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Electric field-mediated gene transfer: characterization of DNA transfer and patterns of integration in lymphoid cells

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ABSTRACT

Southern analysis of individual transfectants generated by electroporation demonstrated a strong preference for the integration of DNA in low copy number even when electroporation was performed in the presence of increasing DNA concentrations. Although transfer of multiple DNA copies was detected at higher DNA concentrations (16 pmoles/ml or greater), the average gene copy number even at 36 pmoles DNA per ml, was only 13. Multiple gene copies were integrated at either a few chromosomal sites, or at a single site within individual transfectants. Restriction endonuclease cleavage data were consistent with a random orientation of molecules within a concatemer, suggesting that the concatemer may have risen via end-to-end ligation of linear molecules, rather than by homologous recombination. Integration of exogenous DNA into the host chromosome occurred preferentially at the ends of the linear molecule. Although the linearization site was lost upon integration, endonuclease sites as close as 18 bp from the linearization site were retained. These data, as well as direct restriction mapping of the transferred genes, indicate that DNA transfer and integration occur without DNA rearrangement. Taken together, these results suggest that electroporation may offer some unique advantages for the transfer of eukaryotic genes.

INTRODUCTION

We and others have shown that electroporation is a simple and efficient way to introduce genes into a variety of cell types resistant to transfection by chemical methods (1-6). In addition, we have also demonstrated that the method can be used to transfer marker genes into human hematopoietic progenitor cells and that the genes are maintained and expressed in the progeny of these cells (7).

Electroporation, therefore, appears to meet some of the criteria for a potential gene therapy protocol, including ease of use and lack of perturbation of the hematopoietic developmental system. A very important question in assessing of the utility of the technique for gene therapy, or more generally for the study of eukaryotic gene expression, is to determine whether the gene of interest is transferred in a functional, non-mutated form

and if its expression is still subject to the normal regulatory constraints of the system.

The mechanisms involved in the processing and integration of DNA transferred by electroporation are difficult to study in the human hematopoietic system due to the inability to obtain sufficient numbers of progenitor cells for nucleic acid analyses. We therefore undertook a systematic study of the patterns of integration of DNA transferred by electroporation into cultured mammalian cells.

MATERIALS AND METHODS

DNA and Cell Lines

The plasmids pSV2gpt and pSV2neo were obtained from the American Type Culture Collection (8), pSV7neogpt* and pSV400dhfr were obtained from H. Potter (2) and H. Dorai (9), respectively. Plasmid DNA was prepared as described earlier (10) except that the DNA was purified through two CsCl gradients prior to use.

The M12 cell line derived from a spontaneous B cell lymphoma of the BALB/C mouse, was obtained from H. Potter (11).

Electroporation

Electroporation was carried out as described earlier using the ISCO 494 power supply (1). All plasmids were linearized by restriction endonuclease digestion and then phenol-extracted and precipitated with ethanol. The linearized DNA was added to the cell suspensions at the concentrations indicated, 10 minutes prior to exposure of the cells to the electric field. For co-transfection, each plasmid was added to the cell suspension at a concentration of 15 pmoles per ml.

Following electroporation, cells were plated at concentrations of $1x10^2$, $2x10^2$ and $4x10^2$ viable cells per well of a 96 well micro-titer plate. Selection for the presence of the Eco gpt gene or the neo gene was performed using 1.5 µg mycophenolic acid (MPA) per ml or 1.5 mg G-418 per ml respectively, while selection for the murine dihydrofolate reductase gene (dhfr) was carried out in the presence of 0.15 µM methotrexate.

Preparation of Genomic DNA

High molecular weight DNA was prepared as described by Perucho et al, (12,13). Briefly, cells were washed twice in phosphate-buffered saline and a cell pellet containing approximately $2x10^7$ cells was resuspended in 1 ml of a solution containing 150 mM NaCl, 10 mM EDTA, 10 mM Tris HCl pH 7.5. SDS was added to a final concentration of 0.5% and the mixture incubated at

65°C for 10 min. Proteinase K was then added to a final concentration of 200 µg/ml and the incubation continued for 4 hrs. at 37°C. The lysates were extracted sequentially with phenol, phenol:chloroform (1:1) and chloroform and then precipitated with ethanol. Following resuspension of the pellet in 500 µl TE (10 mM Tris HCl pH 7.5, 1mM EDTA) DNase-free RNase was added to 100 µg/ml and the solution incubated at 37°C for 1 hr. The RNase was removed by proteinase K digestion and phenol extraction and the cellular DNA was precipitated in 2 volumes of ethanol. After washing in 70% ethanol, the final pellet was suspended in TE, at a final concentration of approximately 100 µg/ml.

