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A vector based on the SV40 origin of replication and chromosomal S/MARs replicates episomally in CHO cells

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ABSTRACT

We have developed an episomal replicating expression vector in which the SV40 gene coding for the large T-antigen was replaced by chromosomal scaffold/matrix attached regions. Southern analysis as well as vector rescue experiments in CHO cells and in *Escherichia coli* demonstrate that the vector replicates episomally in CHO cells. It occurs in a very low copy number in the cells and is stably maintained over more than 100 generations without selection pressure.

INTRODUCTION

At present, only some viral-based vectors, such as SV40-, BPV- or EBV-based vectors, replicate episomally in some eukaryotic cells (see 1–3 for review). The replication origins contained in these vectors require the interaction with virally encoded *trans*-acting factors. These proteins very often lead to transformation of the transfected cells (4). For example the replication of SV40 DNA requires a single viral protein, the large T-antigen, whereas the other factors are supplied by the host cell. Infection with SV40 or transfection with vectors carrying the gene coding for the SV40 large T-antigen can lead to immortalization of primary cells and can induce tumor formation in animals (5–7). Therefore, such vectors are only partially useful for gene transfer into mammalian cells. In order to construct an episomal replicating vector not expressing any viral protein and thus avoiding cell transformation, we now have constructed a vector in which the gene coding for the SV40 large T-antigen was replaced by the scaffold/matrix attached region (S/MAR) from the 5′-region of the human interferon β-gene (8). S/MARs are typically 70% A/T-rich sequences (9) and are often found in association with chromosomal origins of bidirectional replication (10). Furthermore, they play a crucial role in maintaining chromosome structure and functioning in the eukaryotic nucleus (11). Here we show that a vector carrying an S/MAR-element in association with the SV40 origin of replication is replicating at very low copy numbers in CHO cells and is stably maintained without selection for more than 100 generations. This construct represents the first small circular vector replicating episomally in a eukaryotic cell, which does not require any virally encoded *trans*-acting factors for its replication.

MATERIALS AND METHODS

The S/MAR fragment from the 5′-region of the human interferon β-gene was isolated from plasmid pTZ-E20 (8) as a 2.0 kb *Eco*RI/*Bgl*II fragment and ligated into the polylinker of pGFP-C1 (Clontech). A restriction map of the resulting plasmid pEPI-1 is shown in Figure 1a. CHO cells were grown in Ham's F12 medium (Biochrom KG) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2.5 µg/ml amphotericin B (Biochrom KG) and 50 µg/ml gentamicin (Biochrom KG). 3×10^6 CHO cells were electroporated with 5 µg of vector pEPI-1 or pGFP-C1 DNA at a setting of 300 V, 960 µF and 200 Ω. One day after gene delivery transfected cells were selected with 500 µg/ml G418 (Boehringer Mannheim). Stable clones were isolated after 2 weeks and cultured with 250 µg/ml G418. A modified HIRT protocol (12) was used to isolate extrachromosomal DNA of stable transfectants. This DNA as well as chromosomal DNA was digested with various restriction enzymes, separated by agarose gel electrophoresis, blotted and then hybridized with the ^{32}P-labeled pEPI-1 probe using ^{T7}QuickPrime Kit (Pharmacia). *Escherichia coli* DH5α (Clontech) and CHO cells were electroporated with the HIRT-extract from ~10^6 stably transfected CHO cells. *Escherichia coli* transformants were selected using plates containing 30 µg/ml kanamycin. CHO cells were selected with 500 µg/ml G418.

RESULTS

A restriction map of vector pEPI-1 and its functional elements is shown in Figure 1a. CHO cells were transfected with 5 µg pEPI-1 DNA and clones were isolated after 2 weeks of selection with G418. Chromosomal and extrachromosomal DNA was isolated from G418 resistant clones 6–8 weeks after gene transfer. The DNA was digested with various restriction enzymes, blotted and hybridized with a ^{32}P-labeled pEPI-1 probe. No hybridization signal to chromosomal DNA from cells transfected with vector pEPI-1 was observed (results not shown) and in DNA isolated from the HIRT-extract a restriction pattern identical to the original pEPI-1 plasmid DNA (Fig. 1b, lanes 1 and 2) was obtained in all 24 clones analyzed (Fig. 1b, lanes 3–6). In contrast, in CHO cells transfected with the plasmid pGFP-C1 the vector DNA was randomly integrated into the genome (Fig. 1b, lanes 10–12). Conversely, it has been documented extensively that plasmids

