Mol Gen Genet (1994) 242:495–504

MGG



*Original articles*

# Ingested foreign (phage M13) DNA survives transiently in the gastrointestinal tract and enters the bloodstream of mice

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Received: 12 August 1993 / Accepted: 15 October 1993

**Abstract.** Is the epithelial lining of the mammalian gastrointestinal (GI) tract a tight barrier against the uptake of ingested foreign DNA or can such foreign DNA penetrate into the organism? We approached this question by pipette-feeding circular or linearized double-stranded phage M13 DNA to mice or by adding M13 DNA to the food of mice whose fecal excretions had previously been shown to be devoid of this DNA. At various post-prandial times, the feces of the animals was tested for M13 DNA sequences by Southern or dot blot hybridization or by the polymerase chain reaction (PCR). On Southern blot hybridization, the majority of M13 DNA fragments were found in the size range between <200 and 400 bp (base pairs). For the PCR analysis, synthetic oligodeoxyribonucleotide primers were spaced on the M13 DNA molecule such that the sizes of the persisting M13 DNA fragments could be determined. We also extracted DNA from whole blood or from sedimented blood cells of the animals at different times after feeding M13 DNA and examined these DNA preparations for the presence of M13 DNA by dot blot hybridization or by PCR. M13 DNA fragments were found between 1 and 7 h postprandially in the feces of mice. By PCR analysis, fragments of 712, 976, and 1692 bp in length were detected. In DNA from blood, M13 DNA fragments of up to 472 bp were found by PCR between 2 and 6 h after feeding. Dot blot or Southern blot hybridization revealed M13 DNA at 2 and 4 h, but not at 1, 8 or 24 h after feeding. This DNA was shown to be DNase sensitive. M13 DNA was found both in blood cells and in the serum. A segment of about 400 bp of the DNA amplified by PCR from feces or blood was analyzed for its nucleotide sequence which was found to be identical to that of authentic M13 DNA, except for a few deviations. M13 DNA could not be detected in the feces or in the blood of the animals prior to feeding or prior to 1 h and later than 7 h after feeding. These controls attest to the validity of the results and also argue against the possibility that the murine GI tract had been colonized by phage M13. Moreover, M13 DNA-positive bacterial colonies were never isolated from the feces of animals that had ingested M13 DNA. The results of reconstitution experiments suggested that 2 to 4% of the orally administered M13 DNA could be detected in the GI tract of mice. A proportion of about 0.01% to 0.1% of the M13 DNA fed could be retrieved from the blood.

**Key words:** Phage M13 DNA – Dot blot hybridization – Southern blot hybridization – Polymerase chain reaction

Communicated by W. Goebel

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## Introduction

It has been documented in several biological systems that mammalian cells can take up foreign DNA and incorporate it into their genomes. In the past, we have investigated certain aspects of this mechanism by studying the integration of adenovirus DNA into the genomes of mammalian cells (Doerfler 1968; Groneberg et al. 1975; Doerfler et al. 1983; Jessberger et al. 1989; Orend et al. 1994; Tatzelt et al. 1993). In living organisms, the gastrointestinal (GI) tract is constantly exposed to foreign DNA by the incessant flow of partly or completely digested nutrients. The cells of the epithelial lining of the GI tract exhibit high turnover for several reasons, probably not all of them mechanical. It is not known whether DNA ingested by mammals can survive, at least partly, the enzymatic repertoire of the GI system, to what extent cells in the GI tract can incorporate foreign DNA, or whether DNA fragments from nutrients are able to pass from the GI tract into the circulation.

The fate of nucleic acids in the GI tract of mammals, e.g., ruminants (McAllan 1980, 1982; Storm et al. 1983) and rats (Maturin and Curtiss 1977), was first investigated several years ago. The limited sensitivity of the methods then available could not eliminate the possibility that trace amounts of intact DNA fragments could survive passage through the mammalian gut.

Although the available data tend to favor the view that foreign DNA sequences that enter the GI tract in nutrients cannot pass undigested, we have re-investigated the problem using current methods. The most rigorous test applied involves administration of pure circular or linear, double-stranded DNA, either directly or added to food pellets, to mice and screening of their fecal excretions for these DNA sequences at various times after feeding. We have also extracted DNA from whole blood, from isolated blood cells and from serum, and tested it for the presence of the foreign DNA sequences at various times after feeding.

In the present report, we demonstrate that M13 DNA fragments of up to 1692 bp in length survive passage through the GI tract of mice. The M13mp18 genome contains 7250 bp. Upon pipette-feeding of M13 DNA, the animals excrete foreign DNA fragments 1 to 7 h later. When M13 DNA was added to food pellets, DNA fragments were also detected in the feces. The M13 DNA in fecal excretions has been identified by Southern and dot blot hybridizations, and by the polymerase chain reaction (PCR). In fecal excretions of control animals that had not received phage DNA, M13 DNA was never detected. We have also investigated DNA extracted from the blood circulation of mice for the appearance and persistence of M13 DNA. M13 DNA fragments of up to 472 bp in length were detected in the blood of 59 different animals tested between 2 and 6 h after feeding.

The results directly demonstrate that foreign DNA ingested by mammals can survive passage through the GI system and can enter the bloodstream.

## Materials and methods

*Feeding of animals.* Mice of the C57Bl/6 strain between the ages of 3–6 months were fed double-stranded circular M13mp18 DNA in TE (10 m M TRIS-HCl, pH 7.5, 1 mM EDTA) in amounts of 10 to 50 µg in a total volume of 20 µl. In some experiments, M13mp18 DNA was linearized with *Eco*RI prior to feeding. The solution was administered from an Eppendorf pipette tip directly into the oral cavity. In some of the feeding experiments, the M13 DNA sample was applied to the food pellets (Altromin, Lage-Lippe, Germany) offered as diet to the animals. Prior to feeding, fecal samples of each experimental animal were investigated to confirm the absence of DNA sequences with homology to phage M13 DNA. M13 DNA was never found in the feces of mice selected for these experiments.

