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(57) Abstract

The invention relates to the field of genetically engineered viral vectors, more specifically to adeno–associated virus (AAV) vectors, for use in gene therapy. The present invention provides a process for the production of high titer recombinant adeno–associated virus vectors that are essentially free of helper virus such as adenovirus. The invention provides an adeno–associated virus (AAV) packaging cell having been provided with nucleic acid encoding a gene product providing AAV helper function allowing generating recombinant AAV without concomitant helper virus production.

Title: Improved AAV vector production

The invention relates to the field of genetically engineered viral vectors, more specifically to adeno-associated virus (AAV) vectors, for use in gene therapy.

Adeno-associated virus) is a non-pathogenic human parvovirus (reviewed in (Berns, 1990a; Berns, 1990b)). The virus replicates as a single strand DNA of approximately 4.6 kb. Both the plus and the minus strand are packaged and infectious. Efficient replication of AAV requires the co-infection of the cell by a helper virus. Viruses which have been identified to help AAV are the adenoviruses, herpes simplex virus (HSV), cytomegalovirus (CMV) and pseudorabies virus (Berns, 1996). In the absence of a helper virus no substantial replication of AAV is observed. AAV is therefore also classified as a dependovirus. When no helper virus is present, the AAV genome can integrate into the host cell genome. The wild-type virus has a strong preference (70%) for an integration site on the long arm of chromosome 19 (q13.3-qter) (Kotin et al, 1990; Samulski, 1993; Samulski et al, 1991). Following integration, the expression of the virus genes is not detectable. The integrated provirus replicates as a normal part of the host cell genome upon division of the transduced cell and ends up in both daughter cells. This stage of the virus life cycle is known as the latent stage. This latent stage is stable but can be interrupted upon infection of the transduced cell by a helper virus. Following infection of the helper virus, AAV is excised from the host cell genome and starts to replicate. During the early phase of this lytic cycle the rep-genes are expressed. Approximately 12 to 16 hours later the capsid proteins VP1, VP2 en VP3 are produced in detectable amounts and the replicated virus DNA is packaged into virions. A schematic

representation of the AAV-genome and its genes is depicted in figure 1. The virions accumulate in the nucleus of the cell and are released when the cell lyses as a result of the accumulation of AAV and the helper virus (reviewed in Berns, 1990a; Berns, 1990b). Six primate AAV serotypes have been characterised thus far (Berns et al, 1994; Rutledge et al, 1998). The AAV-genome contains two genes rep and cap (Fig. 1). Three promoters (P5, P19 and P40) drive the synthesis of mRNAs coding for 4 Rep-proteins (Rep78, Rep68, Rep52 and Rep40) and three capsid proteins (VP1, VP2 and VP3). The AAV-genome is flanked on both sides by a 145 bp sequence, called the Inverted Terminal Repeat (ITR), which appears to contain all the cis-acting sequences required for virus integration, replication and encapsidation(Lusby et al, 1980; Samulski et al, 1989).

During a productive infection the P5-promoter is activated first and directs the production of Rep78 and Rep68. These proteins are essential for AAV-replication and trans regulation of viral genes. Rep52 and Rep40 are expressed from the P19 promoter and are thought to be involved in the packaging of AAV-genomes (Chejanovsky and Carter, 1989; Smith and Kotin, 1998). The capsid proteins VP1, VP2 and VP3 are produced from a 2.6 kb transcript of the AAV P40 promoter, which is spliced into two 2.3 kb mRNAs by using the same splice donor but two different splice acceptor sites. The splice acceptor sites are located at both sides of the VP1 translation start signal. VP1 is translated from the messenger that uses the splice acceptor directly in front of the VP1 translation initiation codon. VP2 and VP3 are translated from the messenger RNA that is spliced to the acceptor 3' of the VP1 ATG. The proteins VP2 and VP3 are translated from this messenger by use of an ACG translation start (VP2) or a downstream ATG (VP3). Since all three coding regions are in frame, the capsid proteins share a large

domain with an identical amino-acid sequence. VP3 is entirely contained within VP1 and VP2, but the latter two contain additional amino-terminal sequences. Similarly, VP1 contains the entire VP2 protein but carries an additional N-terminal sequence. All three capsid proteins terminate at the same position (Ruffing et al, 1994). The AAV capsid is 20 to 24 nm in diameter (Berns and Bohensky, 1987; Srivastava et al, 1983) and contains approximately 5% VP1, 5% VP2 and 90% VP3. This ratio is believed to reflect the relative abundance of the alternatively spliced messengers and the reduced translation initiation efficiency at the ACG initiation codon for VP2.

Adeno-associated virus vectors can be made by replacing the rep- and cap-gene sequences in the wild type AAV with the sequence of interest. To produce the recombinant virus, concomitant helper virus infected human cells need to be supplied with the rep- and cap-genes through different means. This is routinely done through transfection of a so-called packaging plasmid, providing AAV-packaging function, containing the AAV-rep- and cap-genes but lacking the AAV-ITRs. Recombinant AAV is typically generated by co-transfecting a packaging plasmid together with a plasmid containing the recombinant AAV into helper virus infected cells. The recombinant virus is typically harvested from such cultures 48 to 72 hours after transfection of the cells. Recombinant AAV generated in this way is high titre and can be made essentially free of wild type AAV (Allen et al, 1997; Samulski et al, 1989). Since the cells are also concomitantly infected with a helper virus, usually adenovirus, this helper virus is also produced (Clark et al, 1997; Flotte et al, 1993; Herzog et al, 1997; Monahan et al, 1998; Snyder et al, 1997a). AAV-replication and also packaging can be accomplished in the test tube using a cell free system (Hong

et al, 1992; Hong et al, 1994; Ni et al, 1994; Zhou and Muzyczka, 1998; Ward et al, 1998).

The presence of helper virus in rAAV preparations is not desirable. The concomitant helper virus is a potential pathogen and even minor contaminations of recombinant AAV preparations with the helper virus are not acceptable for clinical use. Several methods are employed to remove the helper virus from the recombinant AAV preparations. In case of adenovirus these include differences in density and temperature sensitivity. AAV particles have a density of 1.41 to 1.45 g/cm^3 whereas adenovirus 2 and 5, the most commonly used helper viruses have a density of 1.33 g/cm^3. With density gradient centrifugation this difference is utilised to separate the two viruses (Clark et al, 1997; Herzog et al, 1997). Differences in temperature sensitivity are also used to remove contaminating adenovirus. Adeno-associated virus particles are more resistant to heat treatment than adenovirus particles. Routinely, recombinant AAV preparations are incubated for 1 hour at 56 ^{0}C. The recombinant AAV is resistant to this treatment whereas the adenovirus helper virus is not (Flotte et al, 1993; Monahan et al, 1998; Snyder et al, 1997a). Although these methods are adequate to remove most of the helper virus they are not ideal for clinical applications of recombinant AAV. One reason is that for clinical applications large amounts of recombinant AAV need to be produced. This implies that also large amounts of helper virus are produced which must then subsequently be removed completely from the rAAV preparation. In addition, the process of validating the absence of the helper virus is difficult.

The invention is directly related to the generation, production and purification of genetically engineered viral vectors designed to introduce and express a gene of interest

in mammalian cells. The present invention provides a process for the production of high titer recombinant adeno-associated virus (AAV) vectors that are essentially free of helper virus such as adenovirus. Several viruses can provide helper functions for AAV. The helper function of adenovirus is momentarily the best characterised. In adenovirus, four regions have been identified that are required for fully permissive AAV infection. These are the E1, E2a, E4orf6 and VA regions. In E1, genes from both the E1a and the E1b region are important. Studies in which the role of these genes has been discovered and characterised are reviewed in (Carter, 1990). HSV can also function as a helper virus for AAV. HSV genes with helper virus function identified so far include the ICP 8 and the IPC 4 genes, the viral DNA polymerase and possibly the viral helicase (Berns, 1996). The invention provides an adeno-associated virus (AAV) packaging cell having been provided with nucleic acid encoding a gene product providing AAV helper function allowing generating recombinant AAV without concomitant helper virus production.

More specifically the invention provides methods, cell lines, recombinant adenoviral vectors and recombinant DNA molecules especially suited for the large scale production of high titer recombinant AAV stocks that are free of replication competent adenovirus. In normal cells AAV replication and packaging is undetectable. However, low level replication and packaging in the absence of helper virus function can be induced. Several methods have been published to induce a productive replicative cycle of AAV on a low scale. These include but are not limited to treatment of cells with cytostatic drugs or UV irradiation (Yacobson et al, 1989; Yalkinoglu et al, 1988) and are not suitable for large scale production of high titer recombinant AAV stocks that are free of replication competent helpervirus. The invention provides a structurally better solution to

completely avoid the generation of helper virus during the production of recombinant AAV. Preventing the generation of the helper virus avoids the requirement for painstaking purification and subsequent validation and testing of the preparations. The invention provides the way to eliminate the generation of helper virus during the production of AAV by eliminating the helper virus requirement of AAV-replication.

Methods to improve the production of recombinant AAV have attracted a lot of attention in recent years. Various ways to express the AAV-genes rep and cap have been found to improve the yield of recombinant AAV over the standard methods (Allen et al, 1997; Conway et al, 1997; Li et al, 1997; Vincent et al, 1997). In addition, the helper virus function has been under study and methods have been found to improve both the quality and the yield of the recombinant AAV preparations (Ferrari et al, 1997; Ferrari et al, 1996; Xiao et al, 1998b). In one aspect of the invention is provided a packaging cell which expresses the adenovirus E2A-gene and additionally required helper functions, where the additionally required helper functions do not posses sequence overlap with the E2A-helper function already present in the packaging cell, leading to the formation of RCA. Preferably the E2A-gene is derived from the adenovirus ts125. In another aspect of the invention is provided a packaging cell which expresses the adenovirus E1-region and additionally required helper functions, where the additionally required helper functions do not posses sequence overlap with the E1-helper function already present in the packaging cell, leading to the formation of RCA. In one particular aspect of the invention the packaging cell comprises the PER cell lines. The PER cell lines have been generated from normal human embryonic retinoblast (HER) cells which were immortalised with a fully characterised plasmid containing the human

