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Expression and rescuing of a cloned human tumour necrosis factor gene using an EBV-based shuttle cosmid vector

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A cosmid vector carrying the Epstein–Barr virus origin of replication, the EBNA-1 gene, the hygromycin phosphotransferase (*hph*) gene and pBR322 sequences has been constructed. This cosmid can replicate autonomously in the nucleus of human tissue culture cells, even when it carries a 35-kb long insert. The cosmid can be rescued from the transfected cells by cloning it directly into ampicillin-sensitive *Escherichia coli*. A gene for human tumour necrosis factor (TNF) cloned into this cosmid vector was introduced in tissue culture cells, where it was transcribed into mature mRNA.
Key words: Epstein–Barr virus/autonomous replication/DNA-mediated gene transfer/rescuing of cosmids from transfected cells

Introduction

Transfection of eukaryotic cells with DNA (Graham and van der Eb, 1973; Wigler *et al.*, 1979) has proven to be a valuable tool in the study of gene expression. In addition to studies in the regulation of expression, the method has been used recently to clone genes coding for cell surface proteins. In several of these cases, the isolation of the genes was achieved by the introduction of total genomic DNA into eukaryotic cells in culture and subsequent screening of the resultant transfected cells using antibodies specific for the particular surface protein (Kavathas and Herzenberg, 1983; Littman *et al.*, 1985).

In all of these cases success depended on the integration of the foreign DNA into the host's chromosomes to generate cells which carried the introduced gene in a stable form. This approach, however, has two disadvantages. Firstly, the expression of the gene becomes dependent on the integration site, which occasionally masks or modifies the control of its expression. Secondly, in those cases where it is desirable to recover the gene, the task becomes tedious and complicated. Therefore, it would be a great advantage if the introduced DNA were to remain unintegrated in the nucleus of the host cell.

We describe in this paper the construction of vectors, cos202 and cos203, which can support their own replication (as well as that of any gene cloned to them) in the nucleus of human cells which express the EBNA-antigen. To test the behaviour of a gene cloned in these vectors, we cloned the tumour necrosis factor gene, introduced it into cells and studied its expression.

Tumour necrosis factor (TNF) is a protein that was thought to play a role in the elimination of emergent tumour cells. TNF is found in the serum of animals which have been given BCG or other macrophage-activating agents and subsequently injected with endotoxin (for review, see Old, 1985). The protein has the ability to cause necrosis of sarcomas, regression of transplantable tumours and the death of several tumour cell lines. In contrast, it leaves most normal cells unaffected and has little species specificity (Helson *et al.*, 1976). In addition, serum containing TNF was shown to cleave *Plasmodium vinckei* from the blood of injected mice (Clark *et al.*, 1981) and to inactivate malaria parasites *in vitro* (Taverne *et al.*, 1981). In the last few years, however, the isolation of the gene in several laboratories and its expression *in vitro* (Pennica *et al.*, 1985; Shirai *et al.*, 1985; Wang *et al.*, 1985; Marmenout *et al.*, 1985) has led to evidence which showed that TNF is a cytokine with a variety of biological activities. These include effects on lipoprotein lipase synthesis and action (Beutler *et al.*, 1985), on gene expression of adipocytes (Torti *et al.*, 1985), on granulocyte activation (Shalaby *et al.*, 1985), on fibroblast growth (Vilcek *et al.*, 1986) and, finally, a complex interaction with products of the interferon gene family (Pannica *et al.*, 1985; Kohase *et al.*, 1986).

We cloned the human TNF gene in a self-replicating vector (cos202) and then introduced it into human cells in culture. The selected recipient cells expressed the recombinant TNF genes as judged by an RNA detection assay.

Results

Construction of cos202 and cos203

The parent molecule for the construction of cos202 and cos203 was a plasmid kindly provided by Dr Bill Sugden (McArdle Laboratory for Cancer Research, University of Wisconsin). This plasmid contains the following elements: (i) the bacterial origin of replication and the ampicillin resistance gene from plasmid pBR322, (ii) the prokaryotic hygromycin B resistance gene *hph* (hygromycin-B-phosphotransferase) (Gritz and Davis, 1983) under a eukaryotic (HSV-TK) promoter and polyadenylation signal (Sugden *et al.*, 1985) and (iii) the Epstein–Barr virus (EBV) origin of replication and the gene coding for the EBNA-1 antigen (Yates *et al.*, 1985).

To this plasmid the following modifications were introduced: the single *Nru*I site upstream from the 5′ end of the *hph* gene was converted into a *Bgl*II site by the addition of a linker to create p202. In order to generate cos202, a cosmid suitable for packaging large stretches of DNA, the single *Nar*I site between the EBV sequences and the *hph* gene was used to introduce a *Pvu*II–*Nru*I fragment from cosmid pLTC (F.Grosveld, personal communication) containing the λ-phage cohesive ends (Figure 1).