Southern Transfer Analysis

Cellular DNA's were digested to completion with the appropriate restriction endonuclease using the cleavage of lambda DNA as an indicator of the extent of the reaction. Approximately 10 ug were then subjected to electrophoresis through 0.6% agarose gels and transferred to nitrocellulose according to Southern (14). The filters were prehybridized in 5 x SSC (1 x SSC is 0.15M NaCl 15mM sodium citrate pH 7.0) containing 0.5% SDS, 100 µg/ml calf thymus DNA, 20 mM sodium phosphate pH 6.5 and 5 x Denhardt's (1 x Denhardt's is 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrollidone) for 6 to 24 hrs at 68°C. Hybridization was carried out in the same solution except that Denhardt's solution was decreased to 1 x and approximately 10^6 cpm/ml of the appropriate nick translated probe (specific activity > 10^8 cpm/µg) was added. Following incubation at 68°C for 18 to 24 hrs, the filters were washed under stringent conditions (0.5 x SSC - 0.5% SDS at 68°C for 1hr.) and exposed to X-ray film.

The intensity of hybridization was quantified by excising the appropriate band from the nitrocellulose filter and counting in a liquid scintillation counter. The copy number of individual transfectants was determined by comparing these results to a standard curve prepared by similar analysis of the copy number standards.

RESULTS

Effect of DNA Concentration on Number of Copies of Genes Transferred

We (1) and others (3,4) have shown that electroporation in the presence of increasing DNA concentrations results in higher levels of transient gene expression as well as in increases in stable transfection frequency. These data suggested that a greater number of cells take up DNA at higher DNA concentrations. In order to determine whether transfected cells also take up

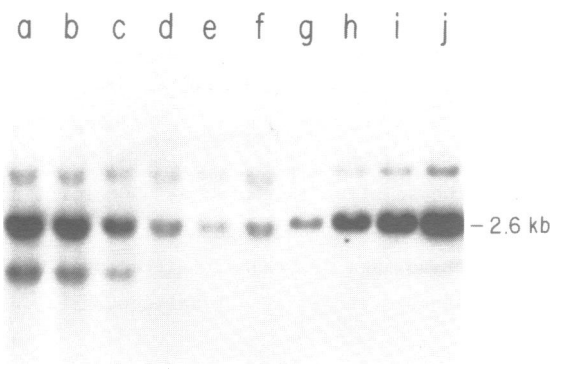


Fig. 1: Southern Analysis of Transfected Cells. M12 cells were transfected with increasing concentrations of pSV7neogpt* linearized with PvuI and plated into 2 wells of a 6 well microtiter plate. Resistant cells were selected in the presence of 1.5 µg MPA per ml or 1.5 mg G-418 per ml for 14 days and then amplified in the absence of selection. Genomic DNA prepared from the amplified cultures was digested with Hind III and EcoRV to isolate a 2.6 kb internal fragment containing the entire neo gene and the 5' end of the gpt gene and run on an 0.8% agarose gel against Hind III - EcoRV digested pSV7neogpt*. Lanes a, b, c, d, e, f show the pattern obtained using 10 µg of genomic DNA isolated from cells transfected with 36 pmoles/ml, 24 pmoles/ml, 8 pmoles/ml, 4 pmoles/ml, 2 pmoles/ml and 1 pmole/ml of pSV7neogpt*, respectively. Lanes g, h, i, j contain 1, 5, 10 and 25 gene copy equivalents of pSV7neogpt* digested with Hind III, and EcoRV. The probe in this case was a Bgl II - EcoRV gpt specific fragment prepared from pSV2gpt (15). The additional bands represent cross-hybridizing fragments present in pSV7neogpt*. These bands were not detected in autoradiograms obtained using a Hind III - NruI neo specific probe prepared from pSV2neo (15).

greater amounts of DNA as a function of DNA concentration the following experiment was performed: M12 cells were electroporated in the presence of 0.2, 1, 2, 4, 8, 16, 24, and 36 pmoles/ml of pSV7neogpt* linearized with PvuI. After determination of cell viability the cells from each DNA concentration point were split into 2 aliquots. One aliquot was plated in 96 well microtiter plates under limiting dilution conditions and transfected colonies arising from single cells were selected in the presence of 1.5 ug MPA per ml. A second aliquot was plated into 2 wells of a 6 well plate and subsequently selected in either 1.5 µg MPA per ml or 1.5 mg G-418 per ml in order to obtain a resistant pooled cell population.

Genomic DNA prepared from the MPA-resistant pooled cell populations was subjected to Southern blot analysis using either a gpt or a neo-specific probe (14,15). Comparison of the intensity of hybridization to copy number standards allowed a determination of the number of gene copies transferred.

Table 1: Effect of DNA Concentration on the Number of Gene Copies Transferred by Electroporation

Gene Copy Number[a] DNA Concentration[b] (μg/ml)	1	5	10	20	40	80	120	180
Pooled cells:	1.0	1.5	1.5	1.0	2.0	6	10	13
Individual colonies:			1.5	2.0	2.0	1.5	1.0	2.0
			1.0	1.0	2.0	1.0	2.0	1.0
			1.5	1.5	1.5	2.0	1.0	2.0
			1.0	1.5	1.0	2.5	1.0	1.5
			1.0	1.0	1.0	3.0	5.0	1.0
					1.0	4.0	1.0	2.0
					2.0	1.0	3.0	10.0
					1.5	5.0	1.0	12.0
					1.0	3.0	6.0	6.0
					1.5	1.5	2.0	1.0

a. determined by band excision and liquid scintillation counting.
b. the indicated concentrations of pSV7neogpt*, PvuI linear were used for electroporation, corresponding to 0.2, 1, 2, 4, 8, 16, 24 and 36 pmoles/ml.

