Transfection of the DNA Polymerase-α Gene

Abstract. *DNA polymerase-α is the major replicative DNA polymerase in animal cells. The gene coding for a mutant DNA polymerase-α was transferred from one cell to another by transfection of DNA from mutant cells. The DNA was isolated from a mutant hamster cell line resistant to aphidicolin, a specific inhibitor of DNA polymerase-α, and transferred into an aphidicolin-sensitive cell line. The resulting transfectants exhibited increased survival in the presence of aphidicolin and contained an aphidicolin-resistant DNA polymerase-α.*

A detailed understanding of DNA replication in bacteria has been dependent on the availability of conditional mutants in DNA synthesis and on the purification of DNA replicating proteins (*1*). Advances in understanding DNA replication in animal cells have been hampered by a lack of DNA replication mutants. The major mammalian DNA replicative enzyme, DNA polymerase-α (Pol-α), is involved in both DNA repair synthesis and mutagenesis (*2–4*). Nevertheless, despite the extensive purification of Pol-α, there is still no consensus on even its primary structure (*5*). Aphidicolin is a tetracyclic diterpenoid mycotoxin that arrests mitosis (*6*) and specifically inhibits its DNA Pol-α in vitro by competition with the substrate deoxycytidine triphosphate (dCTP) (*7*). Aphidicolin has been used to isolate an aphidicolin-resistant (Aph^r) mutant cell line from hamster V79 cells (*2*). This mutant, originally designated aph^r-4-2, was subsequently shown to contain a mutant DNA Pol-α (*3*). We report that the Aph^r phenotype of the mutant can be introduced into aphidicolin-sensitive (Aph^s) wild-type cells by DNA-mediated transfection.

The mutant aph^r-4-2 was obtained at a frequency of 1 in 10^7 clonable cells by growing mutagenized hamster V79 cells in the presence of 1.0 μ*M* aphidicolin (*2*) after mutation was induced with bromodeoxyuridine (100 μ*M*)–black light photolysis followed by ultraviolet irradiation. The mutant cell aph^r-4-2 is slow-growing, has a lengthened *S* phase, is hypersensitive to killing by DNA-damaging agents, and exhibits increased spontaneous mutagenesis and increased mutability after exposure to ultraviolet light (*3, 4*). The mutant cells contain an Aph^r mutant DNA Pol-α, presumably mediated by an alteration in the substrate binding site via a gene mutation, since the mutant enzyme exhibits a tenfold decrease in the Michaelis constant (K_m) for dCTP. These phenotypes indicate a deficit in DNA replication and DNA repair, and a decrease in the fidelity of DNA synthesis. On the basis of fusion experiments (*8*) with Aph^s *gal*3 (*9*) and thioguanine-resistant aph^r-4-2 cells, and on the basis of mixing experiments with DNA Pol-α from wild-type V79 and aph^r-4-2 cells (*3*), the aphidicolin resistance appears to be codominant. We have redesignated aph^r-4-2 as Apr^r-4-2. This codominant phenotype provides a selectable marker for gene transfer of DNA Pol-α.

Reconstruction experiments suggest that Aph^r-4-2 cells can be recovered with 80 percent efficiency by selection in 0.8 μ*M* aphidicolin even in the presence of a vast excess of wild-type cells. Wild-type Aph^s cells [V79 (743x)] could not form colonies ($<2 \times 10^{-7}$) at concentrations of aphidicolin greater than 0.6 μ*M*. The mutant cells, in contrast, formed colonies with high efficiency (70 percent) at 1.0 μ*M* aphidicolin. When a mixture of 50 mutant and 2×10^6 wild-type cells was incubated in the presence of 0.4 to 1.0 μ*M* aphidicolin, the number of viable colonies approximated the sum of the numbers of colonies obtained when the mutant and wild-type cells were incubated separately. In the presence of 0.8 μ*M* aphidicolin, the number of colonies observed in the mixed group was 80 percent of that observed when Aph^r-4-2 cells were incubated alone.

