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Differentiation of Embryonic Stem Cell Lines Generated from Adult Somatic Cells by Nuclear Transfer

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Embryonic stem (ES) cells are fully pluripotent in that they can differentiate into all cell types, including gametes. We have derived 35 ES cell lines via nuclear transfer (ntES cell lines) from adult mouse somatic cells of inbred, hybrid, and mutant strains. ntES cells contributed to an extensive variety of cell types, including dopaminergic and serotonergic neurons in vitro and germ cells in vivo. Cloning by transfer of ntES cell nuclei could result in normal development of fertile adults. These studies demonstrate the full pluripotency of ntES cells.

Stem cells are able to differentiate into multiple cell types, representatives of which might be harnessed for tissue repair in degenerative disorders such as diabetes and Parkinson's disease (*1*). One obstacle to therapeutic applications is obtaining stem cells for a given patient. A solution would be to derive stem cells from embryos generated by cloning from the nuclei of the individual's somatic cells. We have previously cloned mice by microinjection using a variety of cell types as nucleus donors, including embryonic stem (ES) cells (*2–4*). We sought to perform the converse experiment by deriving ES cell lines in vitro from the inner cell mass (ICM) of blastocysts clonally produced by nuclear transfer.

To this end, nuclei from adult-derived somatic donor cells of five strains, including inbred (e.g., 129/Sv and C57BL/6$^{nu/nu}$, nude) and F_1 hybrid (e.g., C57BL/6 × DBA/2) representatives were transferred by microinjection (*5*) to produce cloned blastocysts (Table 1). When plated on fibroblast feeder layers in culture medium (*6*), cloned blastocysts from all five strains tested yielded at least one nuclear transfer ES (ntES) cell line (Table 1) (*8*). Cultures were established from XX embryos derived via cumulus cell nuclear transfer (14.2% of blastocysts) and both XX and XY embryos derived from tail-tip cells (6.5%; Table 1). In total, 35 successfully cryopreserved stable ntES cell lines were produced.

Clonal origin of ntES cell lines was confirmed by polymerase chain reaction (PCR) analysis of polymorphic markers (*8, 9*). The ntES cell morphology of most lines was similar to that of widely disseminated lines such as E14 (*11*). We found no evidence for a pronounced difference in the efficiency of ntES cell line establishment between inbred and hybrid backgrounds (Table 1). All ntES cell lines tested expressed markers diagnostic for undifferentiated ES cells (*12*), including alkaline phosphatase (8) and Oct3/4 (*13*).

Embryonic stem cells have been induced to differentiate in vitro to produce cardiomyocytes (*14*), neurons (*15*), astrocytes and oligodendrocytes (*16*), and hematopoietic lineages (*17*). To assess the pluripotency of ntES cells, we therefore sought (i) to differentiate them in vitro to a wide variety of ectodermal, mesodermal, and endodermal lineages, and (ii) to induce a highly differentiated cell type. We chose a particularly specialized example with therapeutic potential: dopaminergic neurons.

Differentiation of embryoid bodies (*8, 18*) derived from three different ntES cell lines resulted in a mixed population of ectodermal, endodermal, and mesodermal derivatives (*19*). Efficient neural differentiation of ntES cells could be readily induced in each of the seven lines tested. Generation of specific midbrain dopaminergic neurons from ntES cells was achieved with a range of efficiencies by using a multistep differentiation protocol described previously (*15, 20*) (Fig. 1, A and B). One ntES cell line yielded dopaminergic neurons in excess of 50% of the total cell number. The functional nature of these neurons was confirmed by reversed-phase HPLC (RP-HPLC) determination of dopamine release (*21*) (Fig. 1C). Serotonergic neurons were also detected histochemically, although in smaller numbers, and serotonin release was confirmed by RP-HPLC (Fig. 1, D and E).

Two recent reports (*22, 23*) describe a total of five mouse ES cell-like lines derived from the ICMs of cloned blastocysts, although none contributed to the germ line. We characterized the contribution of 19 ntES cell lines to chimeric offspring after ntES cell injection into fertilization-derived blastocysts from the ICR strain (*24*). The contribution of ntES cells to 105 chimeric offspring after 348 blastocyst injections is summarized in Table 1. The contribution can be readily approximated by coat color, because all ntES cell lines are derived from black-eyed strains with dark coat color, whereas ICR is albino (Fig. 2, A and B). ntES cell lines generally contributed strongly to the coats of chimeric offspring (Table 1). This was corroborated for ntES cells derived from a hybrid strain ubiquitously expressing high levels of the reporter transgene, *EGFP* (*25*). All internal organs examined from two EGFP Tg chimeras contained an extensive contribution from the EGFP-expressing ntES cells (*13*).