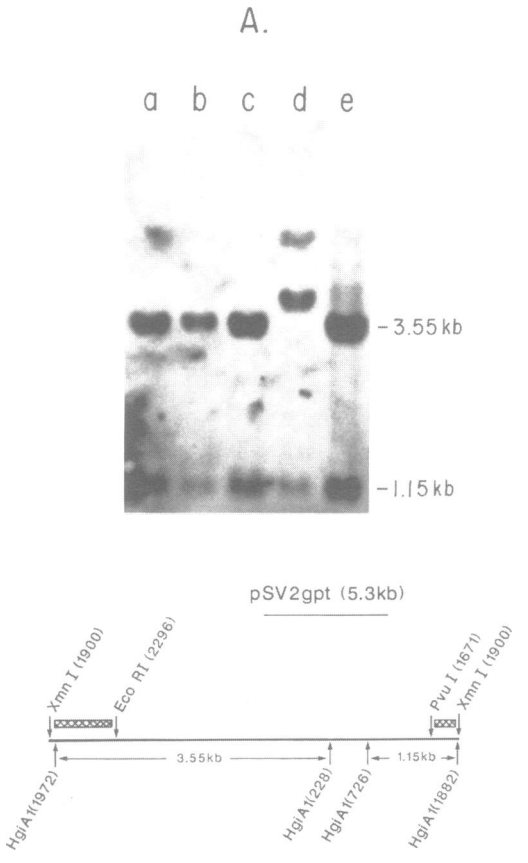
Pooled cell populations rather than transfected colonies were used initially to overcome individual variability and to obtain a more representative assessment. Figure 1 shows a typical autoradiogram of the data and indicates that as the DNA concentration during electroporation is increased the number of gene copies transferred increased. Quantitation of the data shows that greater numbers of genes are transferred only at very high DNA concentrations (Table 1). At DNA concentrations ranging from 0.2 to 8 pmoles per ml, DNA was transferred predominanatly in low or single copy equivalents. Identical results were obtained following G-418 selection.

Individual transfectants obtained from limiting dilution plating were recloned and then also subjected to Southern analysis. A summary of the results is presented in Table 1. As predicted the majority of transfectants obtained following electroporation in the presence of 8 pmoles/ml DNA or less contained single or low copy number inserts. Surprisingly, a significant proportion of the colonies derived from electroporation at higher DNA concentration also contained inserts present in low copy number. Analysis of Hirt supernatants (16) prepared from the pooled cells did not reveal the presence of free plasmid suggesting that all the transferred genes are integrated into the host cell chromosome. The significance of these results will be discussed in greater detail below.

Mechanism of DNA Insertion

In order to examine the structure of the integrated plasmid, genomic DNA was isolated from independant transfectants containing low copy number inserts and from pooled cells transfected at low DNA concentration. Following digestion with a variety of restriction endonucleases the DNA was analyzed by Southern transfer.

As shown in Figure 2A (bottom), digestion of the plasmid pSV2gpt linearized at the XmnI site with the enzyme HgiA1 should yield a 3.55 kb and a 1.15 kb fragment when probed with pBR322 specific sequences. These bands would also be observed in a genomic Southern blot if integration had occured preferentially at the XmnI site and if the HgiA1 sites close to the XmnI site were retained during integration. As shown in Figure 2A, lane e, these are the predominant bands obtained when genomic DNA from a pooled cell population



