human cytomegalovirus DNA). As in this work, the homologous mammalian sequences appeared to be repetitive and evolutionarily conserved. Thus, the human viruses EBV, HSV, CMV and adenovirus types 2, 3 and 12 appear to share the common feature of containing within their genomes sequences which are homologous to conserved repetitive sequences of higher organisms.

Materials and methods

Cells and cell lines

The human lymphoid cell lines Raji, Daudi, Namalwa, Ramos, AW-Ramos and Putko were obtained from G. Klein, Karolinska Institute, Stockholm; BALL-1, Riva, HPB-ALL, Molt-4 and DG75 were from M. Greaves, I.C.R.F., London and the human fibroblast cell line Bu from S. Povey, University College, London. Secondary human fibroblasts were obtained from the Department of Microbiology, University of Gothenburg, tonsils which had been surgically removed from children were from University College Hospital, London, and a biopsy from a normal liver was obtained from Sahlgren's Hospital, Gothenburg.

Ramos (Klein *et al.*, 1975) and DG75 (Goldblum, 1977) are B-lymphoid cell lines derived respectively from American and Israeli cases of EBNA (EBV-associated nuclear antigen) negative Burkitt-like lymphomas. Riva is a cell line estabished from a similar tumour which occurred in England (J. Goldman and M. Greaves, personal communication). BALL-1 (Hiraki *et al.*, 1977) is an EBNA-negative B-lymphoid line whereas Molt-4 (Minowada *et al.*, 1972) and HPB-ALL (Minowada *et al.*, 1978) are EBNA-negative T-lymphoid lines, all derived from the peripheral blood of patients with acute lymphoblastic leukaemia. Molt-4 is an unusual T-cell line in that, unlike most T-cells, it possesses EBV receptors on its cell surface (Menezes *et al.*, 1977). Raji (Pulvertaft, 1965), Daudi (Klein *et al.*, 1968) and Namalwa (Klein and Dombos, 1973) are EBV-positive B-lymphoid cell lines derived from African Burkitt's lymphomas. AW-Ramos (Klein *et al.*, 1975) was established as an EBNA-positive derivative of Ramos by infection with the non-transforming P3HR-1 strain of EBV. Putko (Klein *et al.*, 1980) is a hybrid cell line derived by fusion of the myeloid leukaemia cell line K562 with the EBV-containing, Burkitt lymphoma-derived P3HR-1 line. Bu is a line of diploid human fibroblasts derived from an ovarian teratoma (S Povey, personal communication).

Viral DNA and cloned restriction endonuclease fragments of EBV and Ad2 DNA

Purified DNA from human adenovirus types 2, 3 and 12 was a gift from J.E. Arrand. Plasmid pAT153 containing the *Eco*RI-F fragment of Ad2 DNA was obtained from P.H. Gallimore. Plasmids containing the complete genomes of the papovaviruses BK and JC (Howley *et al.*, 1980) were obtained from D. McCance. Plasmids containing *Eco*RI or *Bam*HI fragments of EBV DNA were described previously (Arrand *et al.*, 1981). All plasmids were propagated in *Escherichia coli* and DNA prepared as before (Arrand *et al.*, 1981). DNA was labelled *in vitro* by nick-translation (Maniatis *et al.*, 1975; Rigby *et al.*, 1977).

Restriction endonuclease cleavage, gel electrophoresis and transfer to nitrocellulose membranes

Restriction endonucleases were purchased from Boehringer, Bethesda

Research Laboratories or New England Biolabs except for *Xho*I, *Bgl*II and *Taq*I which were prepared by standard methods (Roberts, 1976). Conditions for endonuclease digestion and gel electrophoresis have been described elsewhere (Arrand *et al.*, 1974; Rymo, 1979). Complementary strands of subfragments of Ad2 *Eco*RI-F were separated on 5% polyacrylamide gels by the procedure of Maxam and Gilbert (1980). Following elution, the separated strands were allowed to reanneal to convert any cross-contaminating species to the double-stranded form. The single strands were then fractionated separately on 1.5% agarose gels using the methods of Hayward (1972). Fragments were transferred to nitrocellulose membranes essentially as described by Southern (1975).

Preparation and labelling of cellular RNA and hybridisation to immobilised DNA fragments

All procedures for the preparation, labelling and electrophoresis of cellular RNA and for hybridisation to DNA fragments immobilised on nitrocellulose membranes have been described previously (Arrand and Rymo, 1982).

To estimate the degree of mismatch in the hybrids, duplicate membranes containing immobilised DNA fragments were incubated with ^{32}P-labelled cellular RNA, washed in 2 x SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) at 68°C and treated with ribonuclease as described before (Arrand and Rymo, 1982). They were then washed at different temperatures in 0.2 M NaCl, 0.01 M N-tris (hydroxymethyl) methyl-2-aminoethanesulphonic acid, pH 7.4, 50% formamide for ~16 h, rinsed in 2 x SSC, dried and autoradiographed.

Preparation of cellular DNA

Total nucleic acids were isolated by lysis of the cells in 0.5% sarcosyl and digestion with Proteinase K (100 μg/ml) for 3 h at 37°C followed by phenol-chloroform extraction and dialysis. For dot hybridisation, aliquots were denatured by alkali, neutralised and spotted (10 μg of DNA in 5 μl) onto nitrocellulose membranes, which had been pretreated by washing in 10 x SSC and drying at room temperature. The membranes were air-dried and then baked for at least 4 h at 80°C. For restriction enzyme analysis, total nucleic acids were digested with pancreatic RNase A (50 μg/ml) for 60 min at 37°C followed by digestion with Proteinase K in the presence of 0.5% sarcosyl. The DNA was then extracted with phenol-chloroform and ether, concentrated by dialysis against polyethyleneglycol, and finally dialysed extensively against 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM EDTA.

DNA sequencing

The rightmost *Sma*I-*Bam*HI subfragment of EBV *Bam*HI-F (see Figure 3b) was cloned in the *Bam*HI site of plasmid pAT153 after the addition of *Bam*HI linkers (Biolabs) to the *Sma* end.

Restriction fragments of DNA for sequencing by the chemical method of Maxam and Gilbert (1980) were prepared and labelled as previously described (Arrand *et al.*, 1980). Small restriction fragments for sequencing by the enzymatic method of Sanger *et al.* (1977) were first cloned in coliphage M13 using conditions described previously (Arrand and Rymo, 1982). Sequence data were assembled using the computer programs of Staden (1980) and analysed using the SEQ system (Brutlag *et al.*, 1982).

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