As a comprehensive measure of pluripotency, the ability to contribute to the germ line is considered a defining characteristic of ES cells. Chimeric offspring were crossed with the albino strain, ICR. In ongoing experiments, 29 pups have been derived after chimera × ICR crosses as judged by eye and coat color and, where appropriate, EGFP expression (Table 1). Germ line transmission was demonstrated for seven ntES cell lines

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derived from male and female representatives of all mouse progenitor strains. These data confirm that ntES cells contribute to both male and female gametogenesis when derived from either inbred, hybrid, or mutant strains (Table 1), consistent with the universality of the phenomenon among diverse genetic backgrounds.

To determine whether the reprogramming that produced fully pluripotent ntES cells could be reversed, we attempted to re-derive the original nucleus donor cell types in offspring cloned by nuclear transfer from ntES cells (*2*). Nuclei from all ntES cell lines supported development in vitro to the blastocyst stage after microinjection into enucleated oocytes (Table 2). When transferred to pseudopregnant surrogate mothers, blastocysts derived from six of the ntES cell lines developed to term, resulting in a total of 20 live-born pups. Of these, one was derived from the nucleus of a C57BL/6$^{nu/nu}$ (nude, inbred) background, and the remaining 19 from the nuclei of hybrid strains (Fig. 2C; Table 2). Hybrid genomes thus preferentially supported cloning in these experiments. Moreover, 11 (all cumulus-derived females; see Fig. 2C) of the 19 were derived from B6D2F$_1$ ntES cell lines, of which 10 survived to adulthood and are healthy, exhibiting normal fertility (Fig. 2C). The remaining nine, which died perinatally of unknown cause(s), also contained genomic contribution from the hybrid, B6D2F$_1$ (129/Sv Tac × B6D2F$_1$; Table 2), albeit diluted. This possibly reflects a subtle, yet critical contribution made by the hybrid genetic background of B6D2F$_1$. We corroborated the clonal origin of ntES cells and cloned offspring by PCR analysis of polymorphic markers (Fig. 2D).

We have demonstrated that adult-derived somatic cell nuclei can efficiently be used to generate ES cell lines that exhibit full pluripotency; they can be caused to differentiate along prescribed pathways in vitro, contribute to the germ line after injection into blastocysts, and support full development following nuclear transfer. Because ES cells support homologous recombination at a relatively high efficiency, genetic lesions in ntES cells might be repaired by gene targeting or transgenic complementation before they are used to establish germ line chimeras or in cloning. This should facilitate the establishment of germ cells, individuals, and cell lines containing targeted alleles.

Reports of human ES cell-like cell lines (*26*, *27*), coupled to the success of mammalian cloning by somatic cell nuclear transfer, have raised the possibility that ntES cells could provide a source of differentiated cells for human autologous transplant therapy: therapeutic cloning (*28*). In this context, demonstration of the full pluripotency of ntES cells is particularly relevant; for example, adult-derived stem cells are apparently restricted in their range of potential cell fates and may be unable to contribute to all tissues, including hematopoietic lineages (*29*). Indeed, the efficient generation of midbrain dopaminergic neurons in vitro has been achieved to date only with mesencephalic precursors (*30*) and ES cells (*15*), but not from adult-derived cells. In combining ES and nuclear transfer technologies, we have here addressed this limitation and demonstrated the feasibility of the first steps required for the application of cloning to transplant therapy.