adenovirus 5 E1-region (WO 97/00326). The PER cells are specifically useful in preventing the formation of RCA in combination with novel E1-deleted adenovirus vectors (WO 97/00326) which do not posses sequence overlap with the E1-region present in PER cells. In one aspect of the invention, PER cells are supplied with the additionally required helper virus function through infection of an E1-deleted adenovirus that contains no sequence overlap with the E1-sequences already present in the PER cells, leading to the formation of RCA. In another aspect of the invention, the PER cells are provided with the additionally required helper virus function through transfection with plasmid DNA containing the helper virus function encoding genes and which plasmid contains no sequence overlap with the E1 sequences already present in the PER cells, leading to the formation of RCA. An example of such a PER cell, PER.C6 has been deposited under accession number 96022940 ECACC at the Centre for Applied Microbiology Research (CAMR). The most commonly used cell lines for the production of rAAV are HeLa and 293. Although these cell lines are widely used there are several drawbacks attached to them. The HeLa cells are derived from human cancer and thus carry one or more oncogenes in their DNA. It is conceivable that some of the chromosomal DNA is co-packaged with the viral vectors produced on these cells and thus can end up in the target cells in the patient. Compared to HeLa cells, the 293 cells have the advantage that they are not derived from human cancer. However, they are stably transfected with some adenovirus sequences and as a result of that express E1-genes (Graham et al, 1977a). This E1-gene expression is sufficient for the production of recombinant AAV (Herzog et al, 1997; Snyder et al, 1997b; Zhou et al, 1998). However, the 293 cell line has a disadvantage. Not only the E1-region is stably integrated into the DNA of the cells. From the left hand side of the adenovirus genome it is known that the cell line

carries at least adenovirus 5 sequences 1-4344 containing the left hand ITR, the packaging signal, the E1-gene and the gene encoding protein IX (Louis et al, 1997). The presence of more than just the E1-sequences leaves a significant region of overlap on both sides with the most commonly used E1-deleted adenovirus vectors or deletion mutants such as dl312(Snyder et al, 1997b). The region of overlap is sufficient for homologous recombination between the most commonly used E1-deleted adenovirus vectors and the adenovirus 5 sequences in 293. Such a homologous recombination event can lead to the undesired generation of replication competent adenovirus (RCA)(Hehir et al, 1996). Especially for large scale preparations the presence of RCA in E1-deleted adenovirus vector stocks is a problem (Imler et al, 1996; Lochmuller et al, 1994). The invention described in (Ferrari et al, 1996) and WO 96/40240 comprises the transfection of 293 cells with a 35,000 bp DNA fragment isolated from XbaI digested DNA from adenovirus dl309 to provide for the adenovirus helper functions for the production of recombinant AAV. This technique is not ideal since this XbaI fragment has considerable overlap with the adenovirus sequences in 293, enabling the inadvertent generation of replication competent adenovirus. Another disadvantage is that dl309 has an insertion of DNA in the E3-region. Fine tuning of the technique has led to the generation of adenovirus helper plasmids with deletions of adenovirus genes while retaining the helper virus function for recombinant AAV production (WO 97/17458, Ferrari et al, 1997; Li et al, 1997; Xiao et al, 1998a). Using these adenovirus late gene deleted helper plasmids to avoid RCA is in general restricted to 293 cells. As indicated above, this cell line has several disadvantages, one further disadvantage of the 293 cell line is it that only expresses the E1-region and thus additionally requires helper function for efficient and large scale production of

recombinant AAV, which need to be supplied separately. Furthermore, the culturing of 293 cells is considered troublesome.

In another aspect of the invention, a stable packaging cell is provided which expresses adenovirus region E1, and E2a, for example derived from adenovirus ts125. In this preferred embodiment of the invention functional expression of E2a can be timed to optimise the yield of recombinant AAV. The additionally required helper functions are provided in the form of an E1, E2a deleted adenovirus or in the form of plasmid DNA containing the helper virus function encoding genes, whereas the helper adenovirus vector or the plasmid DNA contains no sequence overlap with the helper virus functions already present in the packaging cells of the present invention, leading the formation of RCA. In this preferred embodiment extra E2a helper function may be supplied to the packaging cell provided that the method does not introduce sequence overlap with the E1-region already present in the packaging cells, leading to the formation of RCA.

In a preferred embodiment of the invention, the expression of adenovirus late genes is essentially repressed either by intervention with the transcription of the late genes or by removing one or more of the encoding genes from the DNA encoding the additionally required helper function.

In another aspect of the invention, the cells of the invention are grown to large numbers for the production, harvesting and purification of recombinant AAV. For production of recombinant AAV the cells are supplied with the recombinant AAV DNA, the DNA containing the AAV rep- and cap-genes and DNA containing the helper virus functions. In a preferred embodiment of the invention the AAV rep- and cap-genes are physically linked to the plasmid DNA providing the additionally required helper function such that they are

present on one and the same molecule. The cells can be supplied with the DNA needed for the production of recombinant AAV just prior to the start of recombinant AAV production, in which case, for each production the cells need to be supplied with the DNA through a process. Said process can be any method suitable for the transfection or infection of DNA into large numbers of cells. In a particularly preferred embodiment of the invention the DNA required for the production of recombinant AAV is transfected into PER cells by means of poly(2-(dimethylamino)ethyl-10-4-aminobutyl)phosphazene or other poly(organo)phosphazenes. Alternatively parts of the DNA required for the production of recombinant AAV can be stably integrated into the PER cell chromosomal DNA.

In another aspect of the invention the cells of the invention, recombinant AAV is produced with the packaging cell of the invention growing in suspension cultures using completely defined serum free medium.

In one embodiment of the invention is provided a method for generating a packaging cell containing all necessary helper function for an AAV-reproductive cycle, whereby said helper functions do not contain overlap leading to the formation of replication competent helper virus.
In a preferred embodiment of the invention said packaging cell is stably transformed with the adenovirus E1-region, which region does not contain overlap with additionally required helper functions. In a particularly preferred embodiment of the invention said packaging cell is stably transformed with the E1-region and the E2a-gene. In this particular embodiment of the invention the E2a-function can be switched on or off at will following a signal. In a preferred embodiment of this invention the E2a-gene is derived from adenovirus mutant H5ts125 whereby said signal is a switch in temperature. In another particularly preferred

embodiment of the invention said packaging cell is stably transformed with the adenovirus 5 E1-region, the E2a gene and the adenovirus 5 VA-region (Martinez et al, 1989) or the adenovirus 5 E4orf6 gene, or both. In this particularly preferred embodiment the transcriptional activity of the adenovirus 5 VA-region and/or the adenovirus 5 E4orf6 gene is regulated. Meaning that the transcriptional activity can be switched on or off at will following a signal.

As used herein the term "additionally required helper function" also refers to helper virus functions allowing efficient (large scale) production of recombinant AAV for which the encoding genes are not stably integrated in the DNA of the recombinant AAV producing cell or for which additional expression is desired. Such additionally required helper functions may be provided through any viral or non-viral method able to transfer foreign genetic material into mammalian cells such as but not limited to : poly(organo)phosphazenes, polyethylenimine, calcium phosphate precipitation, electroporation, recombinant, lipid or liposome mediated gene transfer.

In one embodiment of the invention is provided a packaging cell requiring only additional AAV-packaging function and a recombinant AAV-vector for the production of recombinant AAV. Said packaging cell comprises and provides the required adenovirus helper function from stably integrated adenoviral DNA. In one aspect of the invention said helper function is provided by a stably integrated E1-region. In another aspect of the invention said helper function is provided by a stably integrated E1-region and a stably integrated E2a gene. In this particular embodiment of the invention the E2a-function can be switched on or of at will following a signal. In a preferred embodiment of this invention the E2a-gene is derived from adenovirus mutant H5ts125 whereby said signal is a switch in temperature. In

another particularly preferred embodiment of the invention said packaging cell is stably transformed with the adenovirus 5 E1-region, the E2a gene and the adenovirus 5 VA-region (Martinez et al, 1989) or the adenovirus 5 E4orf6 gene, or both. In this particularly preferred embodiment the transcriptional activity of the adenovirus 5 VA-region and/or the adenovirus 5 E4orf6 gene is regulated, meaning that the transcriptional activity can be switched on or off at will following a signal.

The invention provides a cell-culture comprising a cell according to the invention. Large scale production of recombinant vectors for human gene therapy requires an easy and upscalable culturing method for the producer cell line, preferably a suspension culture or other large scale culture such as a bioreactor culture, in medium devoid of any human or animal constituents, i.e. in serum free medium. Several systems have been devised to grow mammalian cells to large numbers. These include but are not limited to roller bottle culture, cell cubes and bioreactors. Each of these systems has advantages and disadvantages. Bioreactors in which cells are grown in suspension are the easiest to standardise and to scale to increasingly larger volumes. However, one drawback is that cells in suspension are not easily transfected. Many different cell culture media are developed to support optimal growth of a large variety of different cells. Most of these media are based on variations of Dulbecco's modified Eagles medium (DMEM) and are supplemented with bovine serum. We have adapted cells according to the invention to suspension cultures using a defined serum free medium. Serum free cultures have the advantage that they are completely defined since they are not dependent on a natural source of serum which can vary in quality and presence of adventitious agents. These serum free media contain additives that replace essential components for cell growth in serum.

The invention also provides a method for producing recombinant adeno-associated virus comprising using a cell or cell-culture according to the invention and provides the use of these adeno-associated virus vectors in gene therapy.

The invention is described on the basis of AAV-2 but it is clear that also other AAV serotypes (such as 1 and 3 to 5)or yet to be discovered serotypes can be adapted for the same purposes. Also dependoviruses common in other species can be used for the same purposes, for instance canine adeno-associated virus is able to infect human cells. In addition human AAV replicates in many mammalian cell types as long as the species specific adenovirus is present, and dependoviruses from other species can be produced with the cells and methods of the present invention using the respective species specific adenovirus. Non-limiting examples of non-primate dependoviruses are avian- canine-, bovine adeno-associated virus (Berns, 1996). For example, it is clear to persons skilled in the art that also adenovirus 1 to 4, 6 to 51 or other human or animal adenoviruses can be manipulated for the same purpose provided that the function of the gene products is comparable. Gene products providing similar AAV helper function but which are derived from different viruses, such as but not limited to HSV, CMV and pseudorabies virus, or are derived from other natural sources or are produced in a synthetic form, can be used for the same purpose.

The invention is further explained in the experimental part of the description and in the drawings without limiting the invention.

Brief description of the drawings

Figure 1. Depicts the structure and the genome organisation of wtAAV.

Figure 2. PER.C6 cells were seeded at a density of 1 x 10^6 cells per 25 cm^2 tissue culture flask and cultured at either 32-, 37- or 39^{0}C. At the indicated time points, cells were counted in a Burker cell counter. PER.C6 grows well at both 32-, 37- and 39^{0}C.