The results summarized in Table 1 indicate that the Aph^r phenotype of Aph^r-4-2 could be transferred by DNA-mediated transfection of wild-type V79 cells. High molecular weight DNA (average size, 50 kilobases) from the mutant or the wild-type cells was precipitated together with calcium phosphate and added to the recipient wild-type cells (*10*). The recipients were incubated without replating in 0.8 μ*M* aphidicolin for 45 days. No colonies were observed in the control group (Table 1). Summing the results from the reconstruction experi-

Table 1. Transfection frequency of the Aph^r, MAX^r, or MAX^rAph^r phenotype. For direct transfection, 0.5×10^6 V79 (clone 743x) cells were plated and incubated in 100-mm plates with 10 ml of growth medium supplemented with 5 percent fetal calf serum (*5*) for 24 hours. Cellular DNA was isolated (*10*); average size was determined by electrophoresis in a 0.3 percent agarose gel, with uncut and Hind III–digested bacteriophage λ DNA used as standards. Donor DNA (20 μg per plate) was precipitated with calcium phosphate (*10*). One milliliter of DNA suspension was added dropwise onto the culture surface after removal of medium. The cells were incubated at room temperature for 10 minutes before addition of 9 ml of growth medium. After 24 hours, the medium was removed, and cells were rinsed once with phosphate-buffered saline and incubated in 10 ml of growth medium for 24 hours to allow phenotypic expression. Aphidicolin-resistant colonies were selected, without replating, in medium containing 0.8 μ*M* aphidicolin for 45 days. Viable clones containing more than 20 cells were isolated with glass cloning cylinders and transferred to 16-mm multiwell plates (Costar No. 3524, Cambridge, Massachusetts). Each clone was tested for viability in medium with or without aphidicolin (0.8 μ*M*). Only clones that survived in 0.8 μ*M* aphidicolin were scored. For cotransfection, plasmid pSV2-gpt was isolated and purified by the alkali lysis method (*15*). Size was verified with a standard pSV2-gpt. The cotransfection procedure was identical to that described by Wigler *et al.* (*12*), except that the recipients (5×10^5 per plate) were incubated for 3 days in growth medium before addition of $CaPO_4$-DNA (micrograms of DNA per plate: pSV2-gpt, 1.25; salmon sperm or Aph^r-4-2, 20). DNA was removed after 24 hours adsorption time and these cells were replated at a 1 to 3 ratio. After 24 hours, the cells were incubated in growth medium containing MAX (*11, 14*) for 10 days, then either in MAX or in 0.8 μ*M* aphidicolin for another 20 days. The frequency is the number of colonies divided by the total number of recipients plated initially. Aph^r and MAX^r are colonies resistant to aphidicolin and MAX, respectively. N.D., not determined.

Donor DNA	Ratio of positive to total plates	Colonies observed (No.)	Apparent frequency per 10^7 recipients		
			Aph^r	MAX^r	MAX^rAph^r
		Direct transfection			
Control					
Buffer	0/12	0	<2	N.D.	N.D.
Salmon sperm	0/15	0	<1	N.D.	N.D.
V79 (wild type)	0/16	0	<1	N.D.	N.D.
Aph^r-4-2 (unsheared, Exp.1)	5/44	9*	4	N.D.	N.D.
Aph^r-4-2 (unsheared, Exp.2)	6/16	6*	8	N.D.	N.D.
Aph^r-4-2 (sheared to 3 ± 2 kb)	0/16	0*	<1	N.D.	N.D.
		Cotransfection			
Salmon sperm	0/8	0	N.D.	<3	<3
Salmon sperm + pSV2-gpt	2/2	28	N.D.	280	<10 (0)*
Aph^r-4-2 + pSV2-gpt	3/4†	52	N.D.	765	147 (10)*

*The null hypothesis that the number of colonies observed is independent of Aph^r-4-2 DNA added was rejected using Fisher's exact test or G statistics (or both) for independents at $P < 0.025$ (*17*). The numbers in parentheses indicate the number of MAX^rAph^r colonies. †1.7×10^5 V79 cells were plated initially.

ments and the three control groups (Table 1), we estimated that the spontaneous frequency of Aph^r colonies in the wild-type recipient cells is less than 1 in 10^7. When unsheared DNA (average size, 50 kb) from Aph^r-4-2 was used as donor DNA, we obtained, in two experiments, nine and six Aph^r colonies, or three colonies per 10^8 recipients per microgram of mutant DNA. However, after DNA of the mutant was mechanically sheared to an average size of 3 ± 2 kb before transfection, no colonies were observed, suggesting that the Aph^r phenotype is conferred by transfer of more than 5 kb of DNA.