*Preparation of M13mp18 DNA.* Double-stranded phage M13mp8 DNA (Hofschneider 1963; Yanisch-Perron et al. 1985) was grown in plasmid form on *Escherichia coli* strain BMH71-18, and phage DNA was purified by standard procedures including equilibrium sedimentation in CsCl density gradients containing 30 µg/ml ethidium bromide (Clewell and Helinski 1972). The dye was eventually removed from the DNA preparation by extraction with buffer-saturated isopropanol.

*Isolation and purification of DNA from fecal samples.* Fecal samples were collected at various times after pipette- or pellet-feeding the M13mp18 DNA solution to mice. Feces were dispersed in 3.33 ml of 10 mM TRIS-HCl, pH 8.0, 2 mM EDTA, 10 mM NaCl, 1% sodium dodecylsulfate (SDS), and 1.33 mg of proteinase K per ml. The suspension was incubated at 37° C overnight, and subsequently nucleic acids were ethanol precipitated at −18° C, pelleted and resuspended in 200 µl of TE. A volume of 50 µl of this solution was added to 0.9 ml of guanidinium thiocyanate (GuSCN) buffer (Boom et al. 1989) consisting of 12 g GuSCN in 10 ml of 0.1 M TRIS-HCl, pH 6.4, 2.2 ml of 0.2 M EDTA, pH 8.0, 0.26 g of Triton X-100, and 40 µl of an aqueous suspension of silica. This solution was kept at ambient temperature for 10 min, shaken on a Vortex for 5 s and briefly centrifuged. The pellet was washed twice in a buffer containing 12 g of GuSCN in 10 ml of 0.1 M TRIS-HCl, pH 6.4, twice in 70% ethanol, and was then dried. Subsequently, the pellet was resuspended in 1 ml of TE, incubated at 56° C for 10 min, and the solution was clarified by centrifugation. The nucleic acids were again ethanol precipitated, resuspended in 0.2 ml of TE and dialyzed for 2 h against TE.

In many of the experiments performed, these extracts were subsequently treated with 10 µg of RNase per ml at 37° C for 1 h. The DNA was then purified again by phenol-chloroform extraction. Prior to use, the RNase was heated to 95° C for 10 min to destroy contaminating DNase.

*Extraction of DNA from total blood, blood cells or serum.* This method has been described elsewhere (Behn-Krappa et al. 1991). Briefly, blood (approx. 1 ml) was drawn by heart puncture of the animals, and clotting was prevented by adding Liquemin (500 units/ml of sample). Different sets of surgical instruments were used for opening the skin or the thorax of the animals to avoid contaminations. In some of the experiments, the blood cells were pelleted by low speed centrifugation, and DNA was extracted separately from the cell pellet and from serum. DNA was extracted by the SDS-proteinase K-phenol/chloroform procedure (Sutter et al. 1978). In other experiments, DNA was extracted as follows. After heparinization of total blood, samples were dispersed in 3.33 ml of 10 mM TRIS-HCl, pH 8.0, 2 mM EDTA, 10 mM NaCl, 1% SDS. After the addition of 4 mg of proteinase K and 4 mg of collagenase to the samples, they were treated identically to the fecal samples. RNase treatment was carried out as described in the preceding section.

*Analysis of the extracted DNA by Southern blotting and hybridization to $^{32}$P-labeled M13mp18 DNA.* For Southern blot analyses (Southern 1975), it sufficed to purify DNA from fecal extracts only as far as the proteinase K step. In all experiments, the alkali blotting procedure was employed (Orend et al. 1991). DNA transferred to Hybond N+ nylon membranes was subsequently hybridized to M13mp18 DNA which was $^{32}$P-labeled by using the random oligodeoxyribonucleotide procedure (Feinberg and Vogelstein 1983).

*Dot blot hybridization.* The DNA to be applied to membranes was boiled in a volume of 100 µl for 10 min in 0.3 M NaOH, quenched on ice and neutralized by the addition of 100 µl of 1 M sodium acetate, pH 7.0. Hybond $N^+$ membranes were wetted in $H_2O$ and pretreated with 1 M sodium acetate before the DNA was adsorbed in dots to the membranes by gravity or by minimal suction. The membranes were then washed in 1 M sodium acetate and in 4 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium-citrate), before baking at 80° C for 2 h. Prehybridization and hybridization to $^{32}$P-labeled M13 DNA proceeded according to standard procedures (Orend et al. 1991).

*Stringent wash conditions.* After Southern or dot blot hybridization, all filters were washed under stringent conditions: three times at 68° C in 2 × SSC, 0.1% SDS and three times at 68° C in 0.1 × SSC, 0.1% SDS. Subsequently, the filters were rinsed, dried and exposed to Kodak XAR5 films for autoradiography.

*Detection of M13mp18 DNA in fecal extracts or in blood samples by PCR.* A modification (Pääbo et al. 1988) of the standard PCR method (Saiki et al. 1987) was employed. In a total volume of 100 µl of 10 mM TRIS-HCl (pH 9.0 at 25° C), 50 mM KCl, 1.75 mM $MgCl_2$, 0.2 mM of each of the four deoxyribonucleoside triphosphates, 0.01% gelatin, 0.1% Triton X-100, 2 mg bovine serum albumin (BSA) per ml, 3 units of *Taq* polymerase were reacted with 95 ng of primers P1 to P7 (see map in Fig. 2) and 15 µl of the TE-dialyzed fecal extract (see above) or DNA extracted from blood or blood cells. The DNA was denatured for 1 min at 95° C, the primers were annealed for 2 min at 66° C, and the polymerase reaction was allowed to proceed for 3 min at 76° C in each of the 35 reaction cycles. Reaction products were analyzed by electrophoresis on 1% agarose gels. The DNA was ethidium bromide-stained, and gel positions were documented by UV photography. The M13mp18 derivation of amplified reaction products was confirmed by Southern blotting and hybridization to $^{32}$P-labeled authentic M13mp18 DNA.