Figure 3. Western blot with 35mg whole cell extract from cell lines generated from PER.C6 transfected with either pcDNA3 (upper panel, lane 1), pcDNA3wtE2A (upper panel, lane 2), pcDNA3tsE2A (upper panel lanes 4-14; middle panel, lanes 1-13 and lower panel lanes 1-12) or PER.C6 cells transiently transfected with pcDNA3tsE2A (upper panel, lane 3) . The blot was probed with an antibody specific for the E2A gene product (B6 aDBP) and visualised using the ECL detection system. All PER.C6tsE2A cell lines express the tsE2A encoded temperature sensitive DBP protein.

Figure 4. The tsE2A expressing cell line PER.C6tsE2A.c5-9 was cultured in suspension in serum free Ex-cell™. At the indicated time points, cells were counted in a Burker cell counter. The results of 8 independent cultures are indicated. PER.C6tsE2A grows well in suspension in serum free Ex-cell™medium.

Experimental part

Materials and Methods

DNA constructs

The packaging plasmid pIM45 (7.3 Kb) contains the AAV-2 *rep* and *cap*- genes (McCarty et al, 1991) and was a kind gift from Dr. S. Zolotukhin. pACV-βgal (8.3 kb) is a plasmid containing a CMV-LacZ expression cassette between AAV-ITRs and was a kind gift from Dr. J.A. Kleinschmidt. Plasmid pIG.E1A.E1B

contains the Ad5 E1a and E1b genes (nucleotides 459 to 3510 of Ad5) under transcriptional control of the human PGK promoter and is described in WO/97/00326.
Plasmid pE2a is another name for plasmid pcDNA3wtE2A described below
Plasmid pE4orf6 was generated by inserting a 929 bp fragment encoding the Ad5 E4orf6 protein into the BamHI site of pCMV/neo (Hinds et al, 1990).

pBr/Ad.Bam-rITR (ECACC deposit P97082122)

In order to facilitate blunt end cloning of the ITR sequences, wild-type human adenovirus type 5 (Ad5) DNA was treated with Klenow enzyme in the presence of excess dNTPs. After inactivation of the Klenow enzyme and purification by phenol/chloroform extraction followed by ethanol precipitation, the DNA was digested with BamHI. This DNA preparation was used without further purification in a ligation reaction with pBR322 derived vector DNA prepared as follows: pBR322 DNA was digested with EcoRV and BamHI, dephosphorylated by treatment with TSAP enzyme (Life Technologies) and purified on LMP agarose gel (SeaPlaque GTG). After transformation into competent *E. coli* DH5a (Life Techn.) and analysis of ampiciline resistant colonies, one clone was selected that showed a digestion pattern as expected for an insert extending from the BamHI site in Ad5 to the right ITR.
Sequence analysis of the cloning border at the right ITR revealed that the most 3' G residue of the ITR was missing, the remainder of the ITR was found to be correct.

pBr/Ad.Cla-Bam (ECACC deposit P97082117)

wt Adeno type 5 DNA was digested with ClaI and BamHI, and the 20.6 kb fragment was isolated from gel by electro-elution. pBR322 was digested with the same enzymes and purified from

agarose gel by Geneclean. Both fragments were ligated and transformed into competent DH5α. The resulting clone pBr/Ad.Cla-Bam was analysed by restriction enzyme digestion and shown to contain an insert with adenovirus sequences from bp 919 to 21566.

pBr/Ad.AflII-Bam (ECACC deposit P97082114)

Clone pBr/Ad.Cla-Bam was linearised with EcoRI (in pBR322) and partially digested with AflII. After heat inactivation of AflII for 20′ at 65 °C the fragment ends were filled in with Klenow enzyme. The DNA was then ligated to a blunt double stranded oligo linker containing a PacI site (5′-AATTGTCTTAATTAACCGCTTAA-3′). This linker was made by annealing the following two oligonucleotides: 5′-AATTGTCTTAATTAACCGC-3′ and 5′-AATTGCGGTTAATTAAGAC-3′, followed by blunting with Klenow enzyme. After precipitation of the ligated DNA to change buffer, the ligations were digested with an excess PacI enzyme to remove concatamers of the oligo. The 22016 bp partial fragment containing Ad5 sequences from bp 3534 up to 21566 and the vector sequences, was isolated in LMP agarose (SeaPlaque GTG), religated and transformed into competent DH5α. One clone that was found to contain the PacI site and that had retained the large adeno fragment was selected and sequenced at the 5′ end to verify correct insertion of the PacI linker in the (lost) AflII site.

pBr/Ad.Bam-rITRpac#2 (ECACC deposit P97082120) and pBr/Ad.Bam-rITR#8 (ECACC deposit P97082121)

To allow insertion of a PacI site near the ITR of Ad5 in clone pBr/Ad.Bam-rITR about 190 nucleotides were removed between the ClaI site in the pBR322 backbone and the start of the ITR sequences. This was done as follows: pBr/Ad.Bam-rITR was digested with ClaI and treated with nuclease Bal31 for

varying lengths of time (2', 5', 10' and 15'). The extend of nucleotide removal was followed by separate reactions on pBR322 DNA (also digested at the ClaI site), using identical buffers and conditions. Bal31 enzyme was inactivated by incubation at 75 °C for 10', the DNA was precipitated and resuspended in a smaller volume TE buffer. To ensure blunt ends, DNAs were further treated with T4 DNA polymerase in the presence of excess dNTPs. After digestion of the (control) pBR322 DNA with SalI, satisfactory degradation (~150 bp) was observed in the samples treated for 10' or 15'. The 10' or 15' treated pBr/Ad.Bam-rITR samples were then ligated to the above described blunted PacI linkers (See pBr/Ad.AflII-Bam). Ligations were purified by precipitation, digested with excess PacI and separated from the linkers on an LMP agarose gel. After religation, DNAs were transformed into competent DH5α and colonies analysed. Ten clones were selected that showed a deletion of approximately the desired length and these were further analysed by T-track sequencing (T7 sequencing kit, Pharmacia Biotech). Two clones were found with the PacI linker inserted just downstream of the rITR. After digestion with PacI, clone #2 has 28 bp and clone #8 has 27 bp attached to the ITR.

<u>pWE/Ad.AflII-rITR (ECACC deposit P97082116)</u>

Cosmid vector pWE15 (Clontech) was used to clone larger Ad5 inserts. First, a linker containing a unique PacI site was inserted in the EcoRI sites of pWE15 creating pWE15.Pac. To this end, the double stranded PacI oligo as described for pBr/Ad.AflII-Bam was used but now with its EcoRI protruding ends. The following fragments were then isolated by electro-elution from agarose gel: pWE15.Pac digested with PacI, pBr/Ad.AflII-Bam digested with PacI and BamHI and pBr/Ad.Bam-rITR#2 digested with BamHI and PacI. These fragments were ligated together and packaged using λ phage packaging

extracts (Stratagene) according to the manufacturer's protocol. After infection into host bacteria, colonies were grown on plates and analysed for presence of the complete insert. pWE/Ad.AflII-rITR contains all adenovirus type 5 sequences from bp 3534 (AflII site) up to and including the right ITR (missing the most 3' G residue).

pWE/Ad.Δ5'.

The construct pWE/Ad.Δ5' is an example of a replicating molecule according to the invention that contains two adenoviral ITRs and all adenoviral sequences between bp 3510 and 35938, i.e., the complete adenoviral genome except for the E1 region and the packaging signal. . pWE/Ad.Δ5' has been made in a cosmid vector background from three fragments. First, the 5' ITR from Ad5 was amplified using the following primers:

ITR-EPH: 5'-CGG-AAT-TCT-TAA-TTA-AGT-TAA-CAT-CAT-CAA-TAA-TAT-ACC-3' and

ITR-pIX: 5'-ACG-GCG-CGC-CTT-AAG-CCA-CGC-CCA-CAC-ATT-TCA-GTA-CGT-ACT-AGT-CTA-CGT-CAC-CCG-CCC-CGT-TCC-3'. The resulting PCR fragment was digested with EcoRI and AscI and cloned into vector pNEB193 (New England Biolabs) digested with the same enzymes. The resulting construct was named pNEB/ITR-pIX. Sequencing confirmed correct amplification of the Ad5 sequences in the left ITR (Ad5 sequences 1 to 103) linked to the pIX promoter (Ad5 sequences 3511 to 3538) except for a single mismatch with the expected sequence according to GenBank (Accession no.: M73260/M29978), i.e., an extra G-residue was found just upstream of the AflII site. This ITR-pIX fragment was then isolated with EcoRI and AflII and ligated to a EcoRI-AflII vector fragment containing Ad5 sequences 3539-21567. The latter fragment was obtained by digestion of pBr/Ad.Cla-Bam (*supra*) with EcoRI and partially with AflII. The resulting clone was named pAd/LITR(Δ5')-

BamHI. The final construct pWE/Ad.Δ5′ was then made by ligating cosmid vector pWE15.Pac (*supra*) digested with PacI to pAd/LITR(Δ5′)-BamHI digested with PacI/BamHI and pBr/Ad.Bam-rITR.pac#2 (*supra*) digested with PacI/BamHI.

pWE/Ad.AflII-rITRΔE2A.

This cosmid is essentially the same as pWE/Ad.AflII-rITR (ECACC deposit P97082116) apart from a deletion of the coding region of E2A. Deletion of the E2A coding sequences from pWE/Ad.AflII-rITR (ECACC deposit P97082116) has been accomplished as follows. The adenoviral sequences flanking the E2A coding region at the left and the right hand side were amplified from the plasmid pBR/Ad.Sal.rITR (ECACC deposit P97082119) in a PCR reaction with the Expand PCR system (Boehringer) according to the manufacturers protocol. The following primers were used:

Right flanking sequences (corresponding to Ad5 nucleotides 24033 to 25180):

ΔE2A.SnaBI: 5'-GGC.GTA.CGT.AGC.CCT.GTC.GAA.AG-3'

ΔE2A.DBP-start : 5'-CCA.ATG.CAT.TCG.AAG.TAC.TTC.CTT.CTC.CTA.TAG.GC-3'

The amplified DNA fragment was digested with SnaBI and NsiI (NsiI is generated in the primer ΔE2A.DBP-start, underlined)

Left flanking sequences (corresponding Ad5 nucleotides 21557 to 22442):

ΔE2A.DBP-stop : 5'-CCA.ATG.CAT.ACG.GCG.CAG.ACG.G-3'

ΔE2A.BamHI: 5'-GAG.GTG.GAT.CCC.ATG.GAC.GAG-3'

The amplified DNA was digested with BamHI and NsiI (NsiI is generated in the primer ΔE2A.DBP-stop, underlined).