In order to increase the sites that could be used for cloning, a blunted pUC19 *Eco*RI–*Hae*III fragment containing several unique restriction sites was introduced into the blunted *Bgl*II site of cos202 (Figure 1).

Construction of HPB-ALL cosmid library using cos202

HPB-ALL is an established human acute lymphocytic leukaemia cell line (Minowada *et al.*, 1978). High mol. wt DNA was isolated from the nuclei of these cells and partially digested with *Sau*3AI. The size-fractionated DNA (35–40 kb) was then cloned

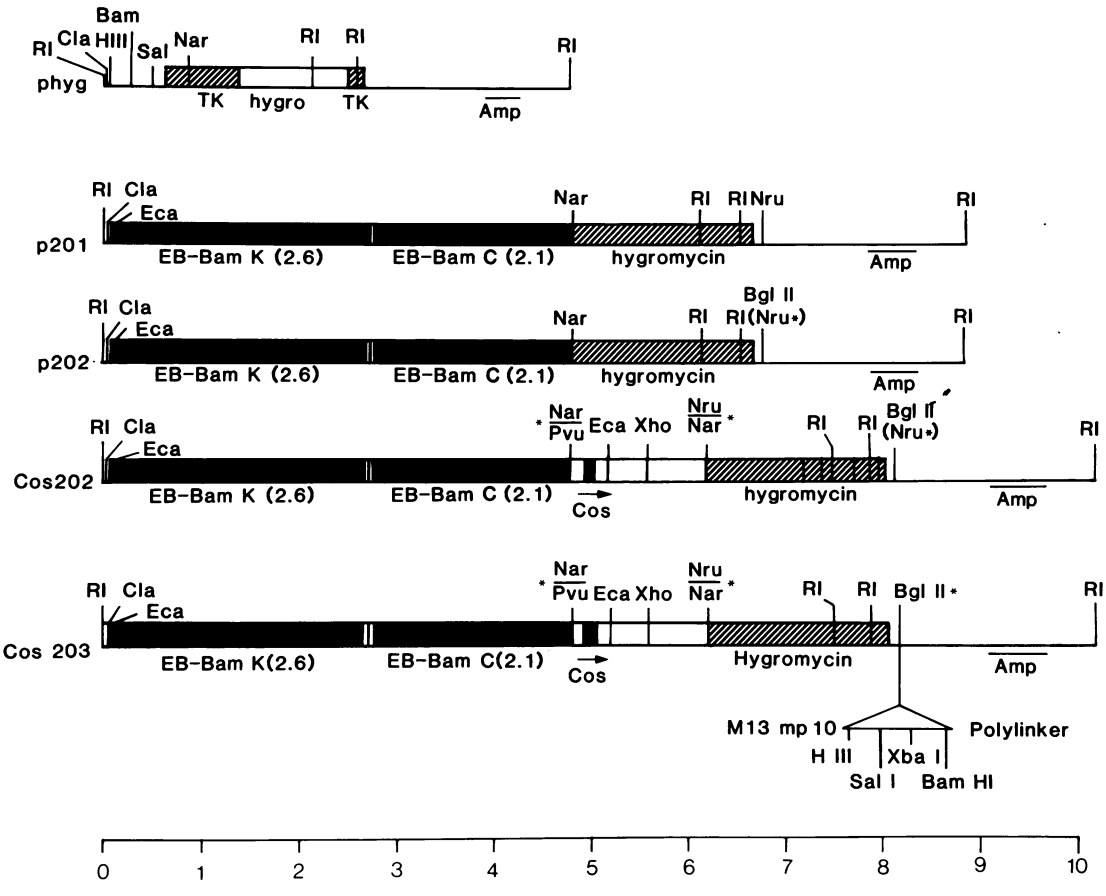

Fig. 1. Structure of cosmid vectors. Cos, phage λ cohesive ends; Amp, β-lactamase gene of pBR322; TK, thymidine kinase gene of herpes simplex virus. Restriction sites marked with * have been destroyed.

into the *Bgl*II cut cos202 and a library of $\sim 10^6$ colonies was generated in *Escherichia coli* ED8767. Random cosmids from this library were isolated and cut with *Eco*RI. All of them were shown to have inserts of ~35 kb of human genomic DNA (data not shown). Assuming random cloning and considering the number of individual colonies, this library represented at least 10 times the haploid genome.

Transfection of human fibrosarcoma cells with total cosmid genomic DNA

The 10^6 colonies of the HPB-ALL cos202 library were grown in bulk and total cosmid DNA was isolated by CsCl gradient centrifugation. The purified cosmid supercoils were subsequently used to transfect the human fibrosarcoma cell line 143/EBNA (see Discussion). This cell line has already been transfected with a plasmid pSVoB-H2.9 containing the G418 (neomycin) resistance gene, the EBNA gene and the transcriptional enhancer of SV40, giving rise to a cell which expresses EBNA in a constitutive manner (Yates *et al.*, 1985).