*Screening of bacterial colonies propagated from fecal pellets for the presence of M13 DNA sequences.* Fecal pellets collected from mice at various times after feeding M13 DNA were suspended in L broth and plated in appropriate dilutions on 13.5 cm diameter agar plates to generate about $10^3$ to $10^4$ colonies per plate. Colonies were transferred to BA85 (Schleicher and Schuell) nitrocellulose membranes (Grunstein and Hogness 1975), and the DNA in these colonies was hybridized to $^{32}$P-labeled M13 DNA to assess possible colonization of fecal bacteria by M13 DNA or phage. Membranes were analyzed by autoradiography. Colonies from the feces of control animals that had not received M13 DNA were screened in the same way.

*Determination of the nucleotide sequence of PCR products.* PCR products were purified with a QIA quick-spin PCR purification kit (Qiagen, Hilden, FRG). DNA was sequenced with a Taq Dye dideoxy terminator cycle sequencing kit (Applied Biosystems) in a Model 373A DNA Sequencer from the same source. Primer P7 (see Fig. 2c) was used in the sequencing reactions.

*Degradation of M13 DNA in blood or in fecal suspensions.* M13 DNA (1 µg) was incubated at 37° C in freshly drawn blood or in a fecal suspension in TE. At various times after starting incubation, DNA samples were prepared, purified (see above), and analyzed for the presence of M13 DNA sequences by the dot blot hybridization procedure.

## Results

### *Questions investigated and experimental approach*

DNA is a stable molecule that can survive extreme environmental conditions and has persisted in organismic remnants for thousands of years (Pääbo et al. 1988). Can DNA also withstand the enzymatic machinery of the GI tract in mammals? The answer to this question has considerable implications, since the digestive tract of all organisms is constantly exposed to many types of foreign DNA often coated with proteins. The epithelial lining of the GI system could thus represent the primary portal of entry for DNA molecules, provided they survived in the GI tract at least in the form of fragments of gene size. To what extent can such foreign DNA be recovered from compartments beyond the GI epithelia, e.g., from the blood circulation of mammals?

In a direct experimental approach to at least some of these problems, male or female C57Bl/6 mice of 3 to 6 months of age, in whose excrements phage M13 DNA had previously never been detected, were pipette-fed microgram amounts of M13mp18 DNA in microliter volumes. In some of the experiments, M13 DNA was added to the food pellets for mice. At various times after the administration of free or food-pellet-associated foreign DNA, the fecal excretions of these animals were examined for the appearance of M13 DNA. Both Southern and dot blot hybridizations, as well as PCR methods yielded positive results. In further work, we also investigated DNA, extracted from whole blood or from blood cell pellets from animals fed with M13 DNA or from negative control animals that had never received M13 DNA, for the presence of M13mp18 sequences. DNA from blood was extracted by conventional methods, and the presence of M13 DNA was investigated by the dot blot or the PCR technique.

Double-stranded circular or *Eco*RI-linearized M13mp18 DNA was chosen for these experiments because we had never found even weak homologies to DNA extracted from the feces of mice which had not been fed with this DNA. Double-stranded DNA was used to simulate the conformation of DNA most likely to be encountered in food remnants.

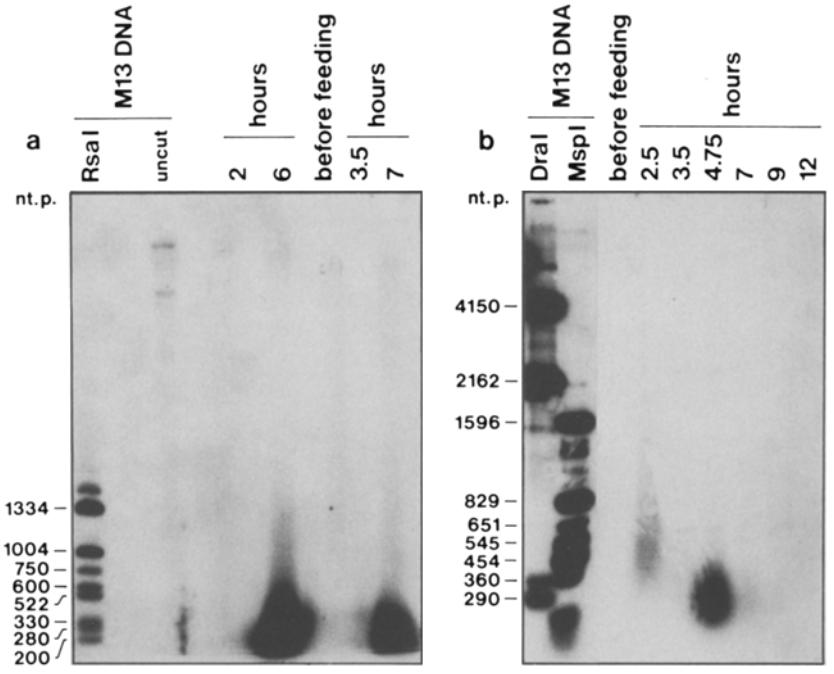


**Fig. 1a, b.** Analysis of transiently detectable M13 DNA in the feces of mice by agarose gel electrophoresis and Southern blotting. Mice were fed 50 μg of phage M13 DNA. At the times after feeding indicated above the autoradiograms, DNA was extracted from the feces of the animals and electrophoresed on a 1.0% agarose gel. As size markers intact and *Rsa*I-cut M13 DNA (**a**) or *Dra*I- or *Msp*I-cut M13 DNA (**b**) were coelectrophoresed. After electrophoresis, DNA fragments were transferred by the alkaline blotting method to Hybond $N^+$ nylon membranes. M13 DNA fragments were visualized by hybridization to $^{32}$P-labeled M13 DNA followed by autoradiography. Panels **a** and **b** show the results of two independently performed experiments using the same procedures

*Detection of M13 DNA in the feces of mice previously fed with this DNA*

The results of Southern blot hybridization analyses of DNA from fecal samples collected from mice 1 to 7 h post feeding, revealed the presence of M13 DNA fragments between <200 and about 1500 bp in length (Fig. 1a, b). At 24 h after feeding, M13 DNA was no longer found. The coelectrophoresed marker DNA fragments provided the scale for estimates of fragment sizes. It was apparent that between 1 and 7 h after feeding M13 DNA, this foreign DNA was excreted in the feces. At later times after feeding, M13 DNA could no longer be detected in the animals' excretions by this method. There was no evidence for long-term survival of uncleaved M13mp18 DNA in the intestines of the animals. In some of these experiments, M13mp18 DNA previously linearized by cleavage with *Eco*RI was fed to the animals. The results of the analyses were similar to those shown in Fig. 1.