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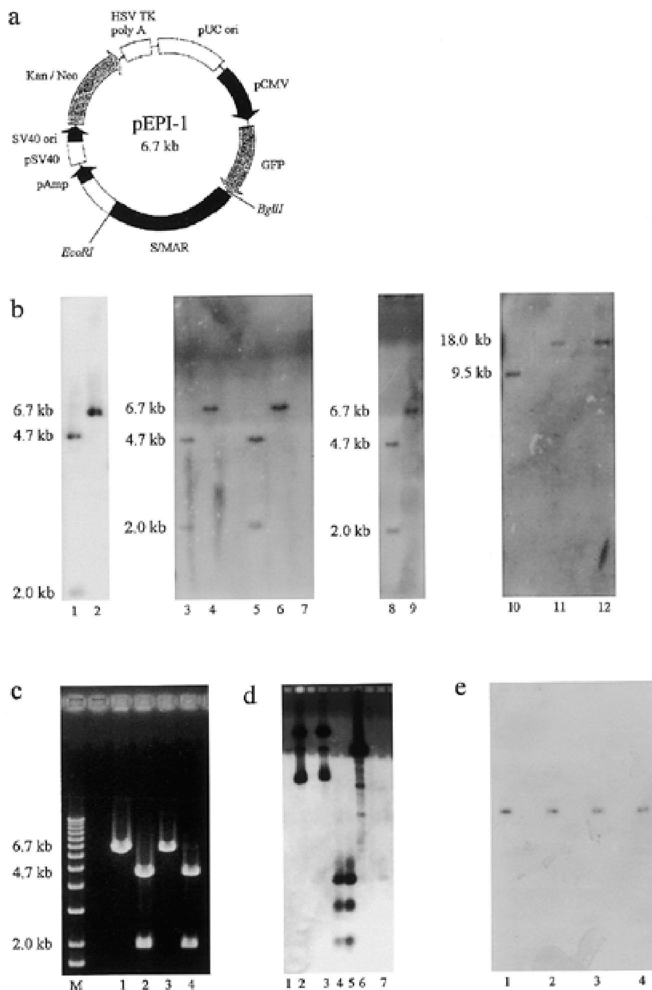

Figure 1. (**a**) Restriction map of pEPI-1. For construction see Materials and Methods. The functional elements of the vector are the pUC origin of replication (pUC ori) for propagation in *E.coli*, the matrix attached region from the 5′-region of the human Interferone β-gene (S/MAR), the promoter of the bacterial ampicillin resistence gene for selection in *E.coli* (pAmp), the SV40 early promoter for selection in CHO cells (pSV40), the SV40 origin of replication (SV40 ori), the kanamycin resistance gene from Tn5 from *E.coli* for selection in *E.coli* and CHO cells (Kan/Neo). (**b**) Restriction analysis of G418 resistant CHO clones. DNA was separated on 0.8% agarose gels, blotted and hybridized with the ^{32}P-labeled pEPI-1 probe. Lanes 1 and 2, restriction digestion of the original vector pEPI-1. Lane 1, *Eco*RI/*Bgl*II digestion, the 2 kb fragment represents the S/MAR; lane 2, *Eco*RI digestion. Lanes 3–6, restriction digestion of CHO clone E2 and E9 transfected with pEPI-1. Lanes 3 and 5, *Eco*RI/*Bgl*II digestion; lanes 4 and 6, *Eco*RI digestion. Lane 7, *Eco*RI digestion of untransfected CHO cells. Lanes 8 and 9, the HIRT-extract from clone E9 was used to re-transfect CHO cells. DNA from these transfectants was isolated from the HIRT-extract and digested with *Eco*RI/*Bgl*II (lane 8) or *Eco*RI (lane 9). Lanes 10–12, CHO cells transfected with pGFP-C1. Chromosomal DNA was isolated, digested with *Eco*RI, separated on a 0.8% agarose gel, blotted and hybridized with ^{32}P-labeled pGFP-C1 probe. Lane 10, clone G1; lane 11, clone G5; lane 12, G8. (**c**) Plasmid rescue experiments in *E.coli*. DNA from the HIRT-extract of clone E9 was used to transfect *E.coli*. Plasmid DNA was isolated and digested with *Eco*RI or *Eco*RI/*Bgl*II. M, 1 kb ladder (Gibco BRL). Lanes 1 and 2, plasmid pEPI-1 digested with *Eco*RI (lane 1) and *Eco*RI/*Bgl*II (lane 2). Lanes 3 and 4, plasmid isolated from *E.coli* digested with *Eco*RI (lane 3) or *Eco*RI/*Bgl*II (lane 4). (**d**) DNA methylation-dependent cleavage assay of CHO clone E9 and plasmid pEPI-1 prepared from *E.coli*. DNA was separated on a 0.8% agarose gel, blotted and hybridized with the ^{32}P-labeled pEPI-1 probe. Lanes 1 and 7, HIRT-extract from 10^7 cells of clone E9. Lane 1, *Nhe*I/*Dpn*I digestion, the 6.7 kb fragment represents the linearized vector; lane 7, *Mbo*I digestion; due to the low copy number of the vector only the largest *Mbo*I fragment is visible on this blot. Lanes 2–6, restriction digestion of the original vector pEPI-1. Lane 2, undigested pEPI-1; lane 3, *Mbo*I digestion; lane 4, *Nhe*I/*Dpn*I digestion; lane 5, *Dpn*I digestion; and lane 6, *Nhe*I digestion. (**e**) Restriction analysis of CHO clone E9 grown without selection pressure after different numbers of generations. DNA was isolated from a HIRT-extract of clone E9, digested with *Eco*RI, separated on 0.8% agarose gel, blotted and hybridized with the ^{32}P-labeled pEPI-1 probe. Lane 1, DNA isolated from cells under selection pressure; lane 2, DNA isolated after 21 days (43 generations); lane 3, DNA isolated after 35 days (71 generations); and lane 4, DNA isolated after 59 days (108 generations).