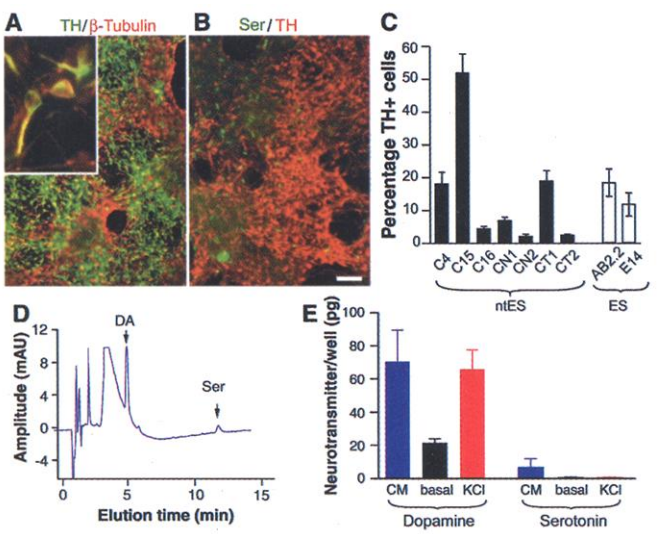

Fig. 1. Dopaminergic and serotonergic differentiation of ntES cells in vitro. Embryoid bodies were plated under conditions favoring CNS selection followed by dopaminergic induction. Images shown are of C15. (**A**) Colocalization of tyrosine-hydroxylase (TH, green) and β-III tubulin (red). The inset shows the morphology of neuritic TH$^+$ cells at higher magnification. (**B**) The presence of serotonergic (Ser, green) and TH (red) neurons. Scale bar = 100 μm. (**C**) Yield of TH$^+$ neurons varied among the cell lines tested, with >50% of total cell number in C15 cells. Other commonly used ES lines (E14, AB2.2) generated a percentage of TH$^+$ cells falling within the range shown by our ntES cells. C4, C15, C16, CN1, CN2, CT1, CT2 represent ntES, AB2.2, and E14 ES cell lines. (**D**) Representative chromatogram showing elution and electrochemical detection of dopamine (DA) and serotonin (Ser) from conditioned medium by RP-HPLC. (**E**) Quantification of dopamine and serotonin release. Neurotransmitter concentration was determined in conditioned medium (CM; 24 hours after last medium change), basal condition (15 min in buffer solution) and upon evoked release (KCl; 15 min in 56 mM KCl buffer). Serotonin release was low under basal and evoked conditions, probably reflecting a lower number of serotonergic neurons.

Table 1. Establishment of ntES cell lines after nuclear transfer from adult-derived cumulus or tail-tip cells and examination of pluripotency after injection into fertilization-derived blastocysts.

Nucleus donor			Establishment of ntES cell via nuclear transfer			In vivo differentiation after ntES cell injection into blastocysts*			
Strain	Sex	Tissue	No. reconstructed oocytes	Blastocyst development (%)	Established ntES cell line (%)†	% normal karyotype‡	No. injected blastocysts	No. chimeras/ offspring	No. germ line transmitting cell lines (black/pups)§
B6D2F$_1$	F	Cumulus	130	57 (43.8)	9 (15.8) [6.9]	67.8 ± 14.1 (6)	129	39/102	1 (5/196)
129/Sv	F	Cumulus	44	13 (29.5)	1 (7.7) [2.3]	51.8 (1)	25	2/15	1 (1/72)
129/Sv	M	Tail tip	88	42 (47.7)	1 (2.4) [1.1]	66.2 (1)	24	17/20	1 (2/127)
129F$_1$	M	Tail tip	182	54 (29.7)	7 (13.0) [3.8]	50.5 ± 16.7 (4)	49	16/25	1 (3/100)
C57BL/6$^{nu/nu}$	F	Tail tip	159	75 (47.2)	5 (6.7) [3.1]	25.8, 31.3 (2)	24	4/22	0
C57BL/6$^{nu/nu}$	M	Tail tip	210	88 (41.9)	4 (4.5) [1.9]	46.1 ± 33.0 (3)	44	16/25	2 (10/119)
EGFP Tg	F	Cumulus	118	50 (42.4)	7 (14.0) [5.9]	10.3 (1)	14	3/13	–
EGFP Tg	M	Tail tip	85	19 (22.4)	1 (5.3) [1.2]	68.8 (1)	39	8/15	1 (8/31)
Total (%) [%]			1016	398 (39.2)	35 (8.8) [3.4]	48.8 ± 20.4 (19)	348	105/237	7 (29/645)

*Data refer to karyotyped ntES cell lines only. †Expressed as % of blastocysts ( ) and of reconstructed oocytes [ ]. ‡More than 50 M-phase cells were examined for each ntES cell line. Number of ntES cell lines examined is shown in parentheses. §Data are shown for ntES cell lines that exhibited germ line transmission in chimeras. Data from nontransmitting chimeras have been omitted.

Table 2. Cloning using ntES cells as nucleus donors.