These results suggested that unprotected double-stranded, circular or linear M13 DMA fed to adult mice can withstand degradation in the GI tract and could be detected in the feces between 1 and 7 h after feeding, although in considerably fragmented form. Similar results were obtained when 50 μg (Fig. 1) or 10 μg (data not shown) of M13 DNA were fed to mice. The time course of appearance and disappearance of M13 DNA precluded the possibility that *Escherichia coli* cells in the gut had become colonized by phage M13 DNA. Moreover, there was no trace of M13 DNA in the feces of the experimental animals prior to feeding.

*Bacteria in the gut of rodents fed with M13 DNA are not colonized by this phage*

Bacteria from feces isolated 4, 24, or 48 h after feeding M13 DNA to mice or from animals that had never received M13 DNA were spread on agar plates and allowed to form colonies at 37° C. These colonies were then screened for the presence of M13 DNA by hybridization to $^{32}$P-labeled phage DNA (Grunstein and Hogness 1975). Positive colonies were never detected in bacterial colonies isolated from control or experimental animals. These negative controls (not shown) demonstrated that, over the course of these experiments, the GI tract of mice did not become detectably colonized by M13 phage. The results did not preclude the possibility that some of the bacteria might have taken up fragments of M13 DNA that did not replicate in the bacteria that formed colonies. However, there is no evidence that bacterial cells isolated from the mice before or after feeding M13 DNA carried this phage or its genome in detectable amounts.

*Amplification of M13mp18 DNA fragments in the feces by PCR*

The application of the PCR method to the detection of M13 DNA in the feces of mice that had previously been fed this DNA provided an alternative means to assess the survival of this DNA in the GI tract. By choosing a primer oligodeoxyribonucleotide pair, P1 and P2, that was located 712 nucleotides apart on the M13 DNA molecule (map in Fig. 2c), a postprandial time course for the survival of M13 DNA was determined. The data in Fig. 2a demonstrated the absence of even trace amounts of M13 DNA in the GI tract of mice before feeding. Between 1.5 h and 6 h after feeding M13 DNA, fragments of the length to be expected for the chosen

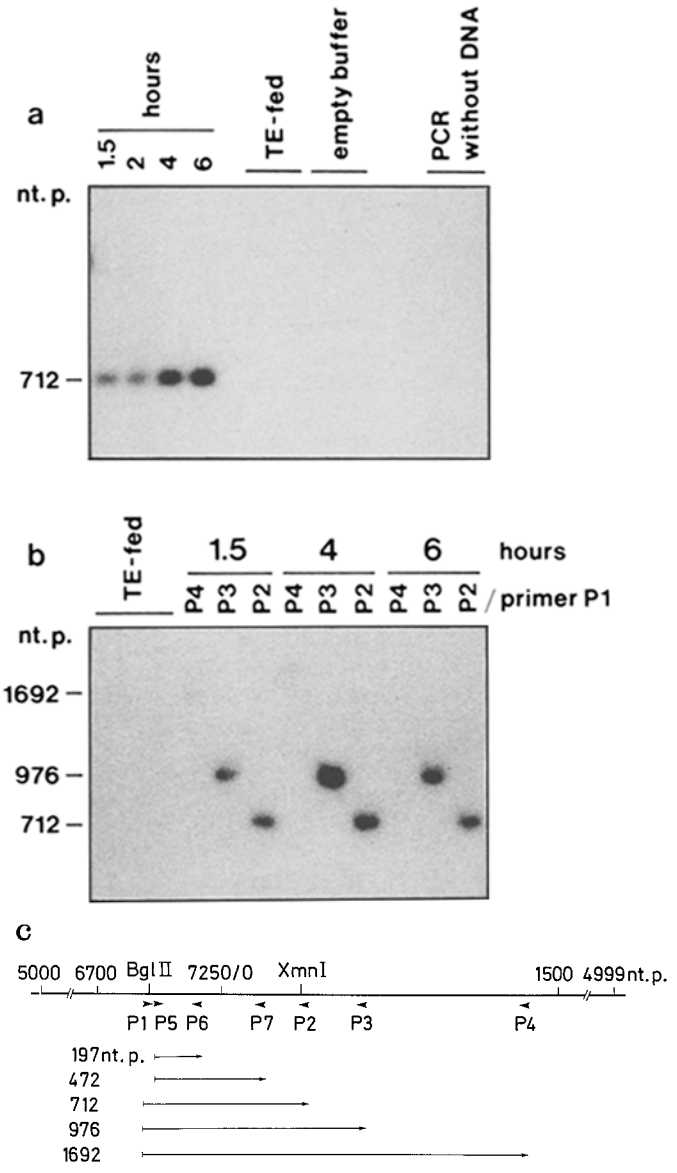


**Fig. 2a–c.** Detection of M13 DNA in the feces of mice by the PCR method. Panel **a**: Animals were fed 50 µg of M13 DNA or TE. At various time intervals after feeding, as indicated, DNA was purified from the feces, was RNase treated and used as substrate for PCR reactions with primer pair P1/P2 (see M13 map in Panel **c**). DNA fragments synthesized were separated by electrophoresis on a 1.0% agarose gel and visualized by hybridization to $^{32}$P-labeled M13 DNA followed by autoradiography. Samples from TE-fed control animals that had never received M13 DNA were identically treated. Negative controls, PCR assays without DNA substrate or buffer controls, were also performed. M13 PCR products were coelectrophoresed as size markers and their lengths were indicated on the left margin of each autoradiogram. Panel **b**: Experimental conditions were similar to those described in the legend to **a**, except that the locations of the primers used for PCR were 712 bp (primers P1/P2), 976 bp (primers P1/P3) and 1692 bp (primers P1/P4) apart (see M13 map in **c**). Panel **c**: The M13 DNA map (linearized between positions 5000 and 4999) indicates the locations of all the oligodeoxyribonucleotide primers (P1 to P7) used in the PCR amplification experiments in this report (see Figs. 2 and 4). Nucleotide positions refer to the published nucleotide sequence of M13 DNA (Yanisch-Perron et al. 1985). P1, 6912 to 6941; P2, 347 to 374; P3, 609 to 638; P4, 1329 to 1354; P5, 6955 to 6980; P6, 7125 to 7152; P7, 149 to 177

primers were readily detectable (Fig. 2a). Treatment of DNA isolated from fecal extracts with DNase eliminated all traces of amplification products. The amplification products in the positive reactions also hybridized to authentic $^{32}$P-labeled M13 DNA as shown by Southern blot hybridization.