Ten μg of cosmid DNA were used to transfect 5×10^5 cells on a 90-mm culture dish by calcium phosphate precipitation (Wigler *et al.*, 1979). Trypsinizing the cells 24 h later and splitting them 1:5 increased the number of resistant colonies 5- to 10-fold.

Selection was applied 48 h after transfection by the addition of hygromycin-B at 150 μg/ml of medium. Hygromycin-resistant colonies appeared within 10–15 days at a frequency of 1 in 500 cells and 40 000 such colonies were generated. Assuming that there is one cosmid in each eukaryotic colony, then 50% of the introduced haploid genome would be represented in this collection of eukaryotic colonies. If there are 10 different cosmids in each eukaryotic colony, then the equivalent of five haploid genomes would be contained in this population of cells.

Rescue of cosmids from the transfected cells

To rescue the cosmids from the transfected cells, individual colonies, or populations from the transfection experiment were grown and the supercoiled DNA was extracted by a modified method used for the isolation of plasmid DNA from bacterial cells (Birnboim and Doly, 1979). If the rescued DNA contained any supercoils of the introduced cosmids, then it should be possible to clone these directly in Amp-sensitive *E. coli* by cosmid/phage packaging (Grosveld *et al.*, 1981).

Small mol. wt DNA from the nuclei (10^7) of two confluent culture dishes yielded ~100–1000 Amp-resistant bacterial colonies. When the plasmid content of these colonies was analysed, it was found that they contained cosmids of ~35–45 kb (Figure 2).

In our hands, supercoiled cosmid DNA isolated from *E. coli* generates in a packaging experiment $\sim 1-2.5 \times 10^5$ colonies/μg of DNA. If we assume the same efficiency of packaging using supercoiled cosmids isolated from the nuclei of the transfected cells, then two confluent plates which give rise to 100–1000 colonies would contain 0.4–4 ng of cosmid DNA, or the equivalent of 10^7-10^8 cosmids, or 1–10 copies/cell nucleus of transfected cell.

DNA isolated from a single eukaryotic colony yielded the same cosmid in 12 of the prokaryotic colonies. In contrast, when populations of eukaryotic colonies were used as a source of super-

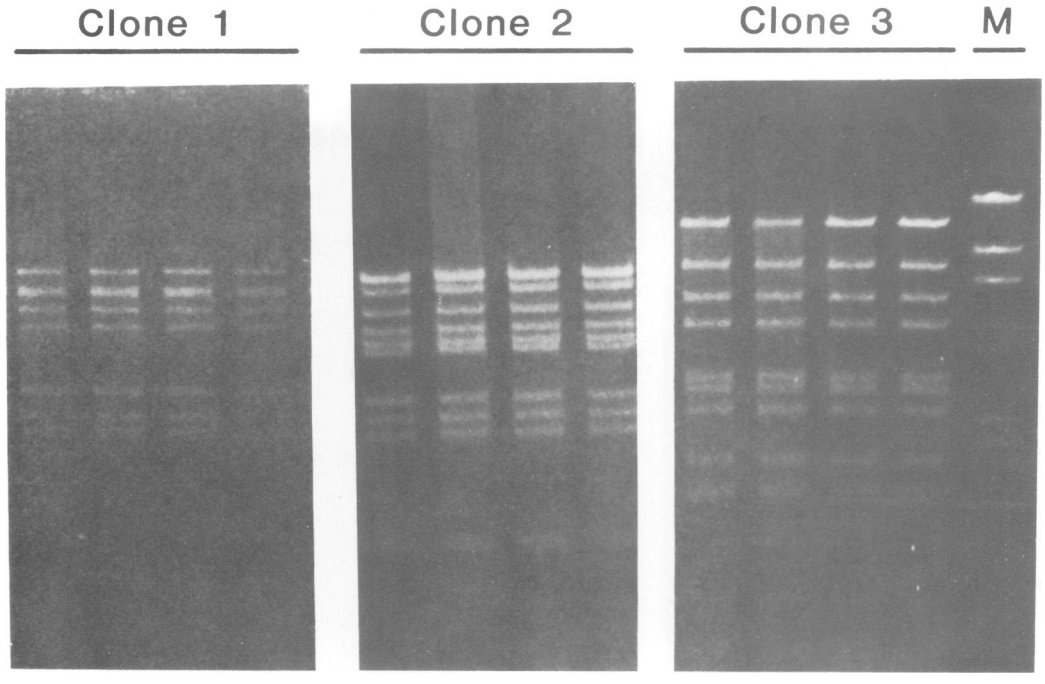

Fig. 2. *Eco*RI restriction digests of cosmids rescued from three independent human fibrosarcoma clones. M, mol. wt marker; λ DNA cut with *Hin*dIII.

