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Fate of polyoma origin of replication after its direct introduction into mice

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Recently we have developed a method for direct introduction of calcium phosphate-precipitated DNA into newborn rats. To examine whether the foreign DNA can replicate, a plasmid containing a polyoma origin of replication was injected into newborn mice. The plasmid was found intact in liver and spleen and able to transform bacteria. The foreign DNA had disappeared by the seventh day after injection. Yet, the plasmid DNA containing the polyoma origin of replication had undergone replication in both the liver and the spleen.

Transfection; Replication; Polyoma

1. INTRODUCTION

DNA-mediated gene transfer is an important tool for studying the function of its different parts. Recently, we have published a method for direct introduction of genes into animals [1]. Thus, DNA (precipitated in calcium phosphate) has been injected into the peritoneum of newborn rats. The injected plasmids, containing eukaryotic genes, have been taken up by the tissues, predominantly the liver and spleen, where the foreign genes have been transcribed and translated [1].

In the present study we have examined whether DNA, introduced into animals by the above mentioned method, can replicate when it includes an origin of replication. If this is true, the method may enable analysis, in whole animal, of DNA origin of replication. We have used the well defined polyoma origin of replication as a model system, since it has been characterized both in cells in culture and in animal tissues. Introduction by injection of polyoma virus into animals [2–4] results in tissue-specific infection and replication of the virus [4]. Alternatively, microinjection of a recombinant plasmid, containing the polyoma origin of replication into mouse fertilized eggs, led to the persistence of the DNA in tissues of the transgenic mice [5]. When the polyoma-containing plasmid DNA was injected directly into animal tissues, it replicated yet underwent reorganization [3]. We now report that a plasmid containing a polyoma origin of replication stays intact and episomal in animal tissues following its injection into the peritoneum. The plasmid DNA persists in the animal tissues for a limited time, yet replication associated events can be detected.

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2. EXPERIMENTAL

2.1. *Animals*

10-day-old Sabra mice were from the Hebrew University breeding centre.

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2.2. *Preparation of calcium phosphate-precipitated DNA and its injection in vivo*

Supercoiled plasmid DNA (30 μg), dissolved in 0.5 ml of 125 mM $CaCl_2$, was added to 0.5 ml of 2× Hepes-buffered saline (280 mM NaCl/1.5 mM Na_2HPO_4/42 mM Hepes, pH 7.1) with continuous air bubbling [6]. After 30 min at room temperature the fine precipitate was collected by 2-min centrifugation at 12000 × *g*. The precipitate was gently dispersed in a small volume of saline and injected intraperitoneally [1].

2.3. *Genomic DNA preparation, Southern and dot-blot hybridization analyses of the DNA*

DNA from mouse liver and spleen was extracted according to Hewish and Burgoyne [7]. Southern blot analysis, utilizing GeneScreen Plus membranes, was performed as described in [8,9]. Prehybridization and hybridization were carried out under conditions recommended by the supplier, using appropriate DNA probes ^{32}P-labeled by nick-translation [10].

2.4. *Bacterial strains, DNA transformation and plasmid extraction*

E. coli bacterial strains used were HB101 (dam^+) and F42/GM48 (dam^-). DNA transformation and plasmid extraction were performed as described in [11].

3. RESULTS AND DISCUSSION

We have previously shown that when DNA is introduced into newborn rats by a new method developed by us, it is distributed in various tissues and is transiently expressed [1]. In the present study, we have examined whether the introduced DNA can replicate. Since the mouse is permissive for polyoma replication we injected this animal with pSV5neo, a plasmid which contains the polyoma origin of replication [12]. DNA was extracted from liver and spleen of treated animals. Dot blot analysis of this DNA revealed 10–100 plasmid copies per liver cell 2 days after injection (fig.1). Similar amounts were found on the third day post-injection (not shown). Yet, no plasmid DNA was detected on the seventh day after injection (fig.1) (failure to detect plasmid sequences indicates that the amount was smaller than one copy

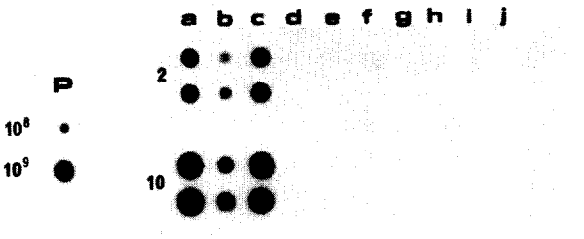

Fig.1. Variation in the copy number of DNA in liver. Plasmid pSV5neo (20 μg), in calcium phosphate precipitates, was injected intraperitoneally into 5-day-old mice (a–c, f–i). Control animals were injected with calcium phosphate alone (d,e,j). Liver DNA extracted after 2 days (a–e) and 7 days (f–j) was analyzed by dot hybridization, using pSV5neo as probe. Hybridization of the plasmid itself at 10^8 and 10^9 molecules per dot is shown in P.

per cell). Southern blot hybridization of DNA extracted from the liver or spleen, demonstrated that the polyoma-containing plasmid was intact on day 2 but undetectable on day 7 after injection (fig.2).