Nucleus donor			Nuclear transfer and in vitro development		In vivo development		
Strain	Sex	No. ntES cell lines	No. reconstructed oocytes	Morula/blastocyst development (%)	Implantation sites/ transferred embryos	Fetuses/ placentae	Live cloned offspring (%)
$B6D2F_1$	F	6	933	386 (41.4)	294/386	15	11 (2.8)
129	F	1	181	26 (14.4)	10/26	0	0
129	M	1	296	146 (49.3)	86/146	22	0
$129F_1$	M	4	712	199 (27.9)	166/196	24	8 (4.0)
$C57BL/6^{nu/nu}$	M	2	675	88 (19.0)*	82/175	2	1 (0.6)
EGFP Tg	M	1	168	46 (27.4)	25/46	1	0
Total (%)		15	2965	803 (29.2)	663/975	64	20 (2.1)

*From 219 reconstructed oocytes, 87 two-cell embryos were transferred to recipient females. Of the 456 other reconstructed oocytes, 88 (19%) developed to morulae/blastocysts.

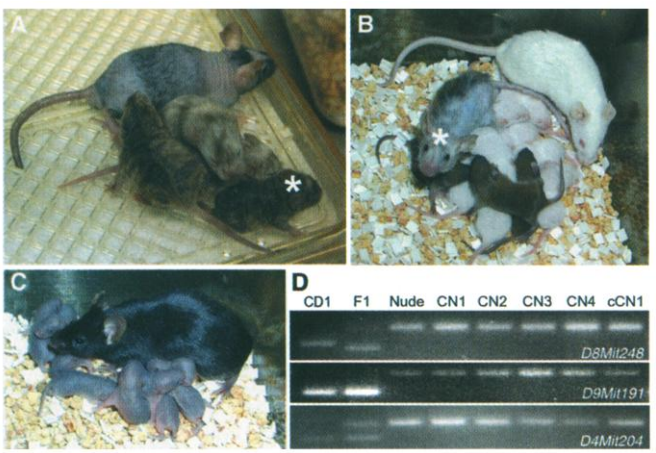

Fig. 2. Totipotency of ntES cells demonstrated in vivo. (**A**) Contribution of $C57BL/6^{nu/nu}$-nude ntES cells (line CN1) to chimeric offspring after injection into ICR × ICR fertilization-derived blastocysts is demonstrated in offspring 14 days after birth in which dark coat color derives from the ntES cell contribution. The adult mouse in the cage is the tail-tip donor used to generate the CN1 ntES line. (**B**) The male indicated with an asterisk in (A) was crossed at 8 weeks with a white (ICR) female, producing a litter containing three dark offspring, confirming the contribution of $C57BL/6^{nu}$ to the germ line. Asterisks in panels (A) and (B) indicate the same male. (**C**) Cloning using ntES cells as nucleus donors, exemplified by a $B6D2F_1$ clone (line C4) shown at 12 weeks with her litter. (**D**) PCR analysis of microsatellite markers in genomic DNA from ntES cell lines (CN1, CN2, CN3, CN4) and cloned offspring (cCN1) confirms the clonal origin of the $C57BL/6^{nu/nu}$ pup derived from line CN1. Polymorphic markers *D8Mit248*, *D9Mit191*, and *D4Mit204* are conserved between genomic DNA from the ntES cell lines and the cloned pup, but differ from those of the ICR surrogate mother (CD1) or ooplast recipient strain $B6D2F_1$ (F1).