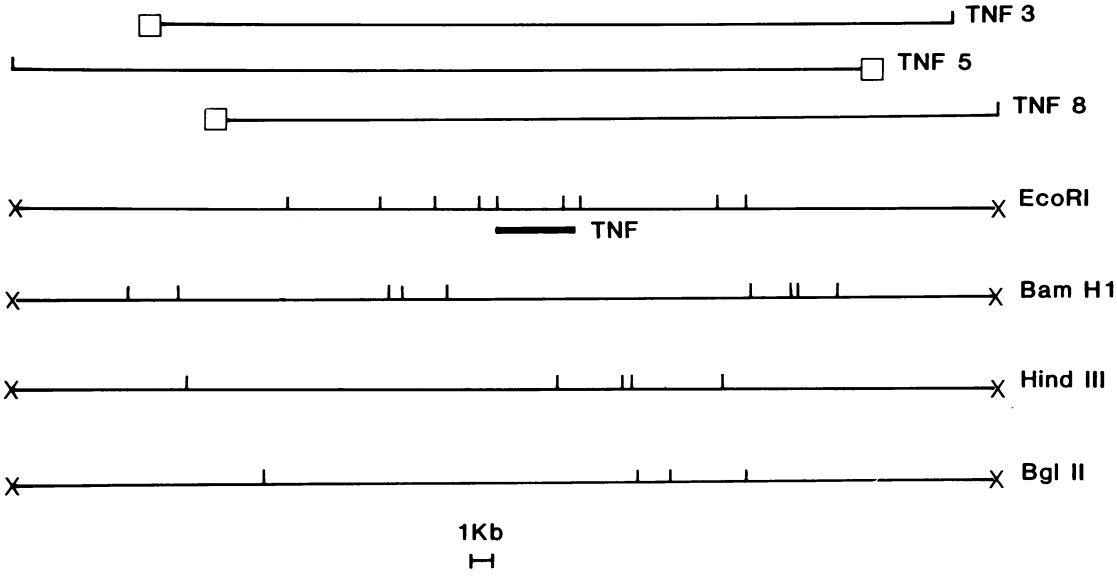

Fig. 3. Restriction map of the TNF gene locus and location of cosmids containing the TNF gene.

coiled DNA, then a variety of cosmids were isolated from the bacterial cells. These experiments showed that it is possible to introduce large cosmids into eukaryotic cells and to rescue them from the nuclei of the transfected cells.

Isolation of TNF gene

To study the expression of gene(s) present on the introduced cosmids, we decided to isolate the TNF gene from the HPB-ALL cos202 genomic library. Two oligonucleotides specific for TNF sequences, 22 and 23 bases long, were synthesized and their 5′ end was labelled using polynucleotide kinase and [γ-^{32}P]ATP. Replica nitrocellulose filters of the cosmid library colonies were hybridized to these probes. Several positive colonies were identified and analysed by restriction mapping.

Southern blots of human genomic DNA cut with *Eco*RI (not shown) and published maps of the human TNF gene (Shirai *et al.*, 1985) suggested that the oligonucleotides should hybridize to a 2.8-kb *Eco*RI fragment. Indeed, 70% of the cosmids contained a 2.8-kb *Eco*RI band which hybridized to both oligonucleotide probes and shared a variety of flanking fragments due to the overlapping nature of the cosmids. Using a variety of restriction endonucleases we were able to construct a map of the TNF locus and three cosmids, TNF3, TNF5 and TNF8, were chosen for further study (Figure 3).

Introduction of TNF cosmids into human fibrosarcoma cells

Human fibrosarcoma cells were transfected with the cosmids TNF3, TNF5 and TNF8 independently, using the calcium phos-