In a previously reported case [3], when polyoma-

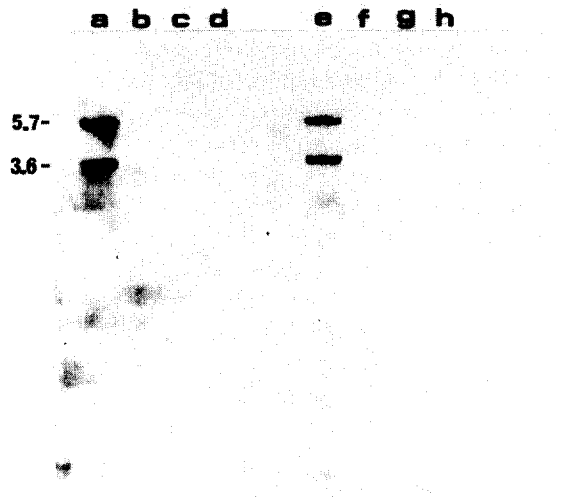

Fig.2. Southern blot analysis of plasmid DNA in liver and spleen. pSV5neo injected as described in fig.1 was extracted from liver (a–d) and spleen (e–h), 2 days (a,e) or 7 days (b–d, f–h) after injection. 30 μg of DNA were cleaved by *Bam*HI, separated by electrophoresis and analyzed by Southern blot hybridization with pSV5neo as probe. The size of the hybridized fragment is given in kb.

containing plasmids have been injected directly into the liver or spleen, the plasmids underwent reorganization excluding prokaryotic sequences. To establish that the DNA injected by our method remained intact, we have rescued it by transforming bacteria with DNA extracted from livers of injected mice. Transformation has been performed using 10 μg total liver DNA. The excess DNA, thus used, resulted in a low yield of bacterial colonies. However, the appearance of colonies resistant to ampicillin was indicative of the insertion of plasmid DNA into the bacterial host. Restriction enzyme analysis has been performed on 4 independent colonies yielding identical restriction pattern. A representative analysis (fig.3) indicates that the transforming plasmid is pSV5neo, i.e. the one which was originally injected into the animals.

Thus the foreign DNA does not undergo a major rearrangement as is evident by its restriction map

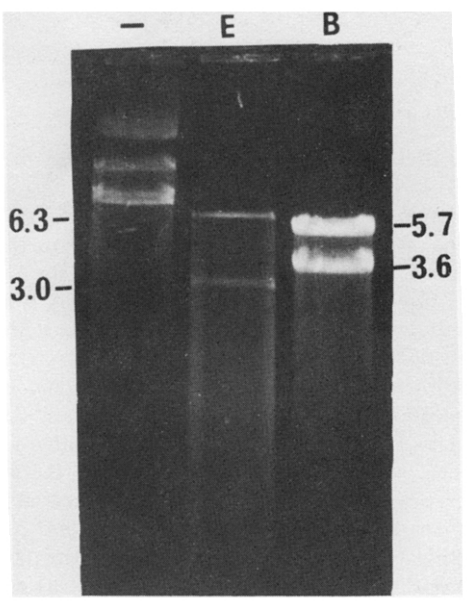

Fig.3. Rescue in bacteria of plasmid extracted from liver. DNA was extracted from the liver of a mouse 2 days after injection of pSV5neo (see fig.2). To rescue the plasmid we have transformed *E. coli* HB101 with 10 μg of liver DNA. Bacterial colonies, resistant to ampicillin, were isolated and their plasmid DNA was extracted and analyzed for size by gel electrophoresis without (–) and with *Eco*RI (E) and *Bam*HI (B) digestion (a representative restriction assay is given). The size of the fragments revealed after ethidium bromide staining is given in kb.

(figs 2 and 3) and is not integrated in the genome since no events of integration were noted in the Southern blot analysis (fig.2), and since it remains supercoiled (as reported before [1]) and could transform bacteria (fig.3). The evidence that the injected plasmid persisted intact and episomal in the animal tissues constitutes a legitimate basis to examine whether the plasmid, which contains a polyoma origin of replication, can replicate in vivo.

Replication can be identified by monitoring changes in the prokaryotic pattern of methylation of plasmid DNA. This method takes advantage of the different methylation sequences in prokaryotes and eukaryotes. Thus, upon replication, in eukaryotic cells, the plasmid loses its prokaryotic methylation pattern [3,13,14]. Consequently, one can take advantage of enzymes which are sensitive to methylation in adenine (methylated only in prokaryotes). Such enzymes are *Dpn*I, which cleaves the sequence GATC