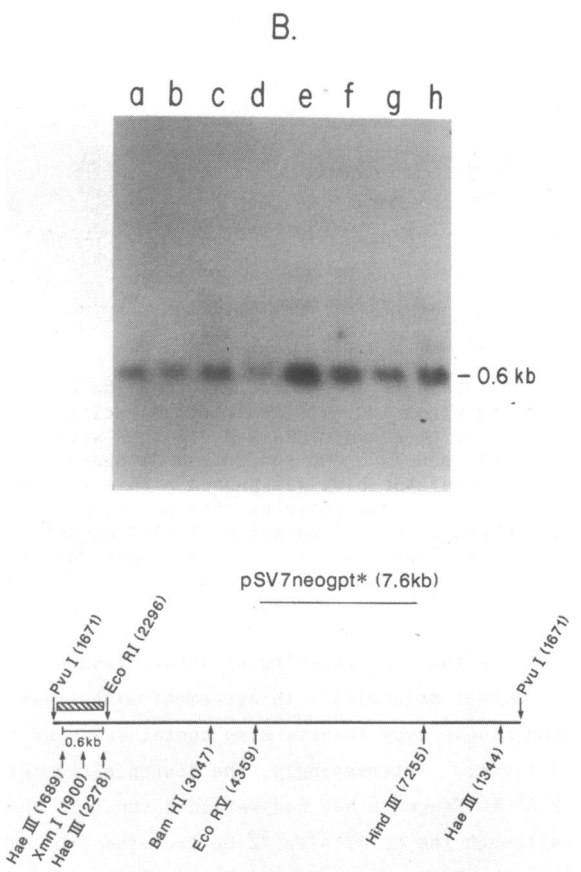


Fig. 2: Hybridization analysis of genomic DNA obtained from low copy number transformants following digestion with restriction nucleases which cleave close to the linear ends. A: M12 cells were electroporated in the presence of 20 µg/ml pSV2gpt, linearized with XmnI. 10 µg of genomic DNA was digested with Hgi A1 and analyzed by Southern transfer using a pBR322 specific probe prepared by digestion of pSV2gpt with EcoRl and PvuI. Lanes a, b, c and d show the pattern obtained with independent single copy transfectants. Lane e shows the pattern obtained with genomic DNA from a pooled cell population. Bottom: Restriction map of pSV2gpt. Cross-hatched boxes show the position of the probe. B: M12 cells were electroporated in the presence of 20 µg/ml pSV7neogpt* linearized with PvuI. Genomic DNA was prepared from MPA resistant colonies and from a pooled cell population and 10 µg were digested with Hae III, and probed with a pBR322 specific probe. Lanes a, b, c, d, f, g, h show the patterns obtained with genomic DNA from independent single copy transfectants and Lane e represents the resistant pooled cells. Bottom: Restriction map of pSV7neogpt*. Cross-hatched boxes show the position of the probe.

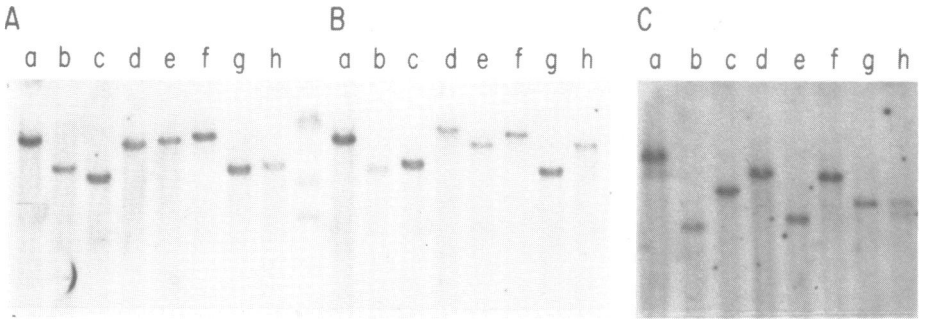


Fig. 3: Hybridization analysis of genomic DNA from low copy number transformants following digestion with restriction nucleases which do not cleave the insert. 10 µg of genomic DNA was digested with SacI (panel A) XbaI (panel B) or EcoRI (panel C) and run on a 0.6% agarose gel. Following transfer to nitrocellulose, the blot was probed with a neo specific probe (15). Lanes a through h show the patterns obtained using independent single copy tranfectants derived by electroporation of pSV7neogpt*, PvuI linear. The unlabelled lane in between panel A and B is lambda DNA digested with Hind III.

is analyzed, suggesting that the majority of integration events had occurred at the ends of the linear molecules. In agreement with these data, 3 out of 4 clones containing single copy inserts also contained these two fragments (Figure 2A, lanes a,b,c). Interestingly, the fourth clone (Figure 2A, lane d) had lost the 3.55 kb fragment but had retained the 1.15 kb piece, indicating that although the HgiA1 site 72 bp from the XmnI site was lost, the site 18 bp from the XmnI site was retained. Integration of the plasmid in the region between the XmnI site and the Eco RI site could lead to the hybridization pattern detected for this clone. We have observed integration at sites other than the linearization site when the DNA used for transfer was nicked.

Digestion of these clones with XmnI resulted in every case, in the detection of fragments larger than the intact plasmid, suggesting that the XmnI site was lost upon integration. The data provide strong support for the contention that following electroporation the integration of linear molecules occurs preferentially through their ends. In addition, the finding that a restriction nuclease site 18 bp away from the linearization site was retained in all the independent transfectants examined, as well as in the uncloned pooled cells, suggests that integration occurs without extensive modification of the ends of the electroporated DNA.