We next selected primer locations further apart (map in Fig. 2c) to evaluate the maximal length of M13 DNA fragments resistant to GI tract degradation that could still be recovered from the feces. The results of PCR analyses revealed that fragments of 712 bp (primer pair P1/P2), 976 bp (primer pair P1/P3, map in Fig. 2c) and 1692 bp (primer pair P1/P4, map in Fig. 2c) could be detected (Fig. 2b).

Other data (not shown) indicated that even after feeding as little as 10 µg of M13mp18 DNA, M13 DNA-specific signals could be elicited on Southern blots of DNA extracted from feces 4–6 h post feeding and amplified by PCR.

*Dot blot hybridization technique*

We also applied the dot blot hybridization technique to detect M13 DNA in the feces of mice previously fed

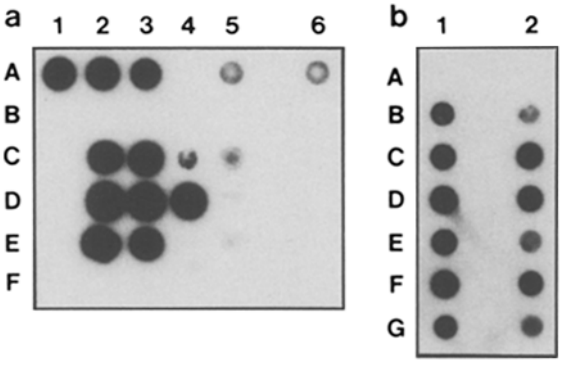


**Fig. 3a, b.** Transient survival of M13 DNA in the feces of mice as demonstrated by the dot blot hybridization technique. Panel **a**: In row A of this dot blot hybridization experiment, 500 (1), 200 (2), 100 (3), no DNA (4), 50 (5), no DNA (6) or 25 ng of M13 DNA was applied to the Hybond N$^+$ membrane. In row B, no DNA was added. In rows C, D and E, DNA extracted from the feces of three different animals was used. Each of these animals had received 50 µg of M13 DNA. In column 1 DNA from feces of the animals prior to feeding was applied. In columns 2 to 6, DNA was used that had been extracted from feces isolated 1, 2.5, 4, 7 or 24 h after feeding, respectively. In row F, fecal DNA samples were used from an animal that had never received M13 DNA. The dot blot procedure was described in Materials and methods. The DNA on the membrane was hybridized to $^{32}$P-labeled M13 DNA. On the dot blot, about one-tenth of the DNA isolated from fecal samples was fixed to the membrane. The maximal signal intensities corresponded to amounts of DNA of about 100 to 200 ng, i.e. about 1 to 2 µg of M13 DNA was present in the total DNA sample isolated from feces. Thus about 2 to 4% of the administered M13 DNA (50 µg) was detected in the feces of mice. Panel **b**: A similar experiment as described in **a** was performed and the mouse was fed 50 µg of M13 DNA. In row A, DNA from the feces of an animal before feeding M13 DNA was used. In rows B to G, DNA was applied that had been extracted from feces collected at 1, 2, 3, 4, 6 and 7 h after feeding, respectively. In column 1, 10%, in column 2, 5% of the DNA extracted at each time point was used

with M13 DNA. The results presented in Fig. 3a, b demonstrated that M13 DNA could be readily detected by this method between 1 and 7 h after feeding. The feces of mice that had not been fed M13 DNA or received TE in mock-feeding experiments were free of M13 DNA (Fig. 3a, b). In Fig. 3a, b, the results of two independent experiments were presented.

We also attempted to quantitate the proportion of the orally administered DNA that survived in the fecal excretions. The results of a reconstitution experiment suggested that of the order of 2 to 4% of the DNA originally administered could be recovered from the feces (for quantitation, see legend to Fig. 3a).

In some of the experiments, 50 µg of M13 DNA was added to the food pellets provided to the mice. Obviously, under this feeding regime, the exact time of ingestion of M13 DNA could not be determined. We hence recorded the times of feces collection after offering the M13 DNA-treated food pellets. By using the dot blot hybridization method, M13 DNA could be detected in the feces of mice at 2, 4.5, 6, and 8 h after presenting the food pellets imbibed with M13 DNA, but not 24 h after pellet exposure (data not shown).

We conclude that the dot blot hybridization procedure allows detection of M13 DNA in the feces of mice fed M13 DNA directly by pipette or administered in food pellets. Fecal excretions are M13 DNA positive between 1 and 7 h, with maximal amounts being apparent from 1 to 4 h after feeding. At later times, M13 DNA can no longer be detected.

*Recovery of M13 DNA from whole blood, blood cells or serum of mice*

A logical extension of the results so far presented was the search for M13 DNA in compartments beyond the GI tract in mice. DNA was extracted from whole blood, from pelleted blood cells or from the serum of animals at various times after feeding 50 µg of M13 DNA to mice. In some experiments, 30 µg or 10 µg of M13 DNA were fed. In the blood of animals that had not received phage DNA, M13 DNA could never be detected by dot blot hybridization or by PCR. In animals that had been fed 50 µg of M13 DNA, M13 DNA fragments of 197 bp (primer pair P5/P6, see map in Fig. 2) could be amplified from whole blood DNA at 2 and 4 h after feeding (Fig. 4). In DNA isolated from animals that had received TE, instead of M13 DNA, M13 DNA was not detectable (Fig. 4). The amplification reaction was also negative in the absence of any template DNA (Fig. 4). In experiments not shown, M13 DNA fragments of 472 bp could be amplified from DNA extracted from whole blood using primer pair P5/P7.

