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POLYMERIZATION OF VECTOR DNA AFTER TRANSFECTION INTO HAMSTER FIBROBLAST CELLS

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We have transformed hamsters lung fibroblasts with two cloned, selectable markers, the widely used pSV2gpt vector and pNE03, which confers resistance to neomycin. Analysis of DNA extracted from both individual and pooled groups of several hundred transformants reveal that in these carrier DNA-free transformation experiments, the vector DNA are found in concatenate forms in the transformants. The copy number of the integrated vectors can vary from a few to several hundred. In addition, the efficiency of transformation is ten times higher in the case of pSV2gpt.

The ability to introduce DNA into animal cells has become an important tool for the study of gene regulation in animal cells (1). Among the various techniques, the calcium phosphate co-precipitation procedure is relatively straightforward and applicable to a variety of cell strains. In the past, DNA mediated gene transfer experiments are generally limited by the availability of specific conditional mutant cells as recipient cells. This limitation excludes many specialized and differentiated animal cells which do not have phenotypes appropriate for applying selective pressure. However, with the construction of vectors which contain dominant genetic markers, selective culture conditions may be readily applied on normal cells. One example

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is the widely used plasmid pSV2gpt (2), in which the bacterial gene coding for xanthine-guanine phosphoribosyl-transferase (XGPRT) is inserted into a pBR322-SV40 recombinant. The expression of this gene allows mammalian cells to utilize xanthine for GMP formation. Another example is the plasmid pNE03 which contains the neomycin resistant coding sequence (3) inserted into pBR322. The expression of this gene is regulated by the Herpes thymidine kinase promoter (4) linked onto the neomycin resistant coding sequence during construction of the plasmid, and confers resistance to antibiotic G418 normally toxic to a variety of animal cells (B. Wold, R. Sweet, R. Axel, personal communication.

Studies on the physical state of the integrated plasmid have been previously described for a few pSV transformants in monkey and mouse cells (2), and recently in CHO cells (5). However, the transformations in the hamster study (5) are performed in the presence of high molecular weight carrier DNA. In this report, we characterize a large number of hamster fibroblast transformants obtained by carrier DNA-free transformation experiments. In addition, we compared the efficiencies of transformation by these two different selectable markers. These observations may be applicable to transformation of other hamster cell lines which constitute a large, valuable pool of somatic cell mutants.

MATERIALS AND METHODS

Cell strains: The Chinese hamster cell lines, WglA and K12 are derivatives of DON cells selected for resistance to azaguanine, and have an HPRT negative phenotype. The spontaneous reversion frequency for the HPRT deficient phenotype is less than 5×10^{-7}. Conditions for cell culture has been reported previously (6).

Transformation: The procedure used has been described by Parker and Stark (7) with the following modifications: (i) the calcium phosphate concentration is 125 mM instead of 250 mM commonly used in transformation experiments for mouse L cells, as we observe that the survival of hamster cells is much better with the lower calcium phosphate concentration, (ii) in order to follow more precisely the linkage relationship of the vectors after transfer into the cells, we have omitted the use of host DNA in all the experiments described here, and (iii) after incubation of cells with the DNA precipitate, the cells are shocked with 15% glycerol in isotonic saline buffer for 3 min at 35°C. For these hamster cells, the glycerol treatment is preferable to DMSO treatment used in other methods (8). After 2 weeks of incubation in the presence of HAT (14 µg/ml hypoxanthine, 0.19 µg/ml aminopterin and 3.9 µg/ml thymidine) or G418 (350 µg/ml), the number of resistant colonies is determined by counting colonies after fixation in 25% methanol and staining with 1% crystal violet. The transformation efficiency is the ratio between the number of resistant colonies to the number of cells seeded per culture dish.

RESULTS

The transformation efficiency obtained using various amounts of plasmid DNA is shown in fig 1. There is a general increase of transformation efficiency up to plasmid DNA concentration of about 15 µg per 100 mm diameter dish. Since no carrier DNA is used in these experiments and the optimum conditions for the formation of the calcium phosphate and DNA co-precipitate is generally around 10 to 20 µg per 100 mm diameter dish (9), the above result is expected. However, the high transformation efficiency achieved by these cell lines is noteworthy. With plasmid pSV2gpt, we routinely observe 200 to 400 HAT resistant colonies per 10^5 cells. When this is compared with 2 to 25 surviving colonies per 10^5 cells as described for monkey (TC7) or 10 to 125 clones per 10^5 cells per mouse (3T6) cells (2), these hamster cells are at least as competent, or slightly better than mouse cells as recipients for Ecogpt transformation. In addition, the pSV2gpt transforms these cells about 10 times more efficiently than pNE03.