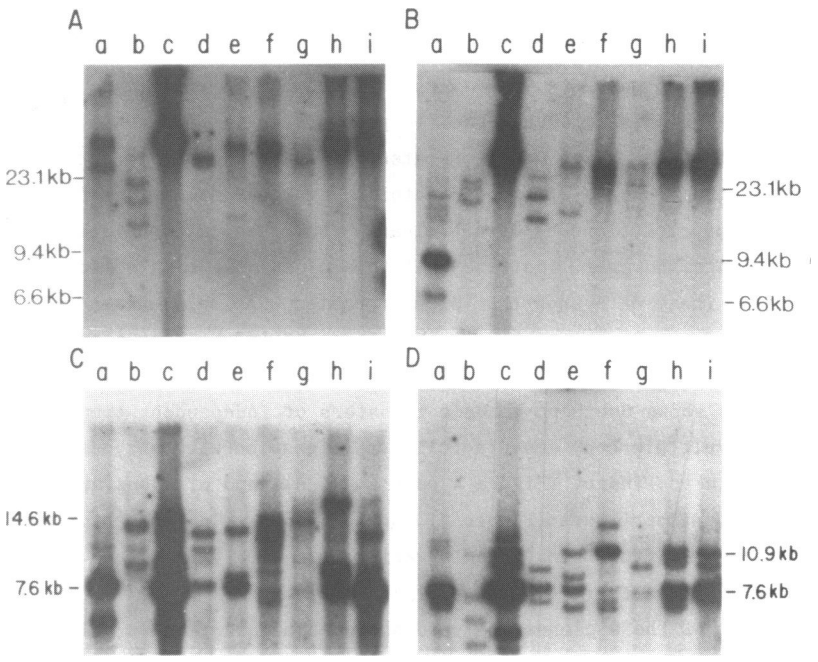


Fig. 4: Restriction digestion and hybridization analysis of genomic DNA isolated from transfectants containing multiple gene copies. 10 µg of genomic DNA was digested with EcoRI (panel A), XbaI (panel B), XmnI (panel C) or Hind III (panel D). Following transfer to nitrocellulose, the blot was probed with the Hind III - NruI neo specific fragment. Lanes a through i show the patterns obtained using independent multiple copy transfectants derived by electroporation of pSV7neogpt*, PvuI linear. The positions of marker bands of lambda DNA digested with Hind III is shown in panels A and B.

To further demonstrate that integration occurs in the absence of DNA rearrangement of the linear ends, DNA isolated from cells transfected with pSV7neogpt* linearized with PvuI, was digested with Hae III and probed with a probe specific for pBR322 sequences. As shown in Figure 2B (bottom), such a probe should detect a 0.6 kb piece if the Hae III site 18 bp away from the PvuI site is retained. The autoradiogram presented in Figure 2B shows that such a fragment was detected in all single copy clones analyzed (Figure 2B lanes a,b,c,d,f,g,h), as well as in a pooled cell population (Figure 2B lane e). The absence of any higher molecular weight bands indicates that DNA integration occurs without rearrangement or other damage to the linear end. The PvuI site however is lost during integration.

Chromosomal Site of Integration

Genomic DNA prepared from 8 clones containing single copy inserts of pSV7neogpt* was digested with enzymes that do not cleave in the insert. Southern analysis using a neo-specific probe shows that in most cases fragments of unique sizes were generated from each clone (Figure 3). The reason for the presence of two bands following EcoRI digestion of DNA from clone 1-6 (panel C, lane h) is not clear. The presence of unique XbaI and SacI fragments, however, indicate that this clone is a single site integrant. These data show that electroporated DNA is integrated at different unique sites in the host chromosome of independant transformants, and suggests that integration is a random process.

Figure 4 shows Southern genomic transfers of independant transformants containing multiple copy inserts following digestion with enzymes which do not cleave in the insert (Figure 4 panels A & B), and with enzymes which cleave in the insert once (Figure 4, panels C & D). The number of gene copies present in the transformants varied from 3 (Figure 4 lane b) to approximately 12 (Figure 4, lane c).

Four out of the 9 clones analyzed showed hybridization to only one band following digestion with enzymes which do not cleave the insert (Figure 4 panels A and B, lanes c,f,h and i), suggesting that integration had occurred at one chromosomal site. In the remaining clones, the transferred DNA appeared to have integrated at more than one chromosomal site, since multiple bands were observed following digestion with enzymes which did not cleave the plasmid (Figure 4 panels A and B, lanes a,b,d,e and g). The possibility that these results were due to incomplete restriction digestion was ruled out by repeated digestion with an excess of enzyme and by inclusion of lambda DNA as a marker in the reaction.

Digestion of the genomic DNA with enzymes which cleave the plasmid once resulted in a very complex pattern of bands (Figure 4 panels C and D). In all cases except one (Figure 4 panels C and D, lane b) digestion of genomic DNA with either Hind III or XmnI produced a band which co-migrated with intact plasmid. The pattern of bands observed for XmnI digestion of clones 4-5 and 6-2 (Figure 4 panel C, lanes f and g) was due to partial digestion. When the experiment was repeated with an excess of XmnI, distinct bands co-migrating with intact plasmid were detected for these clones. The pattern of bands observed for the other clones however, remained unchanged. The data obtained for clone 2-3 in which no plasmid size band was detected, are