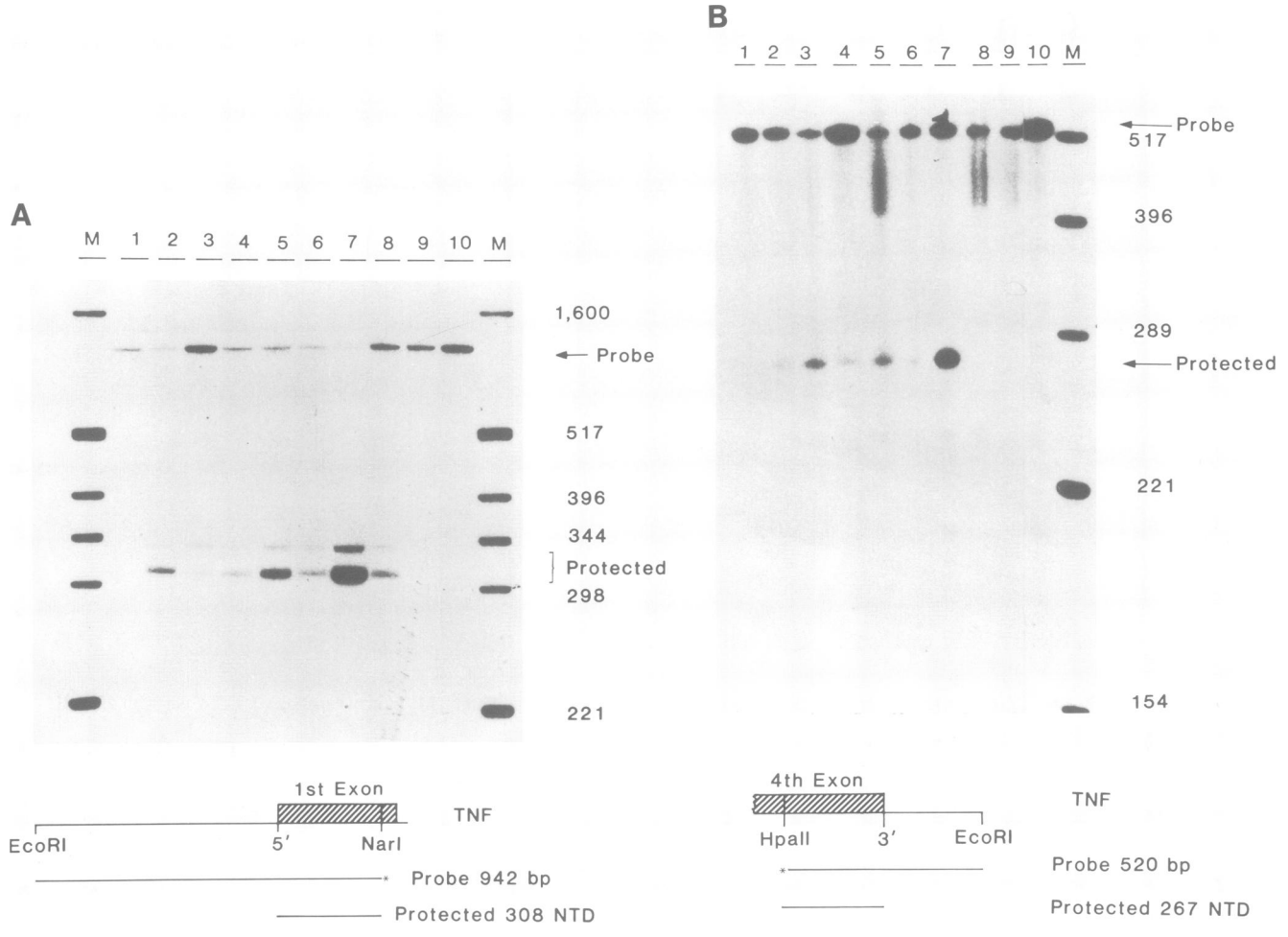

Fig. 4. S1 analysis of TNF gene transcripts in human fibrosarcoma cells **(lane 1)**; in populations of human fibrosarcoma cells transfected with cosmid TNF3 **(lane 2)**, TNF5 **(lane 3)**, TNF8 **(lane 4)**; in human clones isolated from these populations: clone 3.1 **(lane 5)**, clone 3.5 **(lane 6)**, clone 5.2 **(lane 7)**, clone 8.2 **(lane 8)**, clone 8.3 **(lane 9)**; **lane 10**, tRNA; M, mol. wt markers. (The 3′ protected fragment of clone 8.2 [**B, lane 8**] is not evident in the exposure shown in this figure.) **(A)** 5′ end S1 analysis of TNF transcripts. **(B)** 3′ end S1 analysis of TNF transcripts.

phate coprecipitation method. Hygromycin-B resistant colonies were established by the 10th day after transfection. Individual clones were picked from the plates and grown separately, while the rest of the colonies were grown as three distinct populations of cells: HF-TNF3, HF-TNF5 and HF-TNF8.

TNF mRNA analysis in human fibrosarcoma cells

Clones and populations were grown and RNA was isolated using the guanidinium-hydrochloride method of lysing the cells (Chirgwin *et al.*, 1979).

The following probes were used for analysis of the RNA (Figure 4). (i) A 5′ probe from the *Nar*I site in the first exon of the gene to the *Eco*RI site 924 bp upstream. Correctly initiated mRNA protects 308 nucleotides of this probe from S1 nuclease digestion (Nedwill *et al.*, 1985). A second band of minor intensity was observed in all samples. This second band was also observed when we used TNF mRNA isolated from the TNF producing human cell line HL60 (Wang *et al.*, 1985). At present, we do not know whether this band represents an alternative cap site (Kollias *et al.*, 1985). (ii) A 3′ probe from the *Hpa*II site at the 3′ untranslated region of the third exon, to the *Eco*RI site 520 bp downstream. Correctly terminated RNA protects 267 nucleotides of this probe from S1 nuclease digestion (Nedwill *et al.*, 1985).

