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# ADP-ribosylation is involved in the integration of foreign DNA into the mammalian cell genome§

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## ABSTRACT

The most commonly used DNA transfection method, which employs the calcium phosphate co-precipitation of the donor DNA, involves several discrete steps (1,2). These include the uptake of the donor DNA by the recipient cells, the transport of the DNA to the nucleus, transient expression prior to integration into the host cell genome, concatenation and integration of the transfected DNA into the host cell genome and finally the stable expression of the integrated genes (2,3). Both the concatenation and the integration of the donor DNA into the host genome involve the formation and ligation of DNA strand-breaks. In the present study we demonstrate that the nuclear enzyme, adenosine diphosphoribosyl transferase (ADPRT, E.C. 2.4.2.30), which is dependent on the presence of DNA strand breaks for its activity (4,5) and necessary for the efficient ligation of DNA strand-breaks in eukaryotic cells (4,6), is required for the integration of donor DNA into the host genome. However, ADPRT activity does not influence the uptake of DNA into the cell, its episomal maintenance or replication, nor its expression either before or after integration into the host genome. These observations strongly suggest the involvement of ADPRT activity in eukaryotic DNA recombination events.

## INTRODUCTION

Previous studies have demonstrated the involvement of nuclear ADPRT activity in a variety of eukaryotic cellular processes which require the ligation of DNA strand-breaks, possibly because it regulates DNA ligase activity (7,8). These processes include DNA excision repair (6), a number of examples of eukaryotic cellular differentiation (9-17), mitogenic activation of quiescent lymphocytes (18-20), sister chromatid exchange (21,22) and antigenic variation in *Trypanosoma brucei* (23). We have previously predicted that nuclear ADPRT would participate in the mechanism of DNA transfection (24). Here we confirm this prediction, by demonstrating the regulation of DNA-mediated gene transfer in three different mammalian cell lines with four different plasmids by ADPRT activity.

The persistent expression of donor genes (carried by plasmids which lack their own autonomous origins of replication) in proliferating cells usually requires both the end-to-end ligation (concatenation) of the donor DNA

fragments and their subsequent integration into the host genome (3). In the absence of concatenation and genome integration, which both require the ligation of DNA strands, the donor DNA fragments are lost by dilution (and presumably also by degradation) during cell replication, with first order kinetics (25,26). However, the transient expression of the DNA sequences, which can be detected soon after transfection, or the episomal replication of plasmids carrying functional origins of replication, require neither concatenation nor integration into the host genome.

## MATERIALS AND METHODS

### Cells and culture conditions:

NIH/3T3 fibroblasts, *Cos 7* cells and the human fibroblast cell line XP12RO-SV40, were all maintained in Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% foetal calf serum, unless other wise indicated.

### Transient CAT expression assay:

Prior to transfection cells were subcultured at a density of $5x10^5$ in 10cm culture dishes and fed with fresh media 24 hours later. Each sub-confluent plate was then transfected with 2μg of pRSV.*cat* (28), or 2μg of pRSV.*cat* and 2μg of pSV2.*neo* (29), co-precipitated with 18μg or 16μg of carrier salmon sperm DNA (approx. size 20Kb), according to the calcium phosphate procedure of Graham and van der Eb (1,2). The medium was changed 24 hours after transfection. Cells were then assayed for the transient expression of chloramphenicol acetyl transferase (CAT) activity at 42 hours, as described by Gorman *et al* (28). For ADPRT inhibition, the competitive inhibitor 2mM 3-methoxybenzamide, or 2mM 3-methoxybenzoate (the non-inhibitory analogue) were added to the cell cultures either 2 hours prior, or 12 hours after transfection and maintained thus, until the cells were harvested for the assay of CAT activity.