Subsequently, the digested DNA fragments were ligated into SnaBI/BamHI digested pBr/Ad.Sal-rITR to give rise to pBR.Ad.Sal-rITRΔE2A. Sequencing confirmed the exact

replacement of the DBP coding region with a unique NsiI site in plasmid pBR.Ad.Sal-rITRΔE2A.

Next the cosmid pWE/Ad.AflII-rITRΔE2A was generated. The plasmid pBR.Ad.Sal-rITRΔE2A was digested with BamHI and SpeI. The 3.9 Kb fragment in which the E2A coding region was replaced by the unique NsiI-site was isolated. The pWE/Ad.AflII-rITR cosmid was digested with BamHI and SpeI. The 35 Kb DNA fragment, from which the BamHI/SpeI fragment containing the E2A coding sequence was removed, was isolated. The fragments were ligated and packaged using λ phage packaging extracts according to the manufacturers protocol (Stratagene), yielding the cosmid pWE/Ad.AflII-rITRΔE2A.

pVA.

pVA (3.7 kb) is a pUC119 plasmid containing the VAI and VAII region of adenovirus 5 (nucl. 10555 until 11075). The VA-genes of adenovirus 5 were cloned following PCR on isolated DNA from wild type adenovirus 5 using the primers 5'-ACGCGTCGACCTCTGGCCGGTCAGGCGCGCGCAA-3' and 5'-ACGCGGATCCCGCATCTGCCGCAGCACCGGATGC-3'. The PCR was performed using expand long template™ PCR kit (Boehringer) according to the specifications of the manufacturer. The resulting fragment was digested with SalI and BamHI, present in the primers, and ligated into SalI, BamHI digested pUC119.

Cell culture.

293 cells (Graham et al, 1977b) and HeLa cells (Cancer Res. 12:264, 1952) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life technologies Breda, The Netherlands) containing 10% heat inactivated foetal bovine serum at 37 °C and 10% CO_2. Adherent cultures of PER.C6 cells were grown in DMEM supplemented with 10% foetal bovine serum and $MgCl_2$ (10 mM) at 37 °C and 10% CO_2. Suspension cultures of PER.C6 cells were cultured in Ex-Cell™ 525 (JRH Biosciences, Denver,

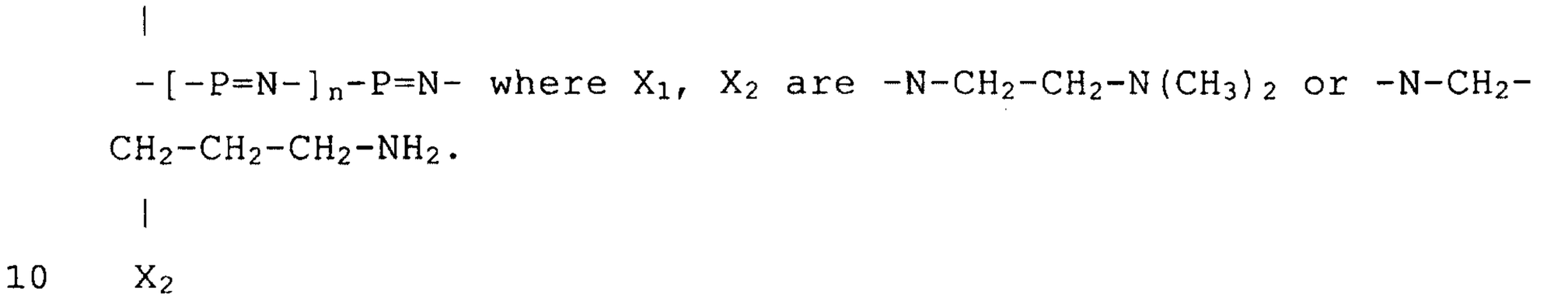
Pennsylvania) supplemented with 1 x L-Glutamin (GIBCO BRL) hereafter called in Ex-Cell™, at 37 ^{0}C and 10% CO_2 in stationary cultures in 6 well dishes (Greiner, Alphen aan de Rijn, The Netherlands) or in Erlenmeyer tissue culture flasks (Corning) during continuous swirling at 100 RPM.

Transfection.

Transfection of monolayer cultures : HeLa cells and 293 cells were transfected using the Calcium Phosphate transfection system (Life technologies, Almere) according the specifications of the manufacturer. Monolayers of PER.C6 cells were transfected using LipofectAMINE™ (Life technologies, Breda) according the specifications of the manufacturer.

Transfection of suspension cultures : PER.C6 cells in logarithmic growth phase were collected by centrifugation (3000g, 5 minutes, rt). The cells were resuspended in transfection mix (described below) at a concentration of 2 x 10^6 cells per ml and incubated for three hours at 37 °C, 10% CO_2. Unless otherwise indicated transfection were performed under continuous shaking (100 RPM). For transfections with DMRIE-C™ (Life technologies, Breda) the transfection mix was made in DMEM according to the specifications of the manufacturer. After a three hour incubation in transfection mix the cells were collected by centrifugation (3000g, 5 min. rt) and resuspended in fresh Ex-Cell™ medium to a final concentration of 10^6 cells per ml. Transfection with FuGENE™ 6 (Boehringer Mannheim) was accomplished with transfection mix made in Ex-Cell™ medium according to the specifications of the manufacturer. After a three hour incubation with transfection mix the cells were diluted with Ex-Cell™ medium to a final concentration of 10^6 cells per ml. Transfection mixes using poly(2-(dimethylamino)ethyl-10-4-

aminobutyl)phosphazene (PPZ) were made as follows. A stock solution of PPZ (2.4 mgram/ml) was made by dissolving the solid compound in Hepes (5 mM, pH = 7.3). The formula of PPZ is :



X_1

|

$-[-P=N-]_n-P=N-$ where X_1, X_2 are $-N-CH_2-CH_2-N(CH_3)_2$ or $-N-CH_2-CH_2-CH_2-CH_2-NH_2$.

|

X_2

Transfection mixes were made by adding the indicated amount of PPZ to 500 ul Ex-Cell™ medium. This solution was mixed with the same volume of Ex-Cell™ containing the indicated amount of DNA. The mixture was incubated for one hour and was subsequently used to resuspend a pellet of 2 x 10^6 PER.C6 cells. The cells were incubated with the transfection mix for three hours and subsequently diluted with Ex-Cell™ medium to a final concentration of 10^6 cells per ml.
Transfected cells were harvested after 48 hours and analysed for β-galactosidase activity.

<u>β-Galactosidase activity assays</u>

Cells were stained for β-galactosidase activity with two different methods. For histochemical analysis and determination of the number of infectious units the following procedure was used. Cells were washed twice with PBS (NPBI, Emmer-Compascuum) and fixed for 10 minutes in 0.2% glutaraldehyde (Sigma, Zwijndrecht, The Netherlands) in PBS. The cells were washed twice with PBS and stained with X-Gal solution (2 mM $MgCl_2.6H_2O$, 5 mM $K_2Fe(CN)_6$, 5 mM $K_4Fe(CN)_6.3H_2O$ and 40 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-b-galactopyranoside, Molecular Probes Europe, Leiden,

The Netherlands) in 0,1 M phosphate buffer pH = 7.4). After overnight staining at 37 °C blue cells were counted under an optical microscope (Olympus CK2-TR). For quantitation of β-galactosidase activity in suspension cultures of PER.C6 the FluoReporter™ LacZ/Galactosidase Quantitation kit (Molecular Probes, Leiden, The Netherlands) was used according to the protocol provided by the manufacturer. β-galactosidase activity of each sample was evaluated by comparing the β-galactosidase activity in 10 ul of cell suspension to a serial dilution of a known concentration of purified β-galactosidase.

Recombinant AAV titration.

HeLa cells were seeded 4 x 10^4 cells per cm^2. The medium was replaced the following day with fresh medium containing serial dilutions of rAAV and adenovirus ts149 (20 pfu/cell). After 4 hours the medium was replaced by fresh medium and the cells were incubated for 24 hours at 37 °C, 10% CO_2 before the β-galactosidase staining. The titer of the recombinant AAV stock was calculated by counting the number of blue cells from the highest dilution giving rise to blue cells and multiplying this number by the dilution factor.

Recombinant AAV production on adherent cells.

Cells were seeded such that they reached 70% confluence the next day. The cells were then transfected with pACV-βgal and pIM45 (ratio 1:4 w/w) and infected with the adenovirus helper virus ts149 (moi = 20), the E1-deleted adenovirus helper virus IG.Ad.MLP.Luc (Vincent et al, 1996) or transfected with the adenovirus helper plasmid DNA. In the latter case the total amount of adenovirus helper gene plasmids was 1,5 times (w/w) more than the total amount of pACV-βgal and pIM45 DNA. When more than one adenovirus helper plasmid was used, equal

amounts (w/w) of the different adenovirus helper plasmids were used. Recombinant AAV production on adherent PER.C6tsE2A.c5-9 were performed as described for the PER.C6 cell line except for some modifications. The cell line was grown at 39 ^{0}C, 10% CO_2. Before transfection the cells were seeded at 39 ^{0}C, 10% CO_2 such that they reached 70% confluency the next day. The cells were subsequently cultured for one day at 32 ^{0}C, 10% CO_2. Next the cells were transfected at 37^{0}C, 10% CO_2 as described for the PER.C6 cell line. Recombinant AAV was harvested 48 hours after transfection. The cells were scraped in their culture medium and subjected to three freeze thaw cycles. The cell debris was centrifuged (2000 RPM, 10 minutes, rt). When using adenovirus ts149 or E1-deleted adenovirus vectors, the supernatants were heat inactivated at 56 °C for 1 hour. When adenovirus DNA fragments were used to complement AAV-production the supernatants were not heat inactivated. All supernatants were filtered (0.45 uM, Millipore) before storage at -20 °C.

Example 1

Generation of producer cell lines for the production of recombinant adenoviral vectors deleted in early region 1 and early region 2A

Here is described the generation of cell lines for the production of recombinant adenoviral vectors that are deleted in early region 1 (E1) and early region 2A (E2A). The producer cell lines complement for the E1 and E2A deletion from recombinant adenoviral vectors in trans by constitutive expression of the E1 and E2A genes, respectively. The pre-established Ad5-E1 transformed human embryo retinoblast cell line PER.C6 (WO 97/00326) and Ad5 transformed human embryo

kidney cell line 293 (Graham et al, 1977b) were further equipped with E2A expression cassettes.