To further demonstrate that the Aph^r clones recovered by transfection were not the result of spontaneous mutation, we carried out cotransfection experiments with pSV2-gpt, a plasmid containing the xanthine guanine phosphoribosyltransferase gene from *Escherichia coli* (*11*). Combinations of DNA from pSV2-gpt with DNA from salmon sperm or the mutant were precipitated together with calcium phosphate and added to wild-type cells (*12*), and MAX^r (*11*) or MAX^{r}Aph^r colonies were selected. No MAX^r or MAX^{r}Aph^r colonies were obtained from cells that had received only salmon sperm DNA (Table 1). Twenty-eight MAX^r colonies were obtained from cells that had received salmon sperm and pSV2-gpt DNA's, yielding a transfection efficiency of 2.8 colonies per 10^5 recipients per 1.25 μg of plasmid DNA. None of these 28 colonies was resistant to 0.8 μ*M* aphidicolin. However, 52 MAX^r colonies were obtained when pSV2-gpt and mutant DNA were transfected together; 10 of these 52 colonies were able to grow in the presence of MAX and 0.8 μ*M* aphidicolin.

From two experiments, we observed a transfection efficiency for the MAX^r-Aph^r phenotype of four colonies per 10^7 recipients per microgram of mutant DNA. This efficiency is ten times higher than that observed in the direct transfection experiments, presumably because the cotransfection selects for a population of recipients that incorporate DNA with high efficiency. The drug-resistant phenotype of transfectants was stable after 28 days of growth in the absence of aphidicolin or MAX (or both). This stability suggests that the functional gene may have been integrated into the recipient's genome (*13*).

Figure 1A shows a comparison of the aphidicolin sensitivities of the wild-type recipient, the original mutant (Aph^r-4-2), and one of the direct transfectants (AphVT-1) grown 28 days in the absence of aphidicolin. The survival fraction of the wild-type V79 recipient was reduced six orders of magnitude by incubation in the presence of 0.6 μ*M* aphidicolin; the survival fractions of both the mutant and transfectant were reduced by less than 40 percent under identical conditions.