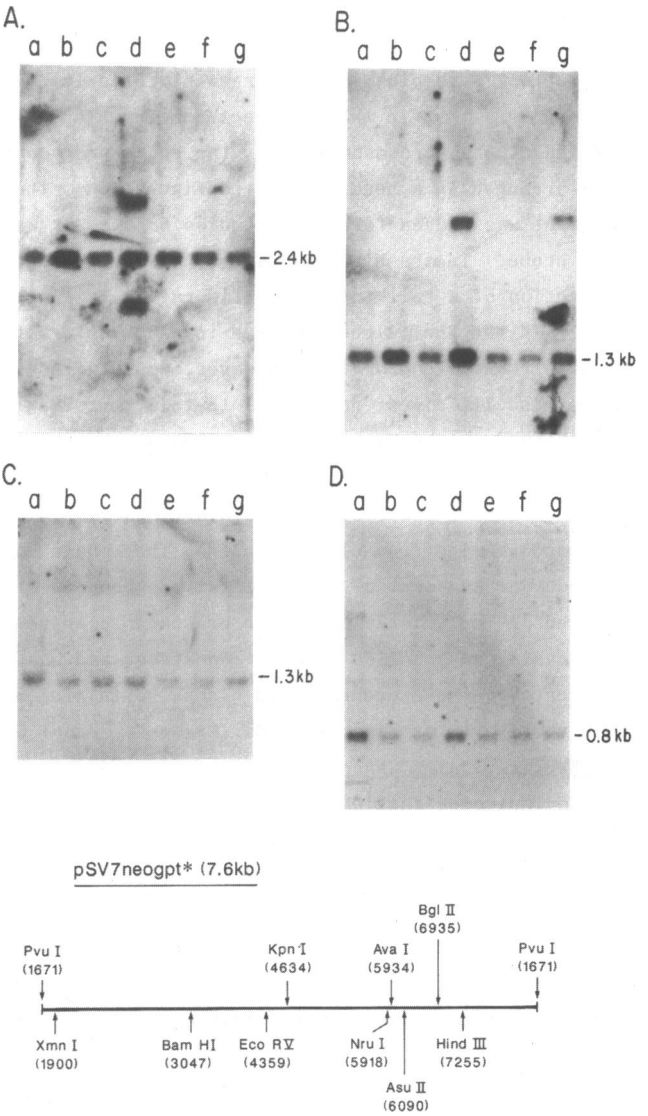


Fig. 5: Restriction nuclease mapping of integrated plasmid DNA. 10 µg of genomic DNA prepared from independent transfectants selected in 1.5 µg/ml MPA, was digested with a combination of restriction endonucleases, run on a 0.8% agarose gel and transferred to nitrocellulose. Panel A: DNA digested with Bgl II/EcoRV and probed with Bgl II - EcoRV gpt specific probe (15). Panel B: DNA digested with Bam HI/KpnI and probed with Bgl II - EcoRV gpt specific probe. Panel C: DNA digested with Hind III/AvaI and probed with Hind III - Nru II neo specific probe (15). Panel D: DNA digested with Bgl II - Asu II and probed with the neo specific probe. Fragment sizes were confirmed by parallel digestion of pSV7neogpt*.

consistent with the integration of three plasmid copies at three independent sites (Figure 4 lane b).

The existence of a 7.6 kb plasmid size fragment following digestion with Hind III or XmnI suggests that in the transfectants in which multiple plasmid copies were inserted at one chromosomal site, the concatemer contained plasmid sequences arranged in a head-to-tail configuration. The Hind III-NruI neo fragment was chosen for these studies because it was found to be the most specific probe. In the Hind III digest however, use of this probe did not allow detection of a tail-to-tail configuration (see map Figure 5, bottom). Similarly in the XmnI digest, a head-to-head conformation would not be detected. A head-to-head conformation however, would give rise to a 10.9 kb fragment in the Hind III digest and a tail-to-tail conformation would result in a 14.6 kb band in the XmnI digest. Fragments of these sizes were detected in the Hind III digest (Figure 4 panel D, lanes c,e,f,h and i) and in the XmnI digest (Figure 4 panel C, lanes c,d,e,f and i). Although the intensity of the bands varied from clone to clone, these data suggest that there may be a random arrangement of molecules within the concatemers. The extra bands observed in the autoradiogram are presumed to arise from fragments which contain the junction between concatemer and host sequences. In addition, DNA rearrangements occurring in clones containing multiple gene copies may also give rise to extraneous bands (Figure 4, panel D, lane f).

Integrity of the Electroporated DNA

Recent evidence indicates that DNA transferred by a number of chemical methods is subject to mutations and rearrangements possibly as a result of passage through the lysozomal compartment (17,18). In a successful gene therapy protocol the gene of interest must be transferred in an intact, unmutated form. In order to investigate the integrity of genes transferred by electroporation and to determine the effect of selective pressure, genomic DNA from 25 single copy transformants was digested with restriction nucleases diagnostic for the marker genes.

Figure 5 shows a representative autoradiogram obtained by digestion of genomic DNA from 7 independent transfectants and probed with either a gpt-specific probe (Figure 5 panels A and B) or a neo-specific probe (panels C and D). Comparison of the results with the restriction map of pSV7neogpt* (Figure 5, bottom) shows that all 7 clones contained the correct size fragments corresponding to a functional gpt gene. These results were not surprising in view of the fact that all clones were selected in MPA and therefore were expected to have a functional gpt gene. The presence of

multiple bands in clone 2-2 (Figure 5, panel A lane d) is confusing but is not due to incomplete digestion. An additional band was also observed following BamHI-KpnI digestion (Figure 5 panel B lane d).

Analysis of the state of the unselected neo genes showed that the neo-specific probe hybridizes in all cases to the size fragments expected for a functional neo gene (Figure 5 panels C and D). Similarly, using a pBR322-specific probe, no rearrangements of the plasmid sequences were detected. Although a much more rigorous analysis will be required to determine whether DNA point mutations have occurred, these results, in conjunction with the finding that all clones were resistant to G-418, suggest that there are no gross DNA rearrangements as a result of DNA transfer by electroporation. The presence of multiple gpt-specific DNA bands in clone 2-2 which was originally identified as a single copy transfectant, however, indicates that DNA rearrangement may occur as a result of continued cell passage and selection.