As shown in Figure 4a and b, TNF mRNA was not detected in untransfected human fibrosarcoma cells (lane 1). In contrast, when RNA from transfected cells was used, the predicted protected fragments were observed in the majority of cases (Figure 4, lanes 2–8). All populations expressed TNF mRNA in this test, while most of the clones showed variable levels of expression with a minority not expressing detectable levels. Taking into account the specific activity of the probe and the amount of RNA used, we estimate that there are two (lane 2), up to 50 (lane 7) copies of mRNA per cell. This analysis showed that the TNF gene is transcribed in the transfected cells, and that the mRNA produced has identical 5′ and 3′ ends to naturally occurring TNF mRNA (D.Kioussis, unpublished data).

Rescue of TNF cosmids from the transfected fibrosarcoma cells

To rescue the TNF cosmids from the transfected fibrosarcoma cells, populations HF-TNF3, HF-TNF5 and HF-TNF8, and clones 3.5, 5.2 and 8.3 were grown and the supercoiled DNA was isolated from their nuclei. The isolated DNA was packaged into phage particles and used to infect *E. coli* cells. Ten to 1000 ampicillin resistant colonies were generated from each DNA preparation and the cosmid DNA was isolated and cut with *Eco*RI.

In 90% of cases, the cosmid rescued from the nucleus of the transfected cells was identical to the one used to transfect the HF cells. On a few occasions, however, the rescued cosmid seemed to differ by one or two *Eco*RI fragments (Figure 5, lane 3). The rearranging fragment was found to contain repetitive

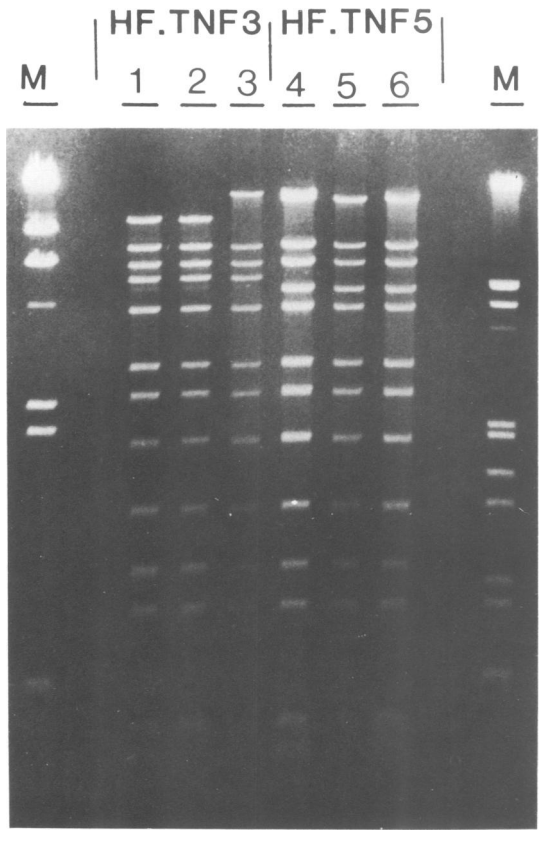

Fig. 5. *Eco*RI restriction digests of TNF cosmids rescued from populations of human fibrosarcoma cells transfected with cosmid TNF3 **(lane 2,3)** or cosmid TNF5 **(lane 5,6)**. The *Eco*RI restriction digests of the cosmid used for transfection are shown in **lane 1**; cosmid TNF3, **lane 4** cosmid TNF5. M, mol. wt markers.

elements when it was hybridized to nick-translated total human genomic DNA (data not shown). It was not possible to follow the fate of the missing DNA fragment from the clones which exhibited rearrangements (Figure 5, lane 3), since it is of a human repetitive nature and was introduced in a human cell. Examples of intact and rearranged rescued cosmids are shown in Figure 5. This experiment showed that we could produce a known cosmid into eukaryotic cells and rescue it back, usually without structural changes.

Discussion

We describe in this paper the construction of cosmid vectors (cos202 and cos203) which can replicate autonomously in the nucleus of eukaryotic cells. Vector cos202 was used to construct genomic libraries and the cosmid DNA from this library was introduced into eukaryotic cells expressing the EBNA-1 antigen (Yates *et al.*, 1985). When these cosmids enter the nucleus of the cell, the EBNA-1 antigen interacts with the ori P segment of EBV which is present on the vector. This interaction is sufficient to allow the cosmid to replicate as a free mini chromosome in the nucleus. Despite the fact that the vector contains the EBNA-1 antigen gene, we found that using the $EBNA^+$ human fibrosarcoma cell line 143/EBNA increased the efficiency of transfection and of rescuing, compared with the efficiency obtained with cultured human cells which were $EBNA^-$. HeLa cells transfected with cos202, without insert, gave a transfection efficiency of $\sim 10^3$ colonies/5 μg DNA/5 $\times$ 10^5 cells. Transfected with cos202 with a 35-kb insert, the efficiency decreased by two orders of magnitude to 10 colonies/5 μg DNA/5 $\times$ 10^3 cells, even when enough DNA was added to the culture to compensate for the fewer copies of vector equivalent present in each μg of DNA, but it was restored to 500–1000 colonies/5 μg DNA/5 $\times$ 10^5 cells when we used HeLa cells which were previously transfected with pSVoB-H2.9 (Yates *et al.*, 1985), and thus had the EBNA gene integrated stably in their genome (D.Kioussis, unpublished data). It is possible that the genomic insert cloned into the vector disturbs the expression of the vector EBNA-1 gene. Alternatively, it is possible that larger cosmids replicate slower in the nucleus of the transfected cells and this leads to fewer copies of EBNA molecules. Any of these events would decrease the chances of stable transformation. Perhaps the addition of a strong constitutive promoter in front of the EBNA gene would be an improvement on the vectors, since this would make the EBNA expression independent of the presence of cloned DNA on the vector.