In further experiments, we routinely used the dot blot hybridization method to demonstrate the presence of M13 DNA in the blood of animals previously fed with 50, 30, or 10 µg of M13 DNA. All DNA samples analyzed were first treated with RNase, and equal amounts of total DNA (about 4 µg) were applied to each position

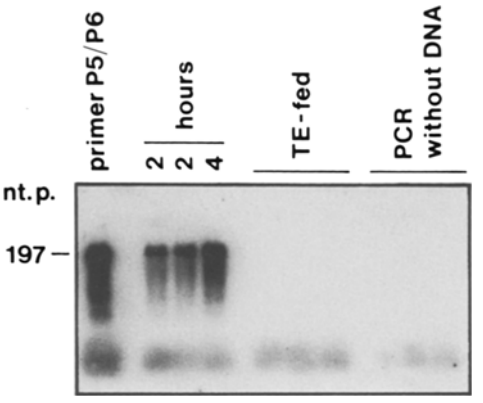


**Fig. 4.** Detection by PCR of M13 DNA in total blood of mice that had received 50 µg of orally administered M13 DNA. At various time intervals after feeding as indicated, about 1 ml of blood was collected by heart puncture from various animals. DNA was extracted from total blood as described under Materials and methods. By using the primer pair P5/P6 (see M13 map in Fig. 2), DNA was amplified by PCR as described under Materials and methods. The amplification products were detected by electrophoresis on a 1.0% agarose gel, Southern blotting to a Hybond $N^+$ membrane and hybridization to $^{32}$P-labeled M13 DNA followed by autoradiography. In parallel, DNA samples from animals that had received TE instead of M13 DNA were analyzed in the same way. No amplification products were detected. PCR amplification did not proceed in the absence of any DNA template (PCR control). PCR products of M13 DNA were co-electrophoresed as DNA size markers

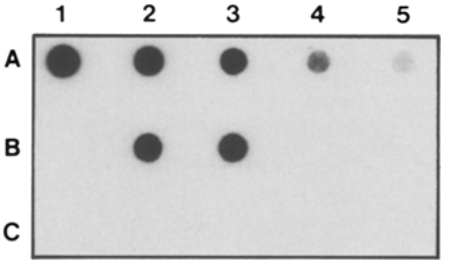


**Fig. 5.** Dot blot hybridization analyses on DNA isolated from total blood of mice that had received 50 µg of DNA. In row A, the results of a reconstitution experiment were shown in which amounts of 20 (column 1), 10 (2), 5 (3), 2 (4), or 1 ng (5) of M13 DNA were fixed to the Hybond $N^+$ nylon membrane. In row B, DNA from total blood of mice that had received TE or 50 µg of M13 DNA was used. In the latter set of experiments, DNA from total blood of mice fed with TE without DNA (column 1) or of blood collected at 2 h (2), 4 h (3), and 24 h (4) after feeding was dotted onto the membrane. In row C, portions of the same DNA samples were treated with DNase (10 µg of DNase per ml for 2 h at 37° C) prior to application to the membrane. The DNA on the membrane was hybridized to $^{32}$P-labeled M13 DNA. Typically 1 ml of blood was obtained from the animals and the DNA was extracted. The signals shown correspond to DNA from 0.25 ml of blood and are equivalent to about 10 ng of M13 DNA. Since 50 µg of DNA were orally fed to the mice, about $10^{-3}$ to $10^{-4}$ (0.1 to 0.01%) of the input was recovered from the blood

on the filters used in the dot blot hybridization experiments. The results presented in Fig. 5 revealed that M13 DNA was present in the blood (cells and serum) of mice between 2 h and 4 h after feeding. In one experiment, M13 DNA was found in the blood of a M13 DNA-fed

mouse 1.5 h after feeding (data not shown). M13 DNA was not detectable in mock-fed animals that had received TE devoid of M13 DNA (Fig. 5) or in the DNA from blood of M13 DNA-fed animals at 24 h after feeding. In a separate experiment, M13 DNA could no longer be found in blood at 8 h after feeding (data not shown). DNase treatment of the DNA samples prior to blotting eliminated the hybridization signals (Fig. 5). M13 DNA extracted from blood could also be identified by Southern blot hybridization (data not shown).

A reconstitution series of hybridization experiments was performed with the amounts of 1 to 20 ng of M13 DNA per slot, in order to estimate the amounts of M13 DNA present in 0.25 ml of blood between 2 h and 4 h after feeding mice 50 µg of M13 DNA. The results shown in Fig. 5 suggested that a fraction of about 0.01% to 0.1% of the 50 µg of DNA originally fed could be recovered from 1 ml of blood. This estimate represented an order of magnitude approximation since only a fraction of the animal's total blood supply could be retrieved by heart puncture.

From the experiments carried out so far, there was no evidence that differences in the sex of the animals or their age within the 3- to 6-month range affected the outcome of any of the experiments reported (see Tables 1, 2).

*Nucleotide sequence determination proves that M13 DNA is recoverable from feces or blood by PCR*

The nucleotide sequence of the PCR product amplified from feces or blood of animals that had been fed with M13 DNA 2 h (blood) or 4 h (feces) previously, was determined. The primer P7 was used in the reaction (Fig. 2c). Over a stretch of about 400 nucleotides, the sequences were found to be identical to the nucleotide sequence of authentic M13 DNA as published elsewhere (Yanisch-Perron et al. 1985). Occasionally, in some of the sequence determinations, a few nucleotide variations were observed. However, these were randomly distributed over the genome in repeated determinations. We therefore surmise that these rare variations are due to infidelities in the PCR reaction.

*Time course of M13 DNA degradation in blood or in fecal suspensions*

M13 DNA was incubated at 37° C with blood or with fecal suspensions, and the persistence of M13 DNA was followed by dot blot hybridization after reextraction of the DNA after various times of incubation (Fig. 6). In blood, 6 h of incubation eliminated all M13 DNA. After 8 h of incubation of M13 DNA with fecal suspensions, about 5% of the phage DNA could still be detected. At later times (10 h), not even traces of M13 DNA could be found. These data are consistent with the results of the experiments in which living animals were fed M13 DNA.