Co-Transfection of Unlinked Sequences

Our previous results showed that marker genes are co-transferred at a high frequency by electroporation. In view of the apparently conflicting finding that DNA is transferred preferentially in low or single gene copy number, co-transfection was re-investigated to determine the effect of the restriction endonuclease cleavage site on the co-transfection frequency.

Plasmids were, therefore, digested at either the same or different restriction nuclease sites and co-transfected into mouse M12 cells. The plasmid pairs used were pSV400dhfr with ppSV2gpt and pSV2neo with pSV2gpt. Following electroporation, the cells were plated in duplicate and one plate was selected in MPA while the other was selected in G-418 or methotrexate. At the end of a two week selection period, the medium was switched to determine which cells had taken up both marker genes. Table 2 show that similar results were obtained independently of which medium is used for the initial selection and indicate that electroporation is an effective way to co-introduce genes, with frequencies ranging from 39% to 77% of transfectants taking up both marker genes. A lower transfection frequency (22%) was obtained when using supercoiled DNA, probably reflecting the overall lower transfection frequency observed with supercoiled DNA.

Similar co-transfection frequencies were obtained whether the plasmids were cut at the same restriction site or at different sites. Thus, if there is a tendency for *in vivo* end-to-end ligation of co-transferred genes as the analysis of multiple copy clones suggests, homologous ends do not appear to

Table 2: Effect of Restriction Endonuclease Cleavage Site on Co-transfection Frequency

Experiment Number	DNA Transfected[a]	Restriction Endonuclease Digest[b]	Percentage Co-transfection[c] MPA Selection[d]	MTX or G-418 Selection[d]
1	PSV400dhfr pSV2gpt	PvuI PvuI	44	39
2	pSV400dhfr pSV2gpt	PvuI XmnI	48	69
3	pSV400dhfr pSV2gpt	NruI PvuI	46	50
4	pSV400dhfr pSV7neogpt*	PvuI PvuI	50	64
5	pSV400dhfr pSV7neogpt*	NruI PvuI	39	42
6	pSV2gpt pSV2neo	EcoRI EcoRI	75	75
7	pSV2gpt pSV2neo	PvuI EcoRI	73	77
8	pSV2gpt pSV2neo	XmnI EcoRI	75	64
9	pSV2gpt pSV2neo	supercoiled	22	23

a. Each plasmid was present at a concentration of 15 pmoles/ml
b. Indicates the enzyme(s) used for linearization.
c. Measured as the number of colonies resistant to both selective media.
d. Indicates first type of selection imposed. Selection was for 14 days then medium switched to second selection for 14 days.

be required. These results are consistent with data obtained using DNA transferred by microinjection (19).

DISCUSSION

We have shown that with the electroporation method of DNA transfer, there is a strong preference for the generation of murine lymphoma transfectants containing plasmid DNA integrated in low copy number at a unique chromosomal site. Similarly Boggs et al (4), reported that approximately 79% of 243 transformed murine erythroluekemia cell colonies analyzed had the exogenous DNA integrated in a single copy at a single site. Our data shows that a concentration of at least 16 pmoles/ml DNA is required before a significant increase in the average number of inserts is detected in an MPA-resistant pooled cell population. Although further increases in DNA concentration do result in a greater number of genes integrated, the average gene copy number is only 13 even at a DNA concentration of 36 pmoles/ml. This is in sharp contrast to the situation observed with calcium phosphate transfection where concatemers containing as many as 50 gene copies are routinely observed (12).

The analysis of individual transfectants obtained following transfection

at different DNA concentrations yielded the surprising result that even when electroporation was performed in the presence of high DNA concentrations, a significant proportion of the cells had integrated the exogenous DNA in a low copy number. The possibility that the findings observed with the pooled cell populations were a result of unintegrated plasmid, was eliminated by examination of the Hirt supernatant fraction, both from the original cell populations, as well as from cell populations analyzed within three weeks after transfection. In no case was the presence of free plasmid detected. Moreover, since the cells were only maintained in selective media for the 2 week period following transfection, and thereafter were amplified for DNA isolation in the absence of selection, the possibility that the culture conditions favored transfectants containing multiple gene copies can also be eliminated.

The data are consistent with the theory that the majority of the cells integrate exogenous DNA at a low copy number even when electroporated in the presence of high DNA concentrations. Analysis of the pooled cell populations, however, indicate that there exists a subpopulation of cells which can take up and integrate multiple DNA copies. The differences between these cells and the majority of cells may be due either to physical or to biological factors, or to a combination of the two. The biological factors may simply reflect the likelihood of periods in the cell cycle during which exogenous DNA can be incorporated into the genome more efficiently, and that at any one time, only a proportion of the cells are in this critical phase. Alternatively, there may also be periods in the cell cycle during which cells are more susceptible to permeabilization by electroporation, perhaps as a result of changes in cell shape or in plasma membrane properties. The existence of inhomogeneity in the electric field could also lead to the definition of a subpopulation of cells, which by virtue of their particular location in the field, are exposed to higher field strengths. This in turn could result in the formation of a greater number of pores and/or pores of larger sizes in these cells, thus allowing the transfer of larger amounts of DNA.