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5. Mouse strains were $B6D2F_1$ (C57BL/6 × DBA/2), 129/SvTac, $129F_1$ (129/SvTac × $B6D2F_1$), nude ($C57BL/6^{nu/nu}$) and EGFP Tg ($B6D2F_2$ × ICR, F_6). Mice 8 to 15 weeks old were used as nucleus donors, with recipient oocytes from 8- to 10-week-old $B6D2F_1$s. Cumulus cells were acutely isolated immediately before nuclear transfer, as described previously (*2*). Tail tip nucleus donors were from 5- to 7-day-old primary cultures presumed to be fibroblasts (*3*).
6. Cloned, embryonic day 3.5 blastocysts were produced by transfer of cumulus or tail-tip cell nuclei from 8- to 12-week-old mice (*2, 3*). Cloned embryos were used to establish nuclear transfer ES (ntES) cell lines essentially as outlined previously (*7*). Briefly, each embryo was placed into one well of a 96-well plate seeded with ICR embryonic fibroblast feeders. After 7 days, colonies of undifferentiated cells were detached by trypsinization and transferred to a 96-well plate containing fresh medium and seeded with fresh embryonic fibroblast feeders. Clonal expansion of undifferentiated ntES cells proceeded after mild trypsinization and sequential transfer to 48-, 24-, 12-, and 6-well plates, and finally into a 12.5 cm^2 gelatinized flask (all in the absence of feeder cell layers) at intervals of 1 to 2 days. ntES cells were isolated and cultured in "DMEM for ES cells" (Specialty Media, Phillipsburg, NJ) supplemented with either 15% heat-inactivated fetal calf serum (FCS) (Hyclone) or 15% knockout serum replacement (Life Technologies), and 1000 U leukemia inhibitory factor (LIF)/ml (Gibco), plus the following (Specialty Media): 1% penicillin-streptomycin, 1% L-glutamine, 1% nonessential amino acids, 1% nucleosides, and 1% β-mercaptoethanol. Cells were split 1:3 or 1:4 every 1 to 2 days. Routine culture was in the absence of feeder cells.
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9. Analysis by PCR was used to confirm the genotypes of strains and cell lines. Primer pairs D4Mit204, D7Mit22, D8Mit248, and D9Mit191 (*10*) (Mappairs, Research Genetics Huntsville, AL) corresponding to microsatellite markers were used to generate a profile of PCR amplimers diagnostic for each genotype. Reactions of 30 μl containing ~50 to 100 ng genomic DNA from ntES cells or tail-tip biopsies were subjected to 34 cycles of PCR (1 min 95°C, 1 min 60°C, 2 min 72°C), and products were separated on a 4% agarose gel (Nusieve 3:1, BMA) before visualization.
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18. Culture conditions for pluripotency assay were as follows. ES cells were plated on uncoated bacterial dishes (2×10^6 cells/10 cm plate) in ES medium for embryoid body (EB) formation as described previously (*15*). Differentiation was induced after trypsinization and transfer to 24-well plates in DMEM containing 10% FCS. Cells were fixed after 9 days' culture in vitro.
19. See Web fig. 2. Supplementary material is available on *Science* Online at www.sciencemag.org/cgi/content/full/292/5517/740/DC1.
20. Induction of dopaminergic differentiation in vitro was as described previously (*15*) with the following minor modifications. Cells were cultured for a longer period during stage III (CNS selection stage), ranging from 9 to 16 days. Concentrations of bFGF, SHH, FGF8 (R&D Systems), and ascorbic acid (Sigma) were 10 ng/ml, 500 ng/ml, 100 ng/ml, and 100 μM, respectively.
21. Reversed-phase-HPLC for the detection of dopamine in neuronally conditioned medium was essentially as described previously (*15*). Samples were collected 7 days after differentiation (stage V), stabilized with orthophosphoric acid and metabisulfite, and subsequently extracted by aluminum adsorption. Separation of the injected samples (ESA Autosampler 540) was achieved by isocratic elution in MD-TM mobile phase (ESA) at 0.7 ml/min. The oxidative potential of the analytical cell (ESA Mod. 5011, Coulochem II) was set at +325 mV. Identical conditions were applied for serotonin detection. Results were validated by co-elution with dopamine or serotonin standards under varying buffer conditions and detector settings.
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a back-crossed EGFP transgenic strain (B6D2F$_2$ × ICR, F$_6$), they were injected into blastocysts derived from the agouti cross B6D2F$_1$ × ICR.

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Molecular Analyses of Oral Polio Vaccine Samples

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It has been suggested that the human immunodeficiency virus (HIV), and thus the acquired immunodeficiency syndrome (AIDS) it causes, was inadvertently introduced to humans by the use of an oral polio vaccine (OPV) during a vaccination campaign launched by the Wistar Institute, Philadelphia, PA, USA, in the Belgian Congo in 1958 and 1959. The "OPV/AIDS hypothesis" suggests that the OPV used in this campaign was produced in chimpanzee kidney epithelial cell cultures rather than in monkey kidney cell cultures, as stated by H. Koprowski and co-workers, who produced the OPV. If chimpanzee cells were indeed used, this would lend support to the OPV/AIDS hypothesis, since chimpanzees harbor a simian immunodeficiency virus, widely accepted to be the origin of HIV-1. We analyzed several early OPV pools and found no evidence for the presence of chimpanzee DNA; by contrast, monkey DNA is present.