The adenoviral E2A gene encodes a 72 kDa DNA Binding Protein (DBP) which has a high affinity for single stranded DNA. Because of this feature, constitutive expression of DBP is toxic for cells. The ts125E2A mutant encodes a DBP which has a Pro→Ser substitution of amino acid 413 (Vliet van der et al, 1975). Due to this mutation, the ts125E2A encoded DBP is fully active at the permissive temperature of 32°C, but does not bind to ssDNA at the non-permissive temperature of 39°C. This allows the generation of cell lines that constitutively express E2A which is not functional and is not toxic at the non-permissive temperature of 39°C, but becomes functional after a temperature switch to the permissive temperature of 32°C.

A. Generation of plasmids expressing wildtype E2A- or temperature sensitive ts125E2A.

pcDNA3wtE2A: The complete wildtype early region 2A (E2A) coding region was amplified from the plasmid pBR/Ad.Bam-rITR (ECACC deposit P97082122) with the primers DBPpcr1 and DBPpcr2 using the Expand™ Long Template PCR system according to the standard protocol of the supplier (Boehringer Mannheim). PCR was performed on a Biometra Trio Thermoblock, amplification program: 94°C for 2 minutes, 1 cycle; 94°C for 10 seconds + 51°C for 30 seconds + 68°C for 2 minutes, 1 cycle; 94°C for 10 seconds + 58°C for 30 seconds + 68°C for 2 minutes, 10 cycles; 94°C for 10 seconds + 58°C for 30 seconds + 68°C for 2 minutes with 10 seconds extension per cycle, 20 cycles; 68°C for 5 minutes, 1 cycle. The primer DBPpcr1: CGG GAT CC*G CCA CC***A TGG CCA GTC GGG AAG AGG AG** (5′ to 3′) contains a unique *Bam*HI restriction site (underlined) 5′ of the Kozak sequence (italic) and start codon of the E2A coding sequence. The primer DBPpcr2: CGG AAT TC**T TAA AAA TCA AAG GGG**

TTC TGC CGC (5′ to 3′) contains a unique *Eco*RI restriction site (underlined) 3′ of the stop codon of the E2A coding sequence. The bold characters refer to sequences derived from the E2A coding region. The PCR fragment was digested with *Bam*HI/*Eco*RI and cloned into *Bam*HI/*Eco*RI digested pcDNA3 (Invitrogen), giving rise to pcDNA3wtE2A.

pcDNA3tsE2A: The complete ts125E2A coding region was amplified from DNA isolated from the temperature sensitive adenovirus mutant H5ts125 (Ensinger and Ginsberg, 1972; Vliet van der et al, 1975). The PCR amplification procedure was identical to that for the amplification of wtE2A The PCR fragment was digested with *Bam*HI/*Eco*RI and cloned into *Bam*HI/*Eco*RI digested pcDNA3 (Invitrogen), giving rise to pcDNA3tsE2A. The integrity of the coding sequence of wtE2A and tsE2A was confirmed by sequencing.

B. Growth characteristics of producer cells for the production of recombinant adenoviral vectors cultured at 32-, 37- and 39^{0}C.

PER.C6 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO BRL) supplemented with 10% Fetal Bovine Serum (FBS, GIBCO BRL) and 10mM $MgCl_2$ in a 10% CO_2 atmosphere at either 32^{0}C, 37^{0}C or 39^{0}C. At day 0, a total of 1 x 10^6 PER.C6 cells were seeded per 25cm^2 tissue culture flask (Nunc) and the cells were cultured at either 32^{0}C, 37^{0}C or 39^{0}C. At day 1-8, cells were counted. Figure 3 shows that the growth rate and the final cell density of the PER.C6 culture at 39^{0}C is comparable to that at 37^{0}C. The growth rate and final density of the PER.C6 culture at 32^{0}C were slightly reduced as compared to that at 37^{0}C or 39^{0}C. No significant cell death was observed at any of the incubation temperatures. Thus PER.C6 performs very well both at 32^{0}C and 39^{0}C, the permissive and non-permissive temperature for ts125E2A, respectively.

C. Transfection of PER.C6 and 293 with E2A expression vectors; colony formation and generation of cell lines

One day prior to transfection, 2 x 10^6 PER.C6 cells were seeded per 6 cm tissue culture dish (Greiner) in DMEM, supplemented with 10% FBS and 10mM $MgCl_2$ and incubated at 37^{0}C in a 10% CO_2 atmosphere. The next day, the cells were transfected with 3-, 5- or 8µg of either pcDNA3, pcDNA3wtE2A or pcDNA3tsE2A plasmid DNA per dish, using the LipofectAMINE PLUS™ Reagent Kit according to the standard protocol of the supplier (GIBCO BRL), except that the cells were transfected at 39^{0}C in a 10% CO_2 atmosphere. After the transfection, the cells were constantly kept at 39^{0}C, the non-permissive temperature for ts125E2A. Three days later, the cells were put on DMEM, supplemented with 10% FBS, 10mM $MgCl_2$ and 0.25mg/ml G418 (GIBCO BRL) and the first G418 resistant colonies appeared at 10 days post transfection. As shown in table 1, there was a dramatic difference between the total number of colonies obtained after transfection of pcDNA3 (~200 colonies) or pcDNA3tsE2A (~100 colonies) and pcDNA3wtE2A (only 4 colonies). These results indicate that the toxicity of constitutively expressed E2A can be overcome by using a temperature sensitive mutant of E2A (ts125E2A) and culturing of the cells at the non-permissive temperature of 39^{0}C.

From each transfection, a number of colonies was picked by scraping the cells from the dish with a pipette. The detached cells were subsequently put into 24 wells tissue culture dishes (Greiner) and cultured further at 39^{0}C in a 10% CO_2 atmosphere in DMEM, supplemented with 10% FBS, 10mM $MgCl_2$ and 0.25mg/ml G418. As shown in table 1, 100% of the pcDNA3 transfected colonies (4/4) and 82% of the pcDNA3tsE2A transfected colonies (37/45) were established to stable cell lines (the remaining 8 pcDNA3tsE2A transfected colonies grew

slowly and were discarded). In contrast, only 1 pcDNA3wtE2A transfected colony could be established. The other 3 died directly after picking.

Next, the E2A expression levels in the different cell lines were determined by Western blotting. The cell lines were seeded on 6 well tissue culture dishes and sub-confluent cultures were washed twice with PBS (NPBI) and lysed and scraped in RIPA (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS in PBS, supplemented with 1mM phenylmethylsulfonylfluoride and 0.1 mg/ml trypsin inhibitor). After 15 minutes incubation on ice, the lysates were cleared by centrifugation. Protein concentrations were determined by the Bio-Rad protein assay, according to standard procedures of the supplier (BioRad). Equal amounts of whole-cell extract were fractionated by SDS-PAGE on 10% gels. Proteins were transferred onto Immobilon-P membranes (Millipore) and incubated with the αDBP monoclonal antibody B6 (Reich et al, 1983). The secondary antibody was a horseradish-peroxidase conjugated goat anti mouse antibody (BioRad). The Western blotting procedure and antibody incubations were performed according to the protocol provided by Millipore. The antibody complexes were visualised with the ECL detection system according to the manufacturer's protocol (Amersham). Figure 3 shows that all of the cell lines derived from the pcDNA3tsE2A transfection express the 72-kDa E2A protein (upper panel, lanes 4-14; middle panel, lanes 1-13; lower panel, lanes 1-12). In contrast, the only cell line derived from the pcDNAwtE2A transfection did not express the E2A protein (lane 2). No E2A protein was detected in extract from a cell line derived from the pcDNA3 transfection (lane 1), which serves as a negative control. Extract from PER.C6 cells transiently transfected with pcDNA3ts125 (lane 3) served as a positive control for the Western blot procedure. These data confirm that constitutive expression of wtE2A is

toxic for cells and that this toxicity can be circumvented by using the ts125 mutant of E2A.

In contrast to PER.C6 cells, the culturing of 293 cells at 39^{0}C is troublesome. Therefore, the transfection of 293 cells with either pcDNA3, pcDNA3wtE2A or pcDNA3tsE2A was performed at 37^{0}C in an atmosphere of 10% CO_2, a semi-permissive temperature for ts125E2A encoded DBP. A day prior to transfection, 293 cells were seeded in, supplemented with 10% FBS and 10mM $MgCl_2$, at a density of 3.6 x 10^5 cells per 6 cm tissue culture dish (Greiner). Five hours before transfection, cells received fresh medium. Cells were transfected with 7.2µg of either pcDNA3, pcDNA3wtE2A or pcDNA3tsE2A plasmid DNA using the Calcium Phosphate Transfection System according to the standard protocol of the supplier (GIBCO BRL). Two days post transfection, cells were put on selection medium, i.e. DMEM supplemented with 10% FBS, 10mM $MgCl_2$ and 0.1 mg/ml G418. The first colonies appeared at day 12 post transfection. As shown in table 2, total number of colonies obtained after transfection of pcDNA3 (~100 colonies) or pcDNA3tsE2A (~25 colonies) was significantly higher than that obtained after transfection of pcDNA3wtE2A (only 2 colonies). These results again show that constitutively expressed E2A is toxic for cells and that this toxicity can be circumvented by using ts125E2A. Moreover, it shows that this is not specific for PER.C6 cells, but that it applies to eukaryotic cells in general (e.g. 293 cells).

D. Complementation of the E2A deletion in Ad5.dl802 by PER.C6 cells constitutively expressing ts125E2A.

The adenovirus Ad5.dl802 is an Ad 5 derived vector deleted for the major part of the E2A coding region and does not produce functional DBP (Rice and Klessig, 1985). Ad5.dl802 was used to test the E2A trans-complementing activity of PER.C6 cells constitutively expressing ts125E2A. Parental

PER.C6 cells or PER.C6tsE2A clone 3-9 were cultured in DMEM, supplemented with 10% FBS and 10mM $MgCl_2$ at 39^{0}C and 10% CO_2 in 25 cm^2 flasks and either mock infected or infected with Ad5.dl802 at an m.o.i. of 5. Subsequently the infected cells were cultured at 32^{0}C and cells were screened for the appearance of a cytopathic effect (CPE) as determined by changes in cell morphology and detachment of the cells from the flask. Table 3 shows that full CPE appeared in the Ad5.dl802 infected PER.C6tsE2A clone 3-9 within 2 days. No CPE appeared in the Ad5.dl802 infected PER.C6 cells or the mock infected cells. These data show that PER.C6 cells constitutively expressing ts125E2A complement in trans for the E2A deletion in the Ad5.dl802 vector at the permissive temperature of 32^{0}C.