Cosmids containing the human TNF gene were isolated from the HPB-ALL cos202 library and were introduced into the human fibrosarcoma cell line 143/EBNA. S1 nuclease analysis demonstrated that the cells produced a TNF mRNA with the expected 5′ and 3′ ends.

Preliminary results indicate that this mRNA is translated into active TNF as judged by the ability of the transfected cells and their medium to kill sensitive L-cells, while L-cells resistant to TNF remain unaffected (J.Taverne and C.Leveton, unpublished data). This observed tumour cell killing activity seems to be abolished by an antibody specific for TNF (kindly provided by Dr Leo Lin of Cetus Corporation). Normally, TNF is synthesized as a long preprotein of 233$\alpha\alpha$. Subsequent cleavage between $\alpha\alpha$76 and $\alpha\alpha$77 liberates a shorter protein of 157$\alpha\alpha$ from the COOH terminus (Pennica *et al.*, 1985). This final protein is secreted and it is the active molecule found in tumour necrosis sera. At the moment, it is not clear whether the activity we observe in the transfected cells and their supernatants is due to the whole preprotein, or that the host cell line is capable of processing the preprotein into its mature form.

As has been observed with other genes, the transfected eukaryotic clones and populations showed variability in the amounts of TNF mRNA that they synthesized. As we discuss below, the introduced TNF gene exists in the nucleus as a free episome, so that its expression cannot be affected by neighbouring sequences or the state of the chromatin at the site of integration. It is possible, in this case, that the variability in expression is due to different copy numbers of the TNF cosmid in the nucleus of the host cells.

When we isolated supercoiled DNA from the nucleus of the TNF expressing clones, we were able to transform *E. coli* bacteria which were subsequently shown to contain a cosmid identical to the cosmid used to transfect the eukaryotic cells. On a few occasions, we recovered a cosmid which showed signs of rearrangement, although the structure of the gene and its flanking sequences remained intact. By hybridizing the TNF cosmids to nick-translated human genomic DNA, it was found that the bands that had undergone rearrangements contained repetitive sequences.

The rearrangement could have occurred in two sites, either in the rescuing *E. coli* bacterium, or in the host eukaryotic cell. The first explanation seems unlikely because the *E. coli* strain used is $recA^-$ and is constantly tested for the appearance of revertants. In addition, when the original cosmid isolated from *E. coli* is repackaged and introduced into the same $recA^-$ cells, it remains unrearranged. It is more likely that rearrangements within the host eukaryotic cells are implicated. It has recently

been established that DNA introduced into tissue culture cells undergoes homologous recombination (Thomas *et al.*, 1986). The cosmids we introduced into the human fibrosarcoma cells carry repetitive sequences, which could be ideal for recombination targets, due to their abundance. At the moment we cannot distinguish whether the recombination occurs among cosmids, or between the introduced cosmids and the repetitive sequences resident in the host's genomic DNA, or both. Whatever the mechanism, it is of note that if the recombination does not remove the EBV origin of replication, the molecules in their majority remain unintegrated. It is possible, therefore, to use the vector as a recombination target or donor of sequences to the genome. The possibility of integration events was checked by Southern blot analysis of the genome of the host cells (D.Kioussis, unpublished data). However, the results were inconclusive, due to the fact that the host cell carries resident plasmid sequences because of an earlier stable transfection with pSVoB-H2.9 (Yates *et al.*, 1985) and the fact that the insert of the cosmid contained a lot of repetitive sequences. Since the integration event could have occurred at any point in the cosmid and our choice of probes was limited, we could not exclude at the moment the possibility that integration may have happened during cell culture. This is a point that will best be followed after the introduction to the eukaryotic cells of single copy DNA cosmids or cosmids with inserts from the genome of an unrelated species. It should be noted that when a smaller insert coding for the human HLA-B7 gene was cloned into the cos203 vector, we observed no rearrangement and a high rescuing efficiency (F.Wilson and D. Kioussis, unpublished data).