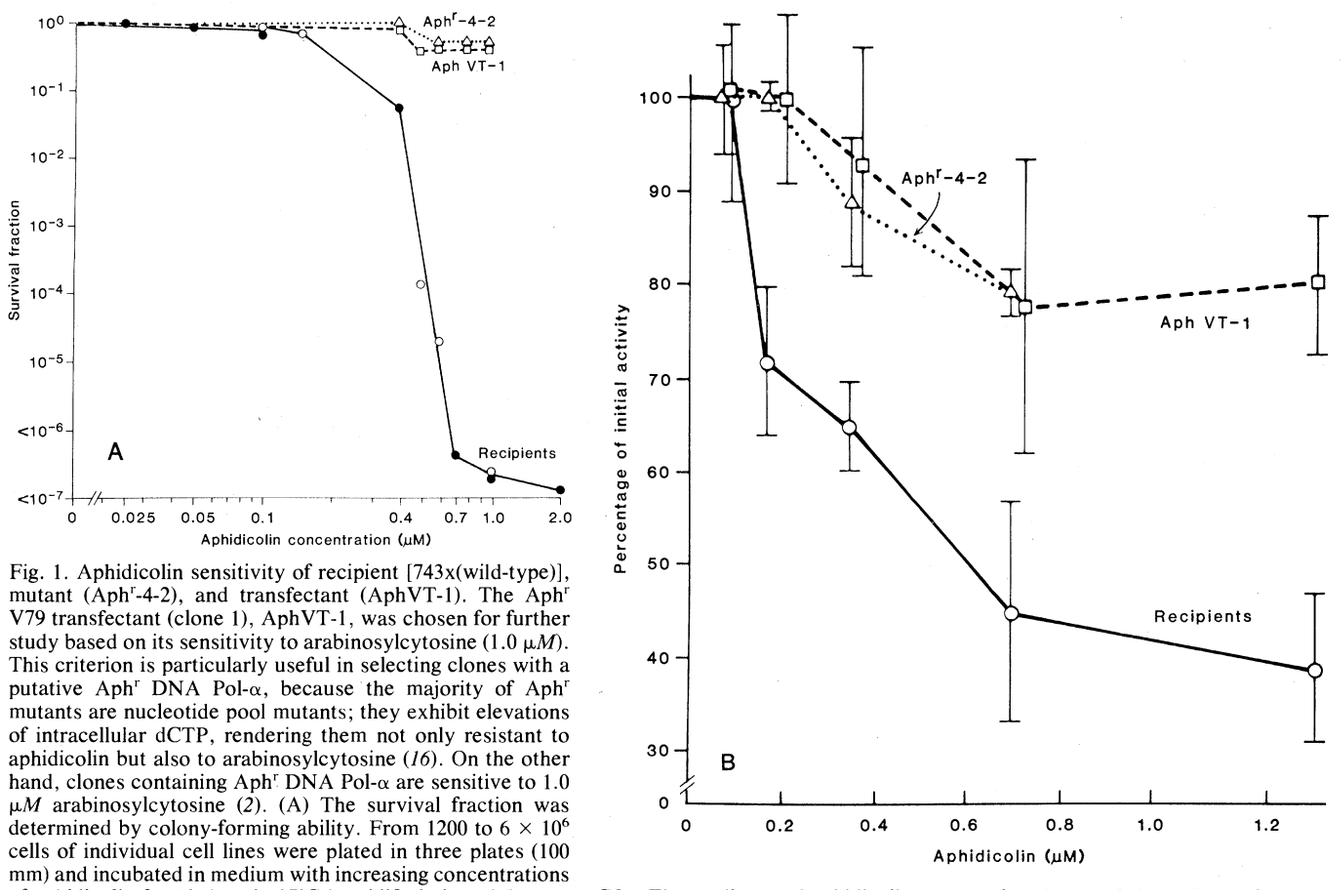

Fig. 1. Aphidicolin sensitivity of recipient [743x(wild-type)], mutant (Aph^r-4-2), and transfectant (AphVT-1). The Aph^r V79 transfectant (clone 1), AphVT-1, was chosen for further study based on its sensitivity to arabinosylcytosine (1.0 μ*M*). This criterion is particularly useful in selecting clones with a putative Aph^r DNA Pol-α, because the majority of Aph^r mutants are nucleotide pool mutants; they exhibit elevations of intracellular dCTP, rendering them not only resistant to aphidicolin but also to arabinosylcytosine (*16*). On the other hand, clones containing Aph^r DNA Pol-α are sensitive to 1.0 μ*M* arabinosylcytosine (*2*). (A) The survival fraction was determined by colony-forming ability. From 1200 to 6×10^6 cells of individual cell lines were plated in three plates (100 mm) and incubated in medium with increasing concentrations of aphidicolin for 10 days in 37°C humidified air and 5 percent CO_2. The medium and aphidicolin were replaced every 2 days. Each plate was rinsed with phosphate-buffered saline and stained with crystal violet stain. Only colonies with a cell number greater than 30 were scored, and the average numbers of colonies are shown. (B) DNA Pol-α activity as a function of aphidicolin concentrations. DNA Pol-α was purified through DEAE-cellulose (DE52) columns from cell extract of approximately 5×10^8 cells (*3*). Aphidicolin sensitivity of the Pol-α from each cell line was assayed in a 50 μl reaction mixture containing 10 μg of activated calf thymus ^{3}H-labeled dGMP DNA (specific activity, 2000 cpm/μg), 50 m*M* tris-HCl (*p*H 8.0), 2 m*M* dithiothreitol, 4 m*M* $MgCl_2$, 100 μ*M* dGTP and dATP (deoxyguanosine and deoxyadenine triphosphates), 3 μ*M* dCTP (deoxycytidine triphosphate), 10 μ*M* [α-^{32}P]dTTP (thymidine triphosphate) (specific activity, 2000 cpm/pmol), and 2.5 μl DNA Pol-α. Incubation was for 20 minutes at 37°C, and acid-insoluble radioactivity was measured after extensive washing. All assays were carried out in triplicate; lines were drawn by connecting the average values, and the bar represents the range of values. One hundred percent activity corresponds to 1.7 pmol, 3.0 pmol, and 1.0 pmol of dTMP incorporated for DNA Pol-α from V79 (recipient), Aph^r-4-2, and AphVT-1, respectively. The ^{3}H-labeled DNA allowed correction for any DNA lost during the extensive washing procedure.