E. Serum free suspension culture of PER.C6tsE2A cell lines.

Large scale production of recombinant adenoviral vectors for human gene therapy requires an easy and upscalable culturing method for the producer cell line, preferably a suspension culture, in medium devoid of any human or animal constituents. To that end, several PER.C6tsE2A clones were brought into suspension culture. As an example, the cell line PER.C6tsE2A c5-9 (designated c5-9) was cultured at 39^{0}C and 10% CO_2 in a 175 cm^2 tissue culture flask (Nunc) in DMEM, supplemented with 10% FBS and 10mM $MgCl_2$. At sub-confluency (70-80% confluent), the cells were washed with PBS (NPBI) and the medium was replaced by 25 ml serum free suspension medium Ex-cell™ 525 (JRH) supplemented with 1 x L-Glutamin (GIBCO BRL), hereafter designated Ex-Cell™. Two days later, cells were detached from the flask by flicking and the cells were centrifuged at 1000rpm for 5 minutes. The cell pellet was resuspended in 5 ml Ex-Cell™ and 0.5ml cell suspension was transferred to a 80 cm^2 tissue culture flask (Nunc), together with 12 ml fresh Ex-Cell™. After 2 days, cells were harvested

(all cells are in suspension) and counted in a Burker cell counter. Next, the cells were seeded in a 125ml tissue culture Erlenmeyer (Corning) at a seeding density of 3 x 10^5 cells per ml in a total volume of 20 ml Ex-Cell™. Cells were further cultured at 125 RPM on an orbital shaker (GFL) at 39^{0}C in a 10% CO_2 atmosphere. Cells were counted at day 1-6 in a Burker cell counter. In Figure 4, the mean growth curve from 8 cultures is shown. PER.C6tsE2A c5-9 performs well in serum free suspension culture. The maximum cell density of approximately 2 x 10^6 cells per ml is reached within 5 days of culture.

Example 2

PER cells as producer cells for recombinant AAV.

PER cells are derived from human retina cells. The retina is not known for its ability to sustain AAV replication. We therefore verified whether PER cells are permissive for recombinant AAV production. PER.C6 cells were transfected using LipofectAMINE™ with the packaging plasmid pIM45, the rAAV-vector pACV-βgal (ratio 10:1 w/w) and infected with adenovirus ts149. Recombinant AAV was isolated after two days and titrated on adenovirus infected HeLa cells. pACV-βgal produced on PER.C6 cells had a titer of 2 x 10^7 infectious units (IU) per ml or 20 IU per cell. The yield of virus per cell obtained with this system is comparable or better then those reported for 293 cell lines with the packaging plasmid pIM45 (Vincent et al, 1997). In parallel we analysed whether the pattern and level of expressed E1-proteins was sufficient for the production of rAAV. To assess this question, PER.C6 cells were transfected with the packaging plasmid pIM45, the rAAV-vector pACV-βgal (ratio 10:1 w/w) and infected with an E1-deleted adenovirus vector IG.Ad.MLP.Luc (Vincent et al,

1996). The yield of rAAV using the E1 deleted adenovirus vector IG.Ad.MLP.Luc was 2 x 10^7 IU/ml and thus the same as with adenovirus ts149. This result indicates that both the pattern and the level of E1-expression in PER.C6 is allowing for the efficient production rAAV.

Viral vector free production of recombinant AAV on PER.C6.

Production of recombinant AAV using adenovirus DNA as a helper rather than the virus has been reported (WO 96/40240, WO 97/17458, (Ferrari et al, 1997; Ferrari et al, 1996; Li et al, 1997; Xiao et al, 1998a)). This work was done exclusively in the 293 cell line. We wanted to determine if also the PER.C6 cell line could be used to produce recombinant AAV. To this end we tested two constructs pWE/Ad.D5' and pWE/Ad.AflII-rITR which contained all adenovirus genes except the E1-region. The construct pWE/Ad.AflII-rITR does, as mentioned contain all adenovirus except E1, however, the promoter of the protein IX gene contains a deletion. pWE/Ad.D5' on the contrary contains the left ITR and the full length protein IX promoter. Recombinant AAV could be produced with both adenovirus DNA fragments. The yields of recombinant AAV were different with both constructs. The yield of recombinant AAV using pWE/Ad.AflII-rITR was significantly higher than using pWE/Ad.D5' (Table 4). One reason for the difference in yield could be differences in expression of relevant proteins from the two different plasmids. Another reason could be that expression of adenovirus 5 protein IX negatively affects recombinant AAV production.

Minimal requirements for recombinant AAV production on PER.C6.

To determine the minimal requirement for recombinant AAV production on the PER.C6 cell line we obtained plasmid clones for the adenovirus genes known to affect AAV production (i.e. E2a, E4orf6 and VA). Together with the E1-region already present in PER.C6, all adenovirus genes known to affect AAV production were thus at hand and they were tested for their effect on recombinant AAV production on PER.C6. In contrast to HeLa cells, PER.C6 cells produce a low but detectable amount of recombinant AAV when transfected with pIM45 and pACV-βgal. Efficient production of recombinant AAV required transfection of additional helper function encoding genes. The highest amount of recombinant AAV was obtained upon transfection of all three expression cassettes or pE2a and pVA (Table 4). Transfection of pE2a alone or together with pE4orf6 resulted in ± 10% of the yield of recombinant AAV compared to all three genes. Transfection of only E4 or only VA did not yield a significant amount of AAV (Table 4). Production on PER.C6tsE2A.c5-9 without the addition of extra adenovirus helper genes already resulted in a titer of 1.3 x 10^3. With the addition of pE4orf6 and pVA alone or in combination with pE2A the yield of recombinant AAV increased. Highest yields were obtained using the construct pWE/Ad.AflII-rITR.

Large scale production of recombinant AAV.

Several systems have been devised to grow mammalian cells to large numbers. These include but are not limited to roller bottle culture, cell cubes and bioreactors. Each of these systems has advantages and disadvantages. Bioreactors in which cells are grown in suspension are the easiest to standardise and to scale to increasingly larger volumes. However, one drawback is that cells in suspension are not easily transfected. Many different cell culture media are

developed to support optimal growth of a large variety of different cells. Most of these media are based on variations of Dulbecco's modified Eagles medium (DMEM) and are supplemented with bovine serum. We have adapted PER.C6 cells to suspension cultures using a defined serum free medium. Serum free cultures have the advantage that they are completely defined since they are not dependent on a natural source of serum which can vary in quality and presence of adventitious agents. These serum free media contain additives that replace essential components for cell growth in serum. We observed that many transfection reagents, specifically the liposomes, perform better when cells were cultured in DMEM than when cells were cultured in serum free Ex-Cell™ medium (not shown). Transfection of PER.C6 cells growing in suspension could, however, be achieved by using the non-liposomal reagent FuGENE™ 6 or by avoiding the contact of the liposome:DNA complex (DMRIE-C) with the Ex-Cell™ medium (Table 5). Alternative transfection agents are poly(organo)phosphazenes. Not much is known about the ability to transfect cells with these agents. To study the use of said agents for the transfection of PER cells in suspension we performed a transfection with increasing amounts of the compound poly(2-(dimethylamino)ethyl-10-4-aminobutyl)phosphazene (PPZ) with a constant amount of DNA. Cells were exposed to the transfection mix for three hours before dilution of the medium. Transfection of the suspension cells as measured by X-Gal staining and fluorometric analysis was dependent on the amount of PPZ added (Table 6 and 7) reaching 5% X-Gal positive cells with 320 ug PPZ (Table 6) and 160 ug PPZ (Table 7). Higher amounts of PPZ in the transfection mix was associated with extensive loss of cells.

Next we assessed the potential of PER cells for the large scale production of recombinant AAV. Large scale production

potential was first evaluated on adherent PER.C6 cells. Ten 170 cm^2 (Greiner) dishes were seeded with 2 x 10^7 PER.C6 per dish in DMEM + 10% FCS. Cells were transfected with pACV-βgal, pIM45 and pWE/Ad.AflII-rITR (2 : 8 : 30 ugram respectively). Virus harvested 48 hours later according to the following protocol. Medium was removed from the cells and the cells were collected by scraping into 4 ml/dish of fresh DMEM. The cell suspension was freeze thawed twice and subsequently incubated with DNaseI (100 ugram/ml) at 37 ^{0}C 30 minutes. The suspension was subjected to two additional freeze thaw cycles after which the cell debri was removed by centrifugation (3000 RPM, 10 minutes). The supernatant was incubated with 13.3 ml of saturated $(NH_4)_2SO_4$ (4 ^{0}C, 10 minutes). The precipitate was removed by centrifugation at 10.000 RPM in an SW27.1 rotor (4 ^{0}C, 15 minutes). Supernatant was incubated with an additional 26.6 ml of saturated $(NH_4)_2SO_4$ and incubated for 20 minutes at 4 ^{0}C. The virus was pelleted by centrifugation at 12.000 RPM in an SW27.1 rotor (4 ^{0}C, 30 minutes). The pellet was resuspended in 5 ml PBS (NPBI) and divided equally over two Quick-Seal Ultra-Clear tubes (Beckman Instruments, Mijdrecht, The Netherlands). The virus suspension was underlayed with an equal volume of OptiPrep (Nycomed Pharma AS, Oslo, Norway). The sealed tubes were rotated (20 minutes, 10 RPM) at an angle of 80 degrees. The virus was separated by density centrifugation (3 hours at 71.000 RPM) in an VTi80 rotor (Beckman Instruments). Fractions were collected (200 ul per fraction) and titrated for the presence of recombinant AAV. Positive fractions were pooled (± 400-600 ul) and diluted with 15 ml PBS (NPBI) and subsequently concentrated with Centriplus 100 and a Centricon 100, respectively (Amicon, Capelle a/d IJssel). The concentrated fraction was titrated and found to have a titer of 8.5 x 10^9 infectious units per ml.

Table 1. Number of colonies after transfection of PER.C6 with E2A expression vectors

plasmid	number of colonies	cell lines established
pcDNA3	≈ 200	4/4
PcDNA3wtE2A	4	1/4
PcDNA3tsE2A	≈ 100	37/45

Table 2. Number of colonies after transfection of 293 with E2A expression vectors

plasmid	number of colonies
pcDNA3	≈ 100
PcDNA3wtE2A	2
PcDNA3tsE2A	25

Table 3. Infection of PER.C6 and PER.C6tsE2A c3-9 with Ad.d1802

	32°C day 2
PER.C6 mock	-
PER.C6 dl802	-
PER.C6ts125c3-9 mock	-
PER.C6ts125c3-9 dl802	Full CPE

Table 4. Adenovirus free production of recombinant AAV on PER.C6 and PER.C6tsE2A.c5-9

Adenovirus helper function plasmid(s)	rAAV ACV-βgal titer (IU/ml) PER.C6	PER.C6tsE2A.c5-9
pWE/Ad.Δ5′	$< 10^3$	8×10^4
pWE/Ad.AflII-rITR	1.3×10^6	9.6×10^5
pE2a, pE4orf6, pVA	3.2×10^5	3.2×10^5
pE2a, pVA	3.2×10^5	4×10^4
pE2a, pE4orf6	2×10^4	6.4×10^5
pE4orf6, pVA	10	1×10^4
pE2a	2.5×10^4	6.4×10^3
pVA	0	1.3×10^3
pE4orf6	0	1.3×10^3
no Ad-DNA	10	1.3×10^3

Table 5. Transfection of PER.C6 cells in suspension.