only containing the S/MAR element used here integrate at elevated copy numbers, especially if they are transfected in circular form (13,14). These results strongly suggest an episomal occurrence of pEPI-1 in CHO cells. To further demonstrate the episomal character of pEPI-1 in CHO cells, the HIRT-extract from ~10^6 transfectants was used for transfection both of CHO cells and *E.coli*. Between 1 and 15 transformants were obtained in these experiments. By control transfection with the vector at various concentrations it could be estimated that this number would be the transfection rate expected if the vector would occur in a copy number below 20 in CHO cells. DNA isolated from a HIRT-extract of transfected CHO clones showed the same restriction pattern as the original vector (Fig. 1b, lanes 8 and 9). Using a HIRT-extract from these re-transfected cells for another transfection, CHO cells again yielded transfectants with the same restriction pattern. Similarily the plasmid from the HIRT-extract could be rescued in *E.coli* and was also found to be identical to the original vector as shown by restriction analysis (Fig. 1c). To further demonstrate that vector pEPI-1 replicates episomally in CHO cells a methylation-dependent cleavage assay was performed (10). DNA from the HIRT-extract isolated from transfected CHO clones was digested with *Dpn*I after linearization with *Nhe*I. As shown in Figure 1d (lane 1) the vector is resistant against digestion with *Dpn*I, while vector DNA isolated from bacteria shows the expected restriction fragments (lanes 4 and 5). In contrast, the vector DNA isolated from the HIRT-extract was digested by *Mbo*I (lane 7) while the bacterial vector was resistant against digestion with this enzyme (lane 3).

The approximate copy number of pEPI-1 in CHO clones was determined by comparing the hybridization intensity of HIRT-extract with that of the original plasmid DNA at defined concentration and estimated to be below 20, in agreement with the above observations. In order to determine plasmid stability, CHO cells were grown in medium lacking G418 for >2 months. During this period of time no significant number of cells died after addition of G418 and the episomal vector still could be detected by Southern analysis (Fig. 1e).

DISCUSSION

Conventional vectors currently used for animal biotechnology suffer from a number of limitations. All available vectors either rely on random integration into the host genome or are only transiently retained. Both fates create serious problems with respect to safety, reproducibility and efficiency. Random integration may lead to insertional mutagenesis and to silencing of the

transgene. Transient expression, on the other hand, implies that repeated treatments would be necessary and this in most cases is not desirable (15). Therefore the ideal vector, especially for gene therapy, should be retained in many cells without integration. A number of virus-based vectors replicate episomally in some mammalian cells (16). However, since replication of these constructs relies on the presence of virus-encoded *trans*-acting factors, which often lead to cell transformation, their use for genetic manipulation of eukaryotic cells is largely limited. We have constructed a vector based on the SV40 origin of replication and replaced the gene coding for the *trans*-acting factor large T-antigen by chromosomal S/MARs. The results obtained by Southern analysis and plasmid rescue experiments strongly suggest that it replicates efficiently and stably extrachromosomally in CHO cells. No random integration was observed in all examined clones. This is clearly distinct from the observation of a head-to-tail integration of highly amplified vectors carrying A/T-rich sequences of a different type (17). While this integration pattern yields the same restriction pattern as the original vector, additional restriction fragments flanking the vectors at the site of integration should have been seen since our plasmid occurs in a very low copy number. A hybridization signal to chromosomal DNA was also not detected. The episomal nature of the construct was further demonstrated by retransfection experiments into CHO cells and *E.coli*, in which the vector could be 'recycled' several times and by its resistency against *Dpn*I cleavage. At present it can only be speculated why this construct acts as an episomal vector. Plasmids carrying only the SV40 origin of replication or S/MARs randomly integrate into the host genome (Fig. 1; 13,14). It therefore could be assumed that the chromosomal S/MAR recruits cellular factors substituting for the function of the SV40 large T-antigen. In fact, it is known that S/MARs bind several *trans*-acting factors required for regulation of a eukaryotic origin of replication and that DNA replication occurs in tight association with the nuclear matrix (11,18). Further experiments are required to determine if this is a general function of S/MARs or a property of this particular element. From a practical point of view a construct consisting of a defined origin of replication in combination with a defined chromosomal DNA sequence may represent a prototype of a safe, reproducible and efficient expression vector for mammalian cells and organisms.

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