### Assay of episomal maintenance and/or replication:

Approximately $5x10^5$ *Cos 7* cells in each 10cm culture dish were transfected with 20μg of the plasmid pSV2.*neo* as described above. Cultures were then maintained in the continuous presence of 5mM 3-aminobenzamide (another ADPRT a competitive inhibitor), or 5mM of one of the non-inhibitory chemical analogues, 4-aminobenzamide, 2-aminobenzamide or benzoic acid. After 36 hours, the episomally maintained (unintegrated) plasmids were recovered in the clear cell lysate by Hirt extraction (30). The total extract from each plate was then used for the transformation of competent *E.Coli* (31). The transformed bacteria were plated on agar plates containing 25μg/ml

kanamycin, and the number of transformed colonies, indicating the number of pSV2.*neo* plasmids recovered from the *Cos* cells, were quantitated 24 hours later.

Eukaryotic Cell Transformation Assay:

Subconfluent mouse NIH/3T3 fibroblasts were transfected with 2μg of either pSV2.*neo* (29), pSV3.*gpt* (29), or pSV0.1, together with 18μg of the carrier salmon sperm DNA. pSV0.1 comprises full length SV40 DNA inserted into the BamH1 site of pAT153. Human XP12R0-SV40 fibroblasts were transfected with 20μg pSV3.*gpt*, or 20μg salmon sperm DNA as described above. Cells transfected with pSV2.*neo* or pSV3.*gpt* received a medium change after 24 hours. 24 hours later cultures were trypsinised and cells from each plate were seeded into 10 plates in the presence of either 500μg/ml Geneticin (G418, Gibco, U.K.), in the case of pSV2.*neo* transfected cells; or in the presence of mycophenolic acid (29), in the case of pSV3.*gpt* transfected cells. The pSV0.1 transfected NIH/3T3 cells were maintained continuously, from 24 hours prior to transfection, in DMEM supplemented with 5% new borne calf serum. For the analysis of the effect of ADPRT inhibition 4mM 3-aminobenzamide, or 4mM 3-aminobenzoate (the non-inhibitory analogue) was added at the time of transfection and continuously maintained thereafter. Geniticin resistant (pSV2.*neo* transfectants), mycophenolic acid resistant (pSV3.*gpt*) colonies, or transformed foci (pSV0.1 transfectants) were counted 2 weeks later.

RESULTS

Inhibition of ADPRT activity, by the competitive inhibitor 3-methoxybenzamide (27), has no detectable effect on the transient expression of chloramphenicol acetyl transferase (CAT) activity in NIH/3T3 cells transfected with either pRSV.*cat* (Fig. 1a), or co-transfected with both pRSV.*cat* and pSV2.*neo* (Fig. 1b). Comparable levels of CAT activity are found in cell extracts from cultures exposed to 2mM 3-methoxybenzamide, either from 2 hours prior to transfection or from 12 hours after transfection up to the time of assay at 42 hours. Therefore, inhibition of ADPRT activity has no effect on the detectable levels of CAT activity in NIH/3T3 cells either during the uptake of transfected DNA into the cells, or during the subsequent period of gene expression prior to integration into the genome. Similarly, ADPRT inhibition does not affect the episomal maintenance and/or replication of the plasmid pSV2.*neo* in transfected *Cos* 7 cells. The extrachromosomal replication of this plasmid in the *Cos* cells is sustained because of the presence of an integrated SV40 genome in the cell and the presence of SV40 origin of