Exp. no[a]	Transfection reagent	DNA (ug)	DNA/lipid (ug/ul)	% Blue	β-Gal.activity ugram/10^6 cells
1	DMRIE-C™	4	1 : 5	10	1.52
	-	-		0	0.0
2	DMRIE-C™	4	1 : 5	20	3.8
	-	-		0	0.0
3	DMRIE-C™	20	1 : 10	ND	0.53
	-	-		ND	0.0
4	DMRIE-C™	20	1 : 20	ND	0.10
	FuGENE™ 6	20	1 : 20	ND	0.34
	-	-		ND	0.0

[a] Experiment 1 was performed in 6 well dishes in stationary cultures. Experiment 2 was performed in 6 well dishes in shaking cultures (100 RPM). During the transfection the cultures where incubated without shaking. Experiments 3 and 4 were performed in Erlenmeyer cultures under continuous shaking (100 RPM).

Table 6. Transfection of PER.C6 cells in suspension.

Transfection reagent	DNA (ug)	PPZ (ug)	% Blue cells	Viable[a] cells/ml	β-Gal. activity (ugram/10^6 cells)
PPZ	4	4	0	9×10^5	0.02
"	4	20	0	9×10^5	0.02
"	4	40	0	7×10^5	0.02
"	4	80	< 0.1	8×10^5	0.02
"	4	160	< 0.1	3×10^5	0.02
"	4	320	5	1.6×10^4	0.50
"	4	640	-	$< 10^3$	-
"	4	1280	-	$< 10^3$	-

[a]Number of viable cells, determined by trypan blue exclusion, two days after transfection.

Table 7. Transfection of PER.C6 cells in suspension

Transfection reagent	DNA (ug)	PPZ (ug)	% Blue cells	Viable[a] cells/ml	β-Gal. activity (ugram/10^6 cells)
PPZ	4	160	5	4×10^5	ND
"	4	200	1	9×10^5	ND
"	4	240	-	$< 10^3$	ND
"	4	280	-	$< 10^3$	ND
"	4	320	-	$< 10^3$	ND
"	4	360	-	$< 10^3$	ND
"	4	400	-	$< 10^3$	ND
"	4	440	-	$< 10^3$	ND

[a]Number of viable cells, determined by trypan blue exclusion, two days after transfection.

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CLAIMS

1. An adeno-associated virus (AAV) packaging cell having been provided with nucleic acid encoding at least one gene product providing AAV helper function allowing generating recombinant AAV without concomitant helper virus production.

2. A cell according to claim 1 wherein said nucleic acid encodes an adenovirus gene product.

3. A cell according to claim 2 wherein said gene product comprises adenovirus E1 or a functional fragment thereof.

4. A cell according to claim 2 wherein said gene product comprises adenovirus E2A or a functional fragment thereof, wherein said E2A is preferably derived from adenovirus ts125.

5. A cell according to anyone of claims 1 to 4 having further been provided with at least one additional nucleic acid encoding additionally required helper function.

6. A cell according to claim 5 wherein said gene product encodes adenovirus E1 or a functional fragment thereof and at least one of said additional nucleic acid encodes adenovirus E2A or a functional fragment thereof, wherein said E2A is preferably derived from adenovirus ts125.

7. A cell according to claim 6 further comprising a nucleic acid encoding adenovirus E4orf6 or a functional fragment thereof.

8. A cell according to anyone of claims 1 to 7 having been derived from a human embryonic retinoblast cell.

9. A cell according to claim 8 comprising a PER.C6 cell having been deposited under accession number 96022940 ECACC at the Centre for Applied Microbiology Research (CAMR) or a cell derived thereof.

10. A cell according to anyone of the claims 1 to 9, comprising cosmid pWE/Ad.AflII-rITR (ECACC deposit p97082116 at CAMR).

11. A cell according to anyone of the claims 1 to 10, comprising cosmid pWE/Ad.AflII-rITRΔE2A.

12. A cell according to anyone of the claims 1 to 11, comprising plasmid pcDNA3wtE2A.

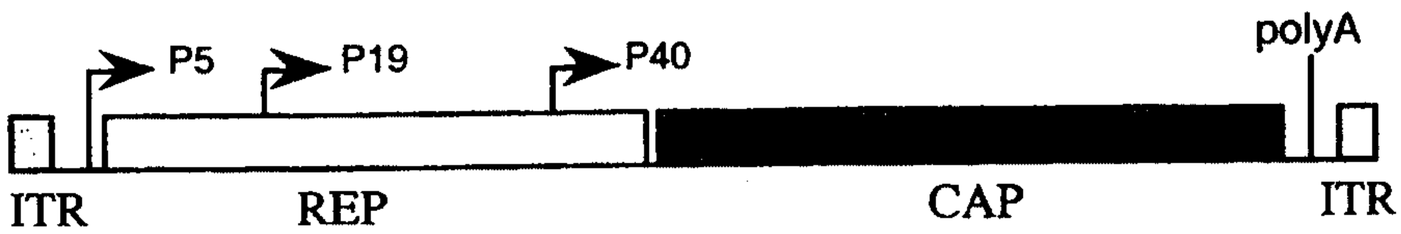
13. A cell-culture comprising a cell according to anyone of claims 1 to 12.

14. A cell-culture according to claim 13 comprising medium devoid of any human or animal constituents.

15. A cell-culture according to claim 13 or claim 14 which comprises a suspension cell culture or other large scale culture.

16. Use of a cell according to anyone of claims 1 to 12 or a cell-culture according to anyone of claims 13 to 15 for producing recombinant adeno-associated virus.

Figure 1. AAV-Genome structure



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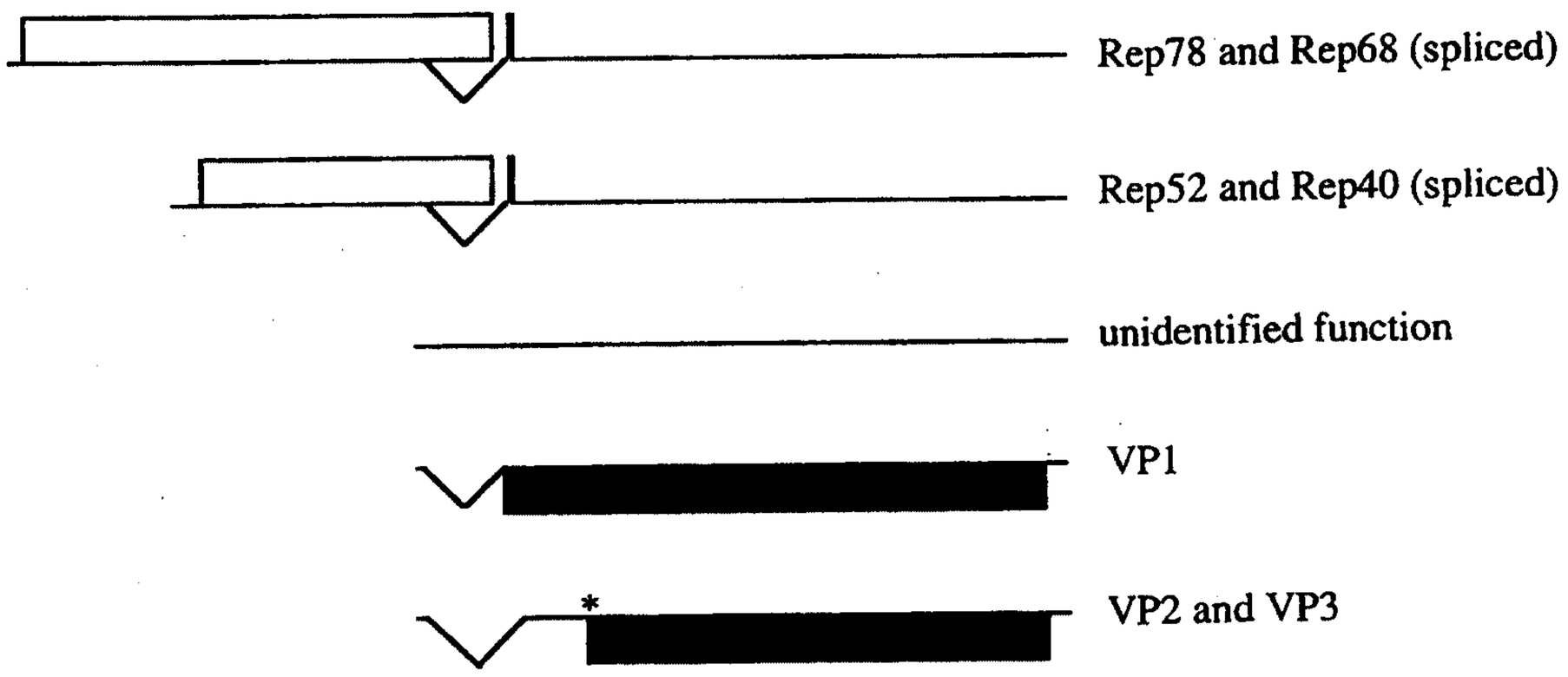