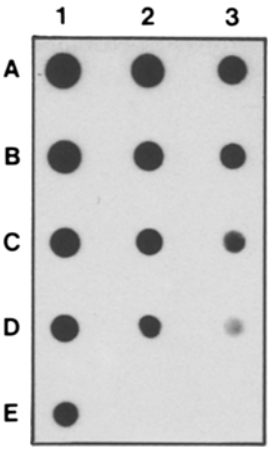


**Fig. 6.** Time course of degradation of M13 DNA incubated with fecal suspensions or in mouse whole blood. Dot blot analyses of M13mp18 DNA (column 1), with fecal suspensions (2) or in blood (3) of mice are shown. Experimental conditions are described in the text. Column 1: 1 µg (row A), 500 ng (B), 200 ng (C), 100 ng (D) or 50 ng (E) of M13mp18 DNA were applied to the membrane in this reconstitution experiment. Column 2: 1 µg of M13mp18 DNA was incubated at 37° C in 100 µl of fecal suspension (200 mg of feces from untreated mice in 2 ml TE) and reextracted 1 h (row A), 2 h (B), 3 h (C), 8 h (D) or 10 h (E) later. Column 3: One microgram of M13mp18 DNA was incubated at 37° C in 100 µl of freshly drawn mouse blood and reextracted 1 h (row A), 2 h (B), 3 h (C), 4 h (D), or 6 h (E) later. Dot blot, hybridization, and wash conditions were as described in the text or in the legends to Fig. 3 or 5

## Discussion

*Conceptual background for experiments*

The organ system most likely to take up foreign DNA is the GI tract, which is constantly exposed to the foreign DNA ingested with nutrients. We have directly tested this possibility by feeding a readily available, well characterized foreign DNA, i.e. circular or linearized phage M13 DNA, either as free DNA or applied to food pellets, to mice in whose feces this DNA was never detected prior to feeding. The feces were then tested for the presence of M13 DNA, and these investigations were extended to the blood of the treated animals. We presume that M13 DNA behaves similarly to many other types of DNA in the GI tract of mice but have not yet investigated this question directly.

*Major findings*

In Tables 1 and 2 all feeding experiments and fecal and blood analyses performed over the past 5 years in our laboratory are listed. Foreign M13 DNA administered to the oral cavity of mice can be detected in the form of fragments of maximally 1692 bp, i.e. about 23% of the total length (7250 bp) of the M13 genome, in the feces of animals between 1 and 7 h after feeding. The majority of fragments of M13 DNA is found in the size range between $<200$ to 400 bp. The time course of appearance and disappearance of M13 DNA in the feces

**Table 1.** Transient survival of orally administered M13 DNA in the feces of mice

| Experiment No. | No. of animals | Sex | Amount of DNA fed | Type of test | Time of test[b] | Results |
|---|---|---|---|---|---|---|
| 1 | 1 | | 50 µg | S | 2, 4, 8 | +Smear[c] |
| 2 | 1 | | 100 µg | S | 2.5 | +Smear[c] |
| 3 | 1 | | 50 µg | S | 6 | +Smear[c] |
| 4 | 1 | | 100 µg | S | 4+8 | +Smear[c] |
| 5 | 1 | | 50 µg | S | 3.75 | +Smear[c] |
| 6 | 1 | | 80 µg | S | 12.5 | +Smear[c] |
| 7 | 2 | | 2×100 µg | S | | − |
| 8 | 1 | | 50 µg | S | 7 | +Smear[c] |
| 9 | 1 | F | 50 µg | S | 4–24 | +Smear[c] Fig. 1a |
| 10 | 3 | F | 2×50 µg | S | 4 | +Smear[c] |
| | | | 1×10 µg | S | 4+6 | +Smear[c] |
| | 1 | F | TE | S | | − |
| 11 | 2 | F | 2×50 µg | S | 2–7 | +Smear[c] Fig. 1b |
| | 1 | F | TE | S | | − |
| 12 | 3 | M | 3×50 µg | PCR | | − |
| 13 | 3 | F | 3×50 µg | PCR | 2–6 | + |
| | 1 | F | TE | PCR | 2–6 | − |
| 14 | 3 | M | 50 µg | PCR | 2–6 | + |
| | | | 30 µg | PCR | 2–6 | + |
| | | | 10 µg | PCR | 2–6 | + |
| | 1 | M | TE | PCR | 2–6 | − |
| 15 | 3 | F | 30 µg | PCR | 2–8 | + |
| | | | 20 µg | PCR | 2–8 | + |
| | | | 10 µg | PCR | 2–8 | + |
| | 1 | F | TE | PCR | 2–8 | − |
| 16 | 5 | M | 1×50 µg | PCR | 1.5–6 | + Fig. 2 |
| | | | 2×30 µg | PCR | 1.5–6 | + |
| | | | 2×10 µg | PCR | 1.5–6 | + |
| | 3 | M | TE | PCR | 1.5–6 | − |
| 17 | 4 | M | 4×50 µg | D | 1.5–7 | + Fig. 3a |
| | 1 | M | 50 µg | D | 1.5–7 | + Fig. 3b |
| | 3 | M | TE | D | 1.5–7 | − |
| 18 | 3 | M | 2×50 µg | D | 2–8 | + |
| | | | | | after 24 | − |
| | | | 1×50 µg[a] | D | 2–8 | + |
| | | | | | after 24 | − |
| | 2 | M | TE | D | 2–24 | − |
| Total | 40 mice fed with M13 DNA; 13 TE controls | | | | | |

These experiments were carried out between 1988 and 1993
[a] This sample was added to food pellets, in all other cases DNA was administered by pipette feeding. [b] Time after feeding (in h). [c] DNA with a length from <200 to 2000 bp hybridized with M13 DNA as a smear. In dot blot hybridization experiments (D) +indicated hybridization to $^{32}$P-labeled M13 DNA. In PCR experiments, the specific amplified band of expected size also hybridized to M13 DNA
F, female; M, male; S, Southern blot analysis; PCR, PCR analysis; D, Dot blot analysis

and in the blood of animals reflects the stability of M13 DNA on incubation with blood or fecal suspension (Fig. 6). The finding that only fragmented forms of M13 DNA can be recovered from the GI tract, the very distinct time course of fecal excretion of M13 DNA, and the absence of M13 DNA-carrying bacteria in the GI tract of DNA-fed animals argue conclusively against the possibility that the murine GI tract is colonized to a detectable extent by phage M13.