The autonomous replication of these cosmids, in conjunction with the high efficiency with which they can transfect human EBNA$^+$ cells in culture, offers an attractive alternative method for cloning single chain, cell surface protein genes. Enough eukaryotic colonies which contain large cosmids can be generated easily and comprise the equivalent of a eukaryotic library. It is generally assumed that most genes, when introduced into the nucleus of cells as naked DNA, can be expressed to a certain degree. If the cosmids carry a gene whose product can be detected on the surface of the transfected cells by a specific antibody, then these cells can be isolated and the cosmid responsible for the expression can be cloned in *E. coli* directly. We are currently employing this approach to clone genes which code for proteins on the membrane of human T-cells.

Materials and methods

Materials

Restriction enzymes (Boehringer, New England Biolabs, Anglian Biotechnology), T4 DNA ligase (Anglian Biotechnology), T4 polynucleotide kinase, Proteinase K (Boehringer) were used according to the manufacturers' recommendations.

Isolation of DNA

High mol. wt DNA from the human T-cell line HPB-ALL was isolated as follows: cell membranes were disrupted in 0.2% NP-40 in 10 mM Tris (pH 7.4), 10 mM $MgCl_2$, 10 mM NaCl, 10 mM $CaCl_2$ and the nuclei were lysed in 1% SDS in 50 mM Tris (pH 7.4), 100 mM NaCl, 10 mM EDTA and incubated at 37°C with 100 μg/ml Proteinase K for 3 h. After two extractions with phenol:chloroform:isoamylalcohol (25:25:1), the DNA was precipitated with two volumes of ethanol. DNA fragments were isolated from gels using low-temperature gelling agarose (Wieslander, 1979).

Construction of the genomic cosmid library

A cosmid genomic library was constructed using cos202 cut with *Bgl*II and human DNA from the HPB-ALL T-cell line. The genomic DNA was cut partially by *Sau*3A and the 35–40-kb size fragments were isolated from a salt gradient. Ligation and packaging of the DNA was performed essentially as previously described (Grosveld *et al.*, 1981). Replica filters were treated for hybridization, while a master copy was kept at −70°C as a 'Hogness' sandwich.

Screening of the cos202-HPB-ALL library using oligonucleotide probes

Two oligonucleotides (23 and 22 bases long) were synthesized and labelled at their 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase (Boehringer) in a buffer containing 25 mM Tris (pH 9.0), 5 mM $MgCl_2$, 5 mM dithiothreitol and 25 μg/ml bovine serum albumin.

The treated nitrocellulose filters were prehybridized in a 65°C shaking waterbath overnight in a solution of 5 × SSC, 50 mM pyrophosphate, 10 × Denhardt, 250 μg/ml denatured salmon sperm DNA, 7% SDS and 10% dextran sulphate (1 × SSC = 150 mM NaCl, 15 mM Na-citrate; 1 × Denhardt = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin). Hybridization was performed at 55°C in the same solution containing the labelled oligonucleotides. The filters were washed at hybridization temperature and to a final concentration of 1 × SSC. The filters were then autoradiographed for 14 days using X-ray Fuji film.

Calcium phosphate DNA transfection of tissue culture cells

Human fibrosarcoma cells 143/EBNA were grown in D-MEM, 10% FCS and seeded at a density of 5 × 10^5/90-mm dish in 10 ml of medium 1 day prior to transfection. Ten μg of cosmid DNA were coprecipitated with calcium phosphate as previously described and applied onto the cells (Wigler *et al.*, 1979). Twenty-four hours later, the medium was replaced and after a further 24 h the cells were split 1:5 and selection was employed by adding hygromycin B to a final concentration of 150 μg/ml.

Isolation of supercoiled small mol. wt DNA from the nucleus of transfected cells

The method used is the one described for the isolation of plasmid DNA from prokaryotic cells (Birnboim and Doly, 1979). The cells (10^7) harvested by trypsinization and centrifugation were resuspended in 200 μl of a buffer containing 50 mM glucose, 10 mM EDTA and 25 mM Tris (pH 8.0). The cells were subsequently lysed by adding 400 μl of 0.2 N NaOH in 1% SDS and the high mol. wt DNA was precipitated by the addition of 200 μl of 3 M KoAC pH 4.8. After centrifugation the supernatant was precipitated with 0.6 volumes of iso-propanol. The pellet was dissolved in 200 μl TNE [10 mM Tris (pH 7.4), 50 mM NaCl, 10 mM EDTA] and the DNase-free RNase was added to the final concentration of 20 μg/ml. After incubation at 37°C for 1 h, the reaction mixture was extracted with phenol and precipitated with two volumes of ethanol.

The final pellet was dissolved in 10 μl of H_2O and used for packaging into *E. coli* as previously described (Grosveld *et al.*, 1981).

Purification of RNA and S1 nuclease analysis

Approximately 10^7 cells were collected and the RNA was isolated by the guanidinium chloride method (Chirgwin *et al.*, 1979). Levels of TNF-RNA were measured by S1 analysis (Berk and Sharp, 1977; Weaver and Weissmann, 1979) using end-labelled probes as indicated in the figures.

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