In the spring of 2000, a committee set up by the Wistar Institute to investigate the "OPV/AIDS hypothesis" (*1, 2*) delivered a total of 14 samples to our laboratory to be analyzed for the presence of chimpanzee DNA (Table 1). The samples were coded by the committee and only after all analyses were concluded and the results transmitted to the committee, were the identity of the samples revealed to us.

Initial experiments showed that DNA could be amplified directly from the OPV samples, whereas extraction procedures resulted in losses of DNA (*3*). In order to estimate how much DNA survived in the 40-year-old vaccine batches, a real-time polymerase chain reaction (PCR) quantitation assay that detects the nuclear *c-myc* gene of mammals was used (*4*). The total amount of DNA in the vaccines as determined by this assay varied from 0 to 7.5 ng/μl (Table 1). For three of the samples, no amplifiable DNA was detected. One of these was a negative control composed of tissue culture medium, one was a late passage of OPV (W Ch 25), and one was an early passage of the OPV (CHAT pool 16 A-5) from the Wistar Institute. All other samples, including CHAT pool 13 that was used in the Congo in the late 1950s, contained detectable DNA. In general, it is noteworthy that no correlation exists between the age of the samples and the amount of DNA retrieved. Thus, the extract of the Sabin I vaccine produced in 2000 contained 5 pg DNA per μl, whereas the CHAT pool 13 produced at the Center for Disease Control (CDC) in 1960 contained 857 pg DNA per μl vaccine.

To identify the source species of the cells used in vaccine production, amplifications of a 151–base pair (bp) portion (with primers) of the mitochondrial (mt) 12*S* rDNA were performed from all 14 samples. The negative control and one late passage of OPV from the Wistar Institute (W Ch 25), where the quantitation failed to detect DNA, yielded no amplification product. All products amplified from the other samples were cloned, and the inserts of 10 clones from each amplification were sequenced. All samples, except CHAT 23 7.7 and the Sabin I vaccine, yielded multiple sequences that differed by up to 20 substitutions within a single amplification. The occurrence of multiple sequences in a single amplification can be a frequent occurrence when mtDNA is amplified. A common reason for this is the amplification of nuclear insertions of mtDNA. Such insertions are numerous and can sometimes dominate amplifications, whereas amplifications of the organellar mtDNA may even be absent, particularly from sources that contain little mtDNA relative to nuclear DNA (*5*). Because intra- or interindividual differences are unlikely in the 12*S* ribosomal gene, which carries little variation within species, we assume that the majority of variation among the clones is due to the amplification of nuclear mtDNA insertions. The multiplicity of mtDNA sequences obtained make the 12*S* rDNA sequences unsuitable for determining the species used for the vaccine production. For example, for CHAT pool 13, seven different sequences were found among 10 sequences determined. Although one of these showed only two differences to mona monkey (*Cercopithecus mona*) while the clone closest to

Table 1. OPV and control samples used in this study. For each sample, the production date (where available), the closest matching species or genera based on the nuclear 28*S* rDNA sequences along with the number of differences, and the DNA concentration (pg/μl sample, averaged over three and two quantitations for the OPV and control samples, respectively) are given. Dashes indicate that no amplification product was seen in two amplification attempts. The OPVs were produced at the Wistar Institute and, where indicated, at the CDC.

Sample number	Nuclear 28S rDNA		DNA (pg/μl)
	Taxon matching	Differences	
	OPV samples		
CHAT 23 7.7	*Macaca*	0	14
WCh-24 57C-40 137-71	*Macaca*	0	53
CHAT Pool 13	*Macaca*	0	344
W Ch 25	–	–	0
CHAT Pool 16 A-5	*Macaca*	0	0
CDC CHAT type I Wy4B-5	*Macaca*	0	47
CDC CHAT 1FL (15 October 1979)	*Homo*	0	7515
CDC CHAT Pool 13 (29 August 1960)	*Macaca*	0	857
Sabin I (20 January 2000)	*Homo*	0	5
	Control samples		
African green monkey	*Chlorocebus*	0	1
Common chimpanzee	*Pan troglodytes*	0	5
Negative control	–	–	0
Macaque	*Macaca*	0	1
Sooty mangabey	*Macaca*	0	6

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