The observation that the foreign DNA fed to mice can be retrieved from their bloodstream has challenging implications. To what extent can this DNA be taken up by cells in the different organ systems? Does foreign DNA resorbed from the gut contribute to mutagenesis and oncogenesis?

Results obtained with the highly sensitive PCR method have to be critically evaluated for the possibility of unintended, haphazardly occurring contaminations with minute traces of the experimental DNA. The negative results of numerous control experiments with DNA samples from the feces or blood from untreated animals or from animals at late times after feeding reassure us that such contaminations can be ruled out. These control experiments have yielded uniformly negative results, although they have been performed in parallel and simultaneously with the positive experiments. Moreover, the transient survival of M13 DNA in feces has been documented by two additional and independent methods – Southern and dot blot hybridizations – which have yielded the same positive results (Figs. 3 and 5). In

**Table 2.** M13 DNA retrieved from blood after oral administration

| Experiment No. | No. of animals | Sex | Amount of DNA fed | Type of test | Time of test | Results |
|---|---|---|---|---|---|---|
| 1 | 5 | M | 1 × 50 µg | PCR | 6 | + |
| | | | 2 × 30 µg | PCR | 6 | + |
| | | | 2 × 10 µg | PCR | 6 | + |
| | 3 | M | TE | PCR | 6 | − |
| 2 | 4 | M | 50 µg | D | 2–4 | + |
| | | | | | 24 | − |
| | 3 | M | TE | D | 4, 6, 24 | − |
| 3 | 5 | F | 30 µg | PCR | 2 | + |
| | | | | | 4–6 | − |
| | 2 | F | TE | PCR | 4, 6 | − |
| 4 | 5 | M | 50 µg | D | 2, 4 | + |
| | | | | | 24 | − |
| | 1 | M | TE | D | 2 | − |
| 5 | 7 | F | 50 µg | D | 2, 4, 6 | + |
| | | | | | 24 | − |
| | 1 | F | TE | D | 2 | − |
| 6 | 4 | F | 50 µg | D | 2, 4 | + |
| | | | | | 1, 8 | − |
| | 1 | F | TE | D | 2 | − |
| 7 | 8 | M | 50 µg | D | 1.5, 2.5, 5 | + |
| | | | | | 24 | − |
| | 3 | M | TE | D | 1.5, 2.5, 5 | − |
| 8 | 3 | F | 50 µg | D | 2 | + |
| | | | | | 1, 24 | − |
| | 3 | F | TE | D | 1, 2, 24 | − |
| 9 | 6 | M | 50 µg | D | 3, 6 | + |
| 10 | 6 | M | 2 × 50 µg | D | 4 | + |
| | | | | | 1.3 | − |
| | | | 2 × 30 µg | D | 4 | + |
| | | | | | 1.3 | − |
| | | | 2 × 10 µg | D | 4 | + |
| | | | | | 1.3 | − |
| | 2 | M | TE | D | 1.3, 4 | − |
| 11 | 2 | F | 50 µg | PCR | 2, 4 | + Fig. 4 |
| 12 | 4 | F | 50 µg | D | 2, 4 | + Fig. 5 |
| | | | | | 24 | − |
| | 1 | F | TE | D | 2 | − |

Totals: 59 mice fed with M13 DNA; 20 TE controls

In all experiments the M13 DNA was pipette-fed. Abbreviations and symbols are as used in Table 1

blood, M13 DNA has also been found by the dot blot technique. Moreover, the PCR products from blood DNA have been unequivocally identified as M13 DNA by direct sequence determination.

*Quantitative evaluation*

The amounts of M13 DNA administered to mice were in the range between 10 to 50 µg. These values must be related to the body weight of mice, (between 10 and 20 g) and thus constitute an approximate fraction of $1/10^6$ of body weight. For humans of average weights between 50 and 80 kg, one would thus have to postulate an intake of about 50 to 80 mg of DNA with the daily food uptake to parallel the situation simulated in the mouse experiments. These assumptions appear reasonable, in particular for nutrients derived from parenchymatous organs rich in cells of animal or plant origin.

How stable is foreign (phage M13) DNA in the organism and can it enter into cells and become integrated into the host's genome? In this context, many questions remain to be investigated, none of which will be technically easy to control. Our data are consistent with the notion that a steady flow of foreign genetic material persists in the GI tract, and some of it can enter the circulation. The present findings must be reproduced with other types of DNA, and these experiments have been initiated.

The implication that a random mixture of DNA including gene fragments or intact genes of animal, plant or microbial origin should have been constantly excreted by innumerable organisms over millenia does not appear startling given the of complexities of evolution. This barrage of linear DNA fragments, i.e. of recombinationally highly active DNA fragments in Nature should mitigate any concerns that one might have had in the past about the biological consequences of experiments carried out

with recombinant DNA over the course of the past two decennia.

*Acknowledgements.* We are indebted to Petra Böhm for expert editorial work, to Irmgard Hölker for the synthesis of oligodeoxyribonucleotides, to Birgit Schmitz for DNA sequencing, and to Monika Arndt for animal care. This research was supported by the Deutsche Forschungsgemeinschaft through SFB274-TP1 and by a grant from the Fritz Thyssen Foundation, Köln.

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### Notes added in proof

From at least 10 different animals sacrificed for blood analyses, feces were taken directly from the animal's rectum. DNA was extracted from these fecal preparations which had not been excreted extracorporeally. In all samples, M13 DNA was detected by dot blot hybridization and by PCR as described. These results exclude the remote possibility that extracorporeal fecal samples might have been contaminated by oral contacts with the animals.

The total number of M13-DNA fed animals analyzed amounts now to 50 for fecal samples plus 16 TE controls, and to 105 for blood samples plus 30 TE controls. The results were as described in the text.

The maximal fragment length of M13 DNA detected by PCR in DNA isolated from blood of M13 DNA-fed mice is now 976 bp.