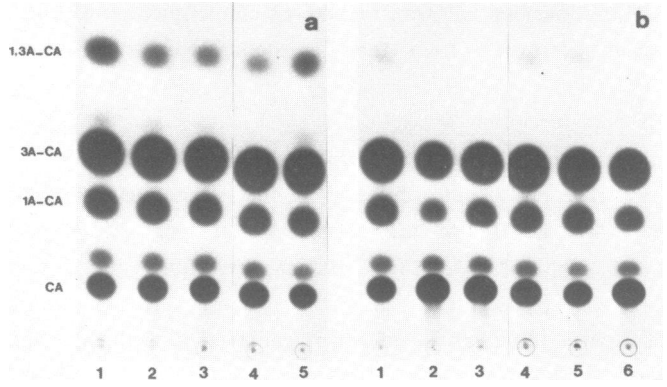


**Fig. 1. The transient expression of CAT activity in transfected NIH/3T3 fibroblasts.** Cells were transfected with either 2μg pRSV.*cat* (a), or 2μg pRSV.*cat* together with 2μg pSV2.*neo* plasmids (b). The cultures were maintained either in the absence of ADPRT inhibitors (lane 1), or in the presence of 2mM 3-methoxybenzamide (lanes 2 and 4), or 2mM 3-methoxybenzoate (the non-inhibitory analogue, lanes 3 and 5). The inhibitors were added either 2 hours prior to (lanes 2 and 3) or 12 hours after transfection (lanes 4 and 5), and continuously maintained until the cells were harvested after 42 hours for the assay of CAT activity. Lane 6 contains 0.1 unit of purified CAT enzyme standard. CA, 1A-CA, 3A-CA and 1,3A-CA refer to the substrate chloramphenicol, and the acetylated products modified in positions 1, 3, or 1 and 3, respectively.

replication in the pSV2.*neo* plasmid. 36 hours after transfection approximately $10^4$ plasmids were recovered in the clear cell lysate of the cells, irrespective of the presence of the enzyme inhibitors, demonstrating that neither the uptake nor the episomal maintenance and/or replication of the plasmid was affected by ADPRT inhibition.

These observations demonstrate that ADPRT activity is not required for processes involved in the uptake of the donor DNA into the cell, in the transient expression of this DNA, or even the episomal maintenance and/or replication of a suitable plasmid in the appropriate host.

By contrast, ADPRT activity is required for the integration of the donor DNA into the genome. This is demonstrated in several ways. ADPRT inhibitors decrease the frequency of stable *neo*$^R$ colonies in NIH/3T3 cells transfected with pSV2.*neo* (Table 1). In control cultures, the frequency of neomycin-resistant colonies was 665 $\pm$ 98 per mg of DNA per $10^6$ transfected cells. The continuous presence of 2mM 3-methoxybenzamide (a non-toxic concentration) almost completely abolished the formation of neomycin-resistant colonies. Inhibition of ADPRT activity for limited periods following transfection reduced the number of resistant colonies, depending on the period of ADPRT inhibition.

**Table 1. Effect of inhibition of ADPRT activity on the formation of *neo*$^R$ colonies in NIH/3T3 cells transfected with pSV2.*neo*.**

| Treatment | Duration | Number of *neo*$^R$ colonies (per μg plasmid DNA per $10^6$ cells ± S.D.) |
|---|---|---|
| Control | | 665 ± 98 |
| 3-methoxybenzamide | 0----------14 | 4 |
| " | 0--2 | 420 ± 85 |
| " | 1--3 | 240 ± 62 |
| " | 3-----7 | 138 ± 43 |
| " | 7-------14 | 560 ± 81 |
| 3-methoxybenzoate | 0----------14 | 593 ± 74 |

Each culture (approximately $5x10^5$ cells) was transfected with 2μg pSV2.*neo*. Cells were then maintained either in the absence of the ADPRT inhibitors (control), or either 2mM 3-methoxybenzamide, or 2mM 3-methoxybenzoate for the indicated duration. The *neo*$^R$ colonies were counted 2 weeks later. Colonies smaller than 1mm in diameter, probably the result of a transient resistance to Geniticin, were excluded from the quantitation.

When 3-methoxybenzamide was present only during the first two days after transfection, it slightly inhibited the formation of *neo*$^R$ colonies. Similarly, the presence of 3-methoxybenzamide later than the seventh day after transfection reduced the number of *neo*$^R$ colonies only marginally. Maximum inhibition of the formation of *neo*$^R$ colonies was observed when 3-methoxybenzamide was present either continuously, or during the third to seventh day after transfection. It is precisely during this time that DNA uptake is probably complete, and integration of the donor DNA into the host genome is taking place. Southern blot analysis of total DNA isolated from NIH/3T3 cells which were transfected with pSV2.*neo* in the absence of ADPRT inhibitors demonstrates that DNA uptake into the cells is complete within the first 24 hours, followed by the concatenation and integration of the donor DNA into the host genome in the subsequent seven days. There is little sign of further DNA integrations into the genome after the first week following transfection (data not shown).