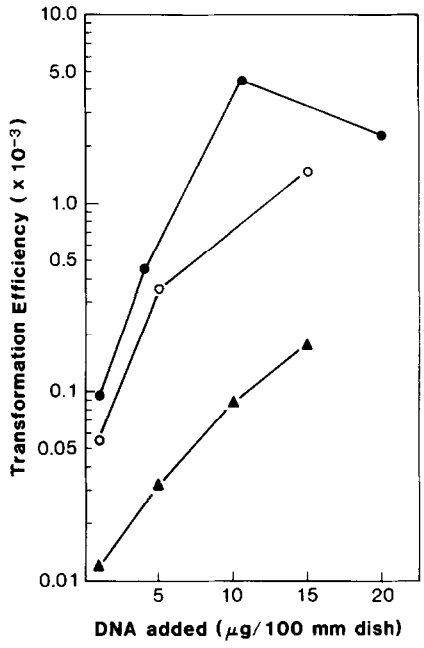

Figure 1. Dosage effect of vector DNA on transformation of hamster fibroblast cell lines with pSV2gpt and pNE03. K12 or WglA cells are mixed with increasing amount of plasmid DNA and treated as described in Materials and Methods. After 2 weeks of growth in the selective medium (either medium containing HAT or G418 at 350 μg/ml), resistant colonies are counted. The efficiency is expressed as the ratio between the number of resistant cell colonies and the number of cells originally seeded per dish. (●—●), K12 cells transformed with pSV2gpt; (o— o), WglA cells transformed with pSV2gpt and (▲—▲), K12 cells transformed with pNE03.

Next, we examine the physical state of these plasmids in the K12 and WglA transformants. The two key questions we would like to address ourselves to are: (i) are the vectors integrated in the host genome, or do they exist as autonomously replicating extrachromosomal elements? and (ii) how many copies of the vectors are taken up by the stable transformants? For this purpose, we isolate individual transformants from WglA and K12 cells, expand them into mass culture and extract genomic DNA according to the procedure described by Pellicer et al. (10). We also prepare extracts from the same cells according to Hirt's method (11). In