DNA polymerase activities of these clones were assayed. We observed no significant variation in the specific activity of DNA polymerase (nanomoles of dTMP per milligram of protein, 20 minutes at 37°C) in mitochondria-free crude extracts from wild-type (2.7), Aph^r-4-2 (1.7), and AphVT-1 (2.4) cells. Figure 1B shows that wild-type DNA Pol-α was sensitive to >0.2 μ*M* aphidicolin. In three separate experiments, the DNA Pol-α activity (mean ± standard error) was decreased by 59 ± 4 percent for wild-type, 13 ± 11 percent for Aph^r-4-2, and 23 ± 5 percent for AphVT-1 by the presence of 0.6 μ*M* aphidicolin (data not shown). Thus the mutant Pol-α is stably expressed in the transfectant.

In cotransfer experiments with the thymidine kinase (*tk*) gene and the β-globin gene into murine thymidine kinase–deficient Ltk$^-$ cells with single selection for *tk*$^+$ transfectants, Wigler *et al.* (*12*) and Huttner *et al.* (*13*) found that more than 75 percent of the *tk*$^+$ transfectants contained the integrated β-globin gene. Similarly, after cotransfection of pSV2-gpt and HeLa DNA into mitomycin-C–sensitive Chinese hamster ovary (CHO) cells, and selection for both MAX^r and mitomycin-C–resistant transfectants, Rubin *et al.* (*14*) have found that 2 of the 800 MAX^r clones (≤0.3 percent) contained human sequences and were mitomycin-C–resistant. With selections for both markers, we found that 20 percent of MAX^r clones were MAX^{r}Aph^r. Because the background frequency was less than 10^{-6} for MAX^r and less than 10^{-7} for Aph^r colonies, the chance to obtain a colony that spontaneously exhibited both MAX^r and Aph^r phenotypes would be less than 10^{-13}. The transfection efficiency of 4 per 10^7 per microgram of mutant DNA is 10^6 times higher than that expected from two independent spontaneous mutations. Taken together, our data indicate that the Aph^r colonies are the result of DNA-mediated gene transfer; a DNA fragment presumably greater than 5 kb, rendering cells aphidicolin resistant, had been transferred via DNA-mediated transfection of V79 wild-type cells. Our studies suggest that Aph^r phenotype can be used as a selectable marker for the transfection of the gene for DNA Pol-α, further providing evidence that Aph^r-4-2 is a mutant with a defect in the gene for DNA Pol-α.

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Ion Channels in Plasmalemma of Wheat Protoplasts

Abstract. *The patch-clamp technique was used to study passive movements of ions through the plasmalemma of wheat leaf protoplasts. This method overcomes the problems inherent in conventional electrophysiological study of plant cells. Changes in conductance were recorded in patches excised from the plasmalemma. Two types of patches were observed: (i) regions of low channel density, where discrete single-channel currents could be resolved and conductance ranged from 10 to 200 picosiemens and (ii) regions of high channel density, where single-channel currents could not be resolved and conductance was on the order of a few nanosiemens. The results indicate a striking similarity between animal and plant cell membranes in the basic phenomena of transport. Moreover, the approach used constitutes a new degree of refinement in the study of processes of regulation, pathology, and toxicity in plants.*

While electrogenic ion pumps, along with other carrier mechanisms, have an important role in plant ion transport (*1*), relatively little is known about the passive ion pathways, in particular about gated channels, such as those observed in animal cells (*2*). One reason for this is the difficulty in using electrophysiological methods to study ion movements in plant cells (*1*). This difficulty stems largely from the specific compartmentalization of the cell. Most of the volume of plant cells is occupied by a vacuole, with a very thin layer of organelle-packed cytoplasm separating the outer membrane (the plasmalemma) from the membrane of the vacuole (the tonoplast). It is, therefore, not a simple task to locate the tip of an inserted microelectrode with respect to the membranes. Until recently, successful attempts at voltage clamping plant cells required the use of large cells from algae, such as *Nitella* (*3*), *Chara* (*4, 5*), and *Hydrodictyon* (*6*). In higher plants electrophysiology has, to our knowledge, been limited to measurements of membrane resistance and membrane potential (*7–13*). Accurate measurements of potentials across the plasmalemma are impeded by the cell wall in series with the plasmalemma. Because of its fixed negative charges, the cell wall adds an unknown voltage component to the measurement.

In this study we examined separately the pathways of passive ion movement in an attempt to distinguish between carrier and gated-channel modes of transport. To overcome the difficulties described above we combined the technique of enzymatic isolation of plant protoplasts devoid of cell walls with the voltage-clamp technique by using a "patch electrode."

Leaf protoplasts were isolated from 7-day-old wheat plants (*Triticum aestivum*)