The continuous presence of 4 mM 3-aminobenzamide (a non-toxic concentration), which is also a competitive inhibitor of ADPRT activity (27), reduced the transformation of NIH/3T3 cells by the plasmid pSV0.1 by approximately ~97% (Table 2). By contrast, 4 mM 3-aminobenzoate (the non-inhibitory analogue of 3-aminobezamide) had no effect on the transformation frequency. Similarly, complete inhibition of cell transformation was observed when the plasmid pSV3.*gpt* (29), which contains the early region of SV40, is

**Table 2. Effect of inhibition of ADPRT activity on the transformation of NIH/3T3 transfected with pSV0.1.**

| Drug Present | Number of plates | Mean number of transformed foci per plate | Foci/μg DNA |
|---|---|---|---|
| None | 4 | 41.5 | 20.8 |
| 3-aminobenzoate | 52 | 37.5 | 18.8 |
| 3-aminobenzamide | 22 | 1.1 | 0.6 |

Sub-confluent NIH/3T3 cells were transfected with 2μg of pSV0.1. Cultures were then maintained either in the absence of the inhibitors or the continuous presence of 4mM 3-aminobenzoate or 4mM 3-aminobenzamide. Foci of transf3ormed cells were counted 10-14 days after transfection. The difference between the plates containing 3-aminobenzamide and either of the other two sets of plates was statistically significant with $p<0.001$ (Student's t test).

used in the presence of 3-aminobenzamide (data not shown). The inhibition of the appearance of transformed foci in the presence of ADPRT inhibitors is not due to the suppression of the transformed phenotype because the addition of 4mM 3-aminobenzamide to cells which had already been transformed morphologically by the SV40 plasmid, inhibited neither the growth nor the expression of the transformed phenotype in these cells. Neither 4mM nor 10mM 3-aminobenzamide affected the cloning efficiency of NIH/3T3 cells which was about 32% both in the presence and absence of the inhibitors.

The regulation of DNA transfection by ADPRT activity is also evidenced in experiments with a human fibroblast cell line (XP12RO-SV40) transfected with the plasmid pSV3.*gpt* and then selected for the bacterial *Eco.gpt* gene in the presence of mycophenolic acid. In 9 plates (each containing approximately $10^6$ cells to which 20μg of pSV3.*gpt* were added), there was an average of 114 colonies per plate, while in 7 plates to which 4mM 3-aminobenzamide had also been added, there was an average of 15 colonies per plate; a suppression of 87% of the transfection efficiency. This difference is significant with $p<0.01$. No spontaneous mycophenolic acid-resistant colonies were observed in these studies when the cells were grown for 14 days and no colonies were seen when cells were transfected with the carrier salmon-sperm DNA only.

## DISCUSSION

These studies demonstrate that the integration of transfected DNA into the host cell genome requires ADPRT activity. By contrast, ADPRT activity is not required for the uptake, nor for the expression of the donor DNA either prior to or subsequent to its integration into the host genome. ADPRT

activity is also not required for the episomal replication and/or maintenance of plasmids carrying autonomous origins of replication. The persistent expression of genes introduced into eukaryotic cells by the calcium phosphate transfection procedure is dependent upon their integration into the host chromosomes, a process which involves DNA strand ligation (3,25,26). Therefore, the integration of donor DNA molecules into the host genome may need ADPRT activity be because the latter regulates the ligation of DNA strand-breaks, which is necessary in this process both at the stage of concatenation and of integration into genomic DNA. These observations begin to elucidate the molecular steps involved in DNA transfection. They also provide a potentially useful approach for the inhibition of DNA transfection.

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