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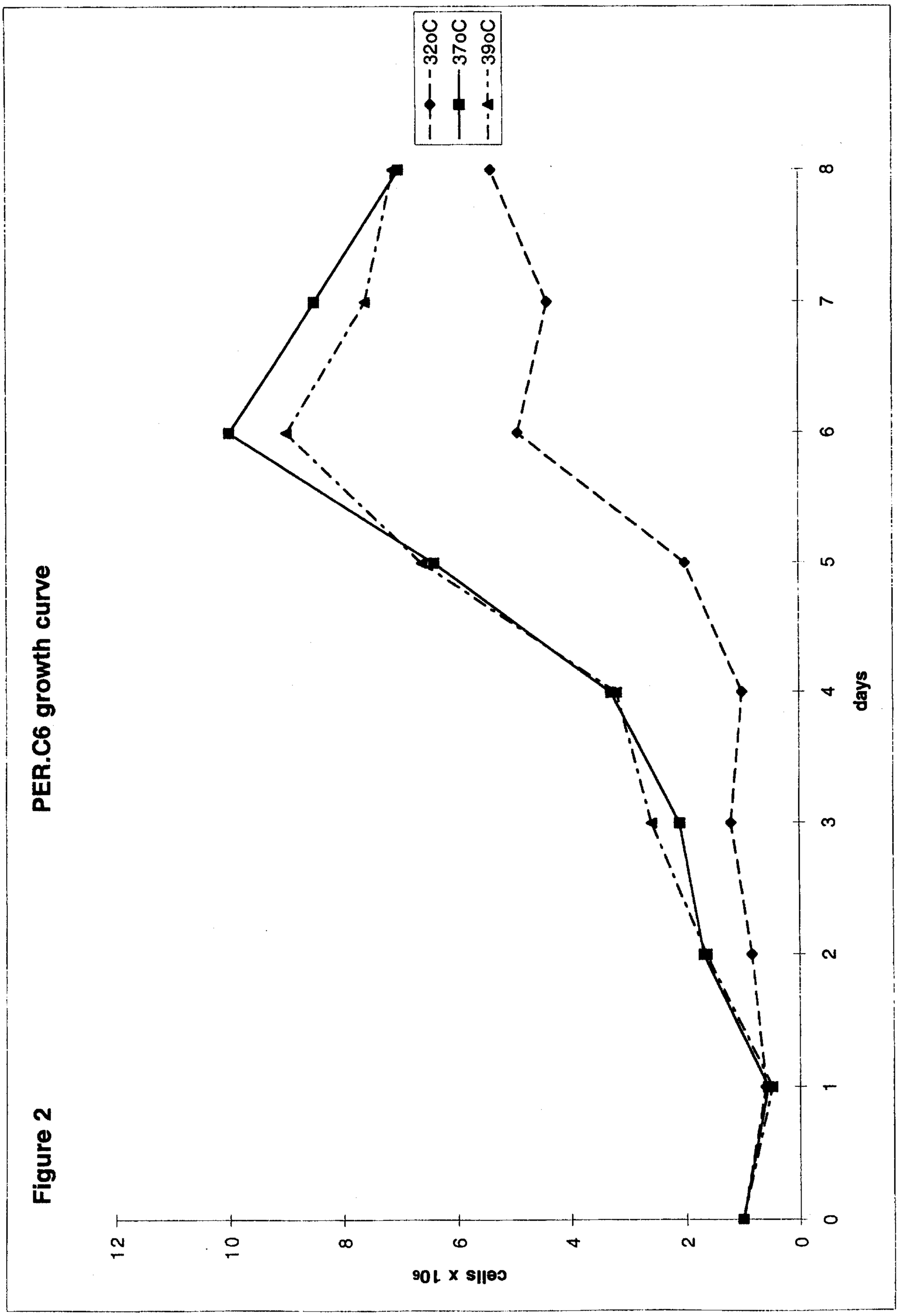
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37oC
39oC
PER.C6 growth curve
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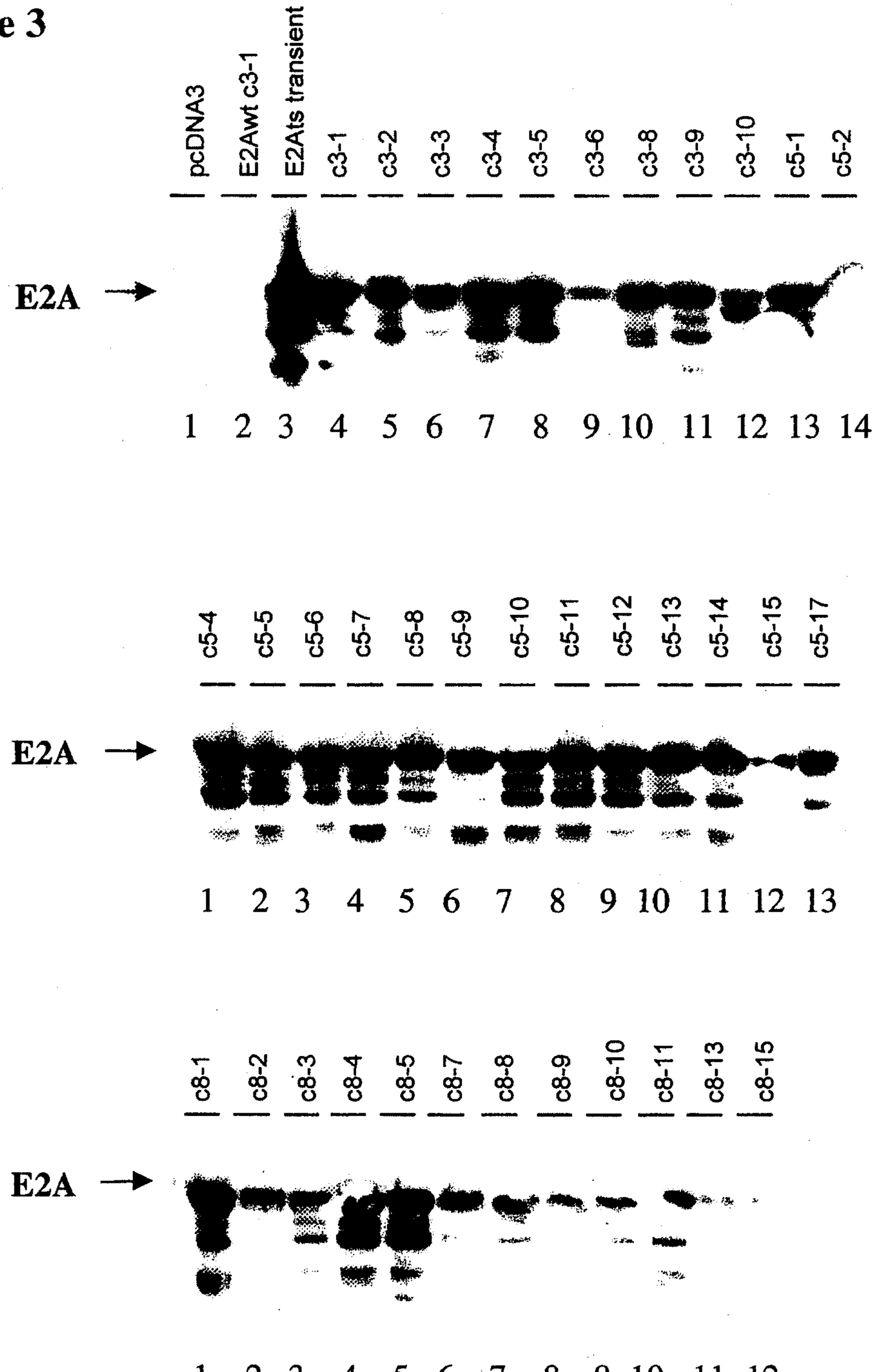
8
10
12
1
3
5
7

Figure 2

Figure 3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c5-6
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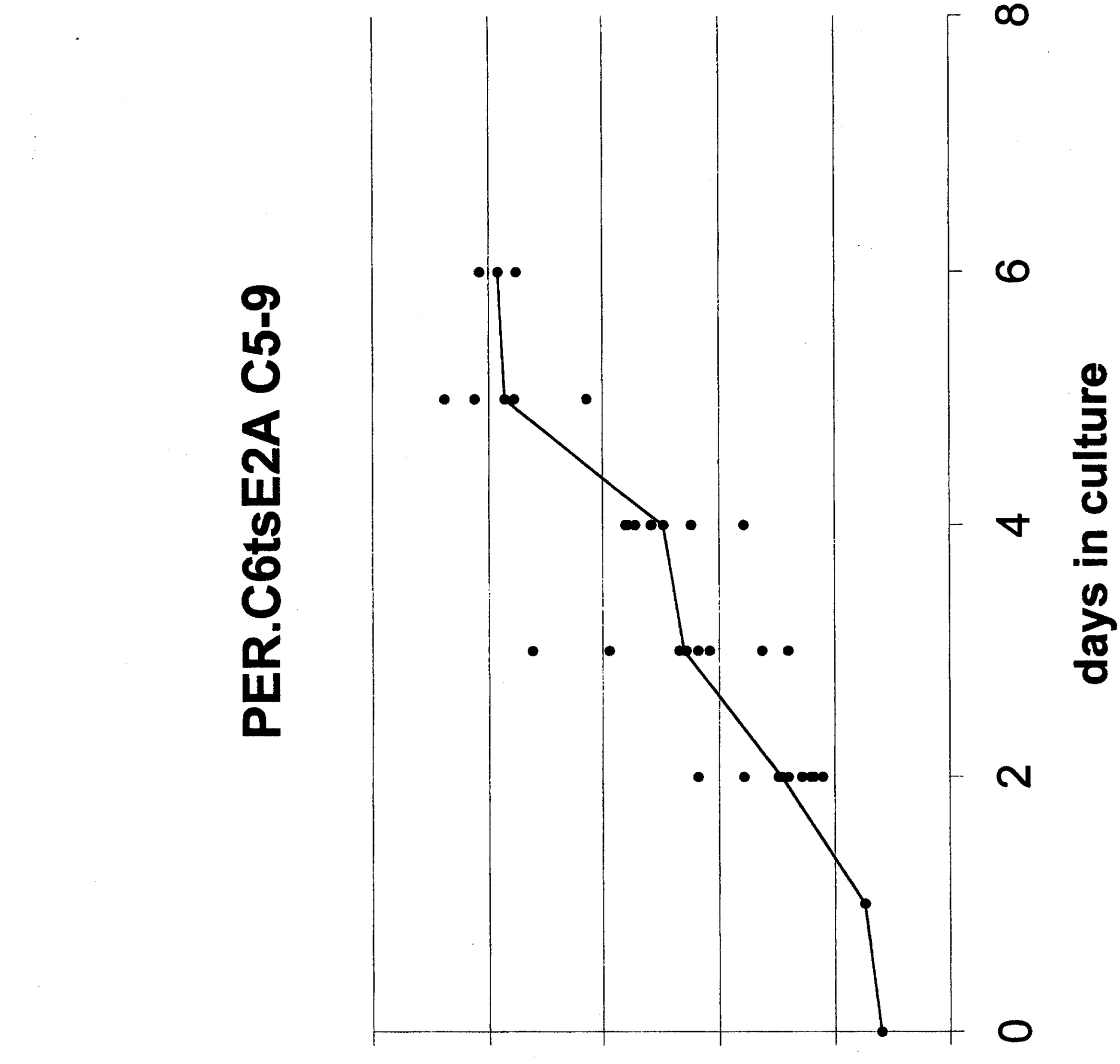
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cells/ml
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00
0
2
4
6
8
days in culture

Figure 4