We (1) and others (3,4) have reported that electroporation in the presence of increasing DNA concentrations results in higher levels of transient gene expression as well as in an increase in stable transformation frequency. The data presented here show that even at high DNA concentrations the majority of cells integrate DNA in low copy number and suggest that the increase in gene expression is due mostly to a greater number of cells taking up DNA. The question of whether the rate limiting factor is the introduction

of foreign DNA into cells or whether it is the integration of this DNA which is limiting is currently under investigation. We are also investigating whether all the genes in transfectants containing multiple copies are equally active. Preliminary data using both a gpt enzyme assay, as well as an immunoassay for expressed protein, indicate that in general, cells containing a larger number of genes express larger amounts of protein, but a direct correlation between gene copy number and expression levels cannot be drawn.

Restriction endonuclease digestion of transfectants containing multiple gene copies revealed a complex series of bands. Taken together, the results indicate that exogenous DNA can integrate at more than one chromosomal site within the same cell, resulting in one case, in a transfectant containing three genes each integrated in a unique location. When the multiple copies were integrated at the same chromosomal site, the restriction endonuclease pattern obtained with enzymes which cleave once in the insert, was more consistent with a random configuration of the concatemers than with the head-to-tail configuration which has been observed both for microinjection and for calcium phosphate-mediated transfection (19,12).

In studies using microinjected DNA, formation of the head-to-tail concatemer has been shown to proceed via homologous recombination; even when using linear plasmids, the linearization site was preserved in at least 90% of the molecules during concatemer formation (19). The findings of a random configuration for the concatemer introduced by electroporation, in combination with the observation that the site used for linearizing the plasmid (in this case PvuI) was lost in the concatemer (data not shown), suggest that alternative mechanisms may exist for the formation of concatemers of electroporated DNA. This situation appears to resemble the case observed when large quantities of linear plasmids were micro-injected into a single cell. Under these conditions, random end-to-end ligation of the plasmids occurred, and the ensuing transformants contained concatemers with randomly oriented molecules mixed in with molecules oriented in a head-to-tail configuration (19). Further experiments showed that any two DNA fragments can be ligated together in mammalian nuclei irrespective of the sequence or topology at their ends. Thus homologous ends are not required for *in vivo* ligation (19).

Whether the co-transfection of two unlinked markers proceeds via an *in vivo* end-to-end ligation or by homologous recombination is not known. The results presented here however, indicate that whatever the mechanism, it neither requires, nor is influenced by, the presence of homologous ends. Preliminary results indicate that approximately 50% of co-transfectants

containing both gpt and dhfr genes show increased gpt activity following amplification of the dhfr sequences in methotrexate. These data suggest that both co-integration, as well as separate integration events, occur during co-transfection, and support the conclusions obtained by analysis of transfectants containing multiple gene copies of a single plasmid.

Re-investigation of the co-transfection frequency supports our earlier conclusions that unlinked genes are efficiently co-transferred by electroporation (1). Southern hybridization analysis demonstrating that genes are transferred preferentially at low copy number, however, suggests a discrepancy with these results. The reasons for this apparent discrepancy which has also been observed by Boggs et al (4), are not clear, but may be related to the possibility that at the higher total DNA concentrations (100 µg/ml or 30 pmoles/ml) used for co-transfection, a larger proportion of transfectants contain low copy number (2-3 genes) rather than single copy inserts. The distinction between single copy and low copy number cannot always be easily made by Southern transfer analysis.

Restriction endonuclease digestion of genomic DNA containing integrated plasmids strongly suggests that single linear molecules are inserted into the host genome through their ends. In the process of insertion, the restriction sites at the ends of the molecule are lost, but sites as close as 18 bp from the ends remain intact, indicating that integration occurs without extensive DNA rearrangement. These results are in agreement with data obtained by microinjection of DNA where it was similarly found that integration occurred exclusively at the ends of a linear molecule in the absence of extensive rearrangements (19). These observations imply that with electroporation as with microinjection or retrovirus-mediated DNA transfer, the structure of the integrated gene can be accurately predicted.

In contrast to this, Boggs et al (4), report that following electroporation, over 30% of the individual transfectants analyzed contained plasmid DNA which had inserted at a site other than BstX1 site used for linearization of the plasmid. We observed similar results when the DNA used for electroporation was nicked prior to transfer. We, therefore, routinely purified our plasmid DNA through two CsCl gradients to ensure that the preparation contained only superhelical molecules prior to linearization.

Analysis of 25 independent transfectants containing low copy number inserts showed that both the selected gene, as well as unselected sequences, are transferred by electroporation in the absence of any gross DNA rearrangements. Moreover, the genes were maintained in a stable functional

form with continued cell culture over a period of 6 months in the absence of selective pressure. These studies in addition to the results showing that the ends of the linear plasmid molecules are essentially unrearranged, lend strong support to the contention that DNA is transferred by electroporation in an unmutated form. Additional, more detailed studies are in progress to determine the nature of mutations, if any, suffered by DNA following electroporation. It is expected that such studies will provide insights into the mechanisms of DNA transfer and processing during electroporation.

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