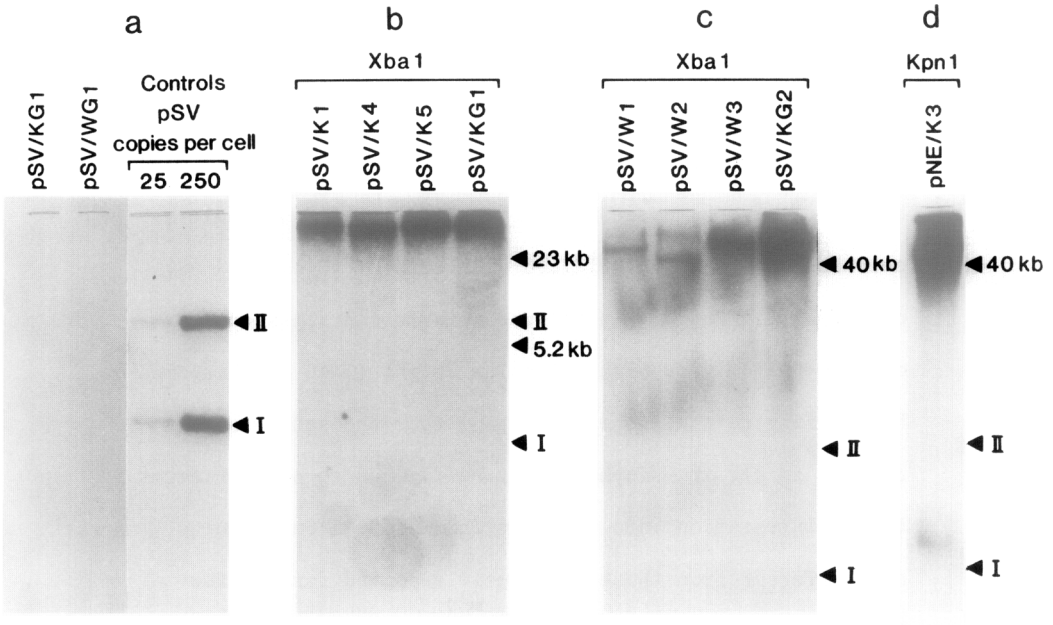

Figure 2. Hybridization of transformant DNA with vector probe. (a) Hirt supernatant is extracted from 1 x 10^6 cells of a pooled group of ∿300 pSV-transformed K12 cells (pSV/KG1) and from 3 x 10^6 cells of a pooled group of ∿300 pSV-transformed Wg1A cells (pSV/Wg1) and hybridized to ^{32}P labeled pSV2gpt. The positions of supercoil (form I) and relaxed circle (form II) of pSV2gpt are indicated in the control lanes where known amount of pSV2gpt DNA is applied. (b) 15 μg of genomic DNA extracted from three individual pSV transformed K12 cells (pSV/K1, pSV/K4 and pSV/K5) and one pooled group of pSV transformed K12 cells (pSV/KG1) are digested with Xba 1, electrophoresed on 1% agarose gels, blotted and hybridized with ^{32}P labeled pSV2gpt. (c) 15 μg of genomic DNA extracted from three individual pSV-transformed Wg1A cells (pSV/W1, pSV/W2, pSV/W3) and one pooled group of pSV transformed K12 cells (pSV/KG2) are digested with Xba 1, electrophorsed on 0.6% agarose gel, blotted and hybridized with ^{32}P labeled pSV2gpt. (d) 15 μg of genomic DNA extracted from an individual pNE03 K12 transformant (pNE/K3) is digested with Kpn 1, electrophorsed on a 0.6% agarose gel, blotted and hybridized with ^{32}P labeled pNE03.

some cases, we pool several hundred transformants from a single dish and grow them up as a group. The Hirt lysates are directly applied onto agarose gels, transferred to nitrocellulose paper by the method of Southern (12) and hybridized with ^{32}P-labeled pSV probe. The results are shown in fig 2a. As in the case of monkey, mouse and CHO cells (2,5), there is no detectable pSV DNA in any of the Hirt extracts of the

transformants being tested. Assuming that the vector sequences are all integrated into the host genome, we digest the genomic DNA preparations with restriction enzyme Xba 1 or Kpn 1 which does not cleave the plasmids. The restricted DNA is electrophoresed on agarose gels, blotted onto nitrocellulose paper and hybridized with either ^{32}P-labeled pSV or pNE03 (fig 2b-d). In all the transformants tested, we observe DNA of very high molecular weight (> 40 kilobases) hybridizing to the probes, implying that the vector sequences in all transformants may be ligated to polymer forms before integration into the host chromosome. Our results are different from those previously reported for the few pSV transformants isolated from monkey, mouse and CHO cells (2,5). There, the Xba digestion patterns yield more variable fragment sizes most of which are of lower molecular weights.

To determine the vector copy number and whether they exist as tandem repeats Wg1A or K12 transformant genomic DNA is cleaved with EcoR1, which cuts pSV at a single site, and hybridized with ^{32}P labeled pSV probe, five out of seven individual transformants show a predominant hybridizing band at 5.2 kb (kilobase), the size of unit length of pSV (fig 3 a,c). Interestingly, all pooled groups of K12 pSV transformants show a predominant hybridizing band at 5.2 kb (fig 3b). From quantitating the intensity of hybridization signal, we estimate that in the cases of pSV/K1 and pSV/W1, there are at most five copies of the vector sequence per cell. Most other individual and group transformants have an average of twenty to fifty copies of pSV per cell. In the case of one individual transformant, pSV-16-2/K1, the average vector copy number per cell is as high as seven hundred. Similar range of copy number is observed for

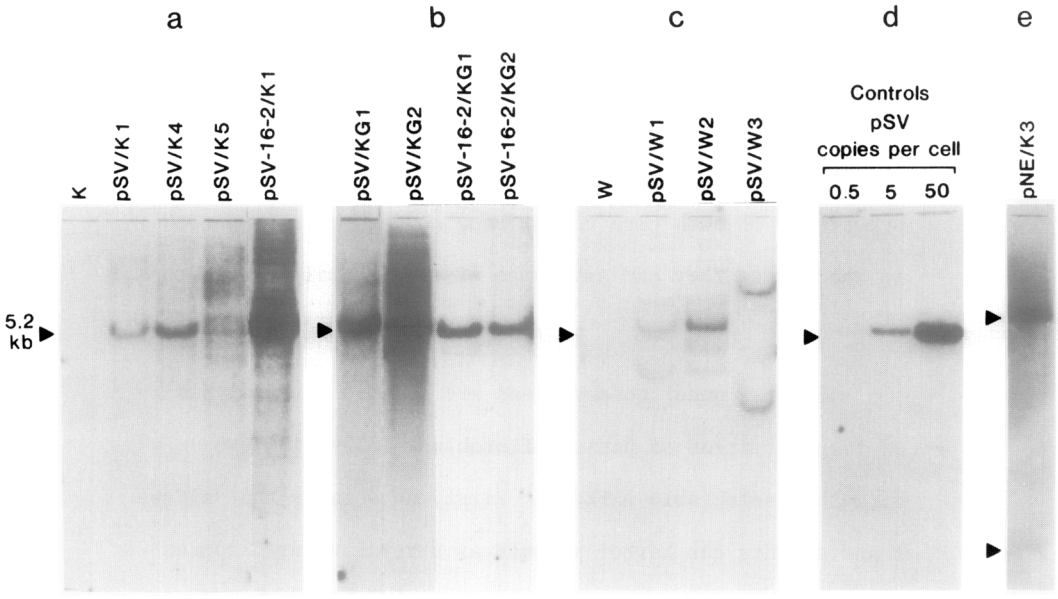

Figure 3. Analysis of integration pattern of vector DNA in hamster transformants. The hybridization probe used for (a-d) is ^{32}P labeled pSV2gpt; (e), ^{32}P labeled pNE03. In all sample lanes, 15 µg of high molecular weight genomic DNA is digested with EcoRI and applied onto 1% agarose gel. (a) DNA extracted from K12 cells (K) and three individual pSV transformed K12 cells. pSV-16-2/K1 refers to a K12 transformant which is co-transformed with pSV2gpt DNA and a Charon 4A clone containing a hamster gene (14). Because of the intensive hybridization of the pSV-16-2/K1 DNA to the probe, the autoradiogram shown has been exposed for a shorter time than the rest of the autoradiograms. (b) DNA extracted from four groups of pooled pSV transformed K12 cells. (c) DNA extracted from WglA cells (W) and three individual pSV transformed WglA cells. (d) known quantitities of pSV2gpt DNA is applied to serve as controls for calibration of hybridization signal. (e) DNA is extracted from an individual pNE03 K12 transformant. EcoRI cleaved pNE03 twice, generating two fragments, which are conserved in the vector DNA extracted from this transformant. The copy number of vector is estimated to be around 500 when compared to pNE03 standards (data not shown).

pNE03 transformants, with one individual transformant, pNE/K3, harboring at least five hundred copies of the vector (fig 3e). It is likely that a small fraction of the hamster transformants contains a very high vector copy number. Therefore, the pooled samples of several hundred transformants represent a group average of vector copy number and hybridization pattern. In similar carrier DNA-free transformation experiments performed in monkey and mouse cells

using pSV as vector, Mulligan and Berg (2) reported vector copies in the one to five range for the four transformants studied. It is not clear if the difference between the two observations is cell-type specific, or is due to the rare occurrence of such high vector copy number transformants, in which case they can simply be missed in small sample pools.

DISCUSSION

Several novel observations are made in our analysis of transformation of hamster fibroblast cells with two different selectable markers. First, we observe that a few transformants can harbor as much as several hundred copies of the vector DNA. The fact that most transformants retain unit length vector DNA suggest that they are present as tandem repeats. The mechanism that leads to exact polymerization of large number of vector DNA is not known, although the phenomenon we observe resembles that reported for tandem duplicates of SV40 after infection into nonpermissive cells (13). Since this also occurs in pNE03 transformants, which does not contain any SV40 sequence, the mechanism is independent of any SV40 sequence involvement. A very useful fact found in one of these transformants (pSV-16-2/K1) which have taken up about seven hundred copies of vector DNA is that a cloned hamster gene used in the co-transformation with pSV2gpt is also present in a highly amplified form in the recipient cells (A. S. Lee., unpublished result). It is now possible to study the effect of greatly increased gene numbers on the regulated expression of that particular gene.

Second, the ten-fold higher transformation efficiency using pSV2gpt as opposed to pNE03 is intriquing. This phenomenon may be due either to the higher tolerance of

the resistant colonies in HAT than in the G418 medium or to the effect of different promoters. Another possibility is that since pSV2gpt contains the origin of SV40 replication whereas pNE03 does not, the origin of SV40 replication may facilitate gene replication resulting in higher integration events. The exact mechanism which causes this differential efficiency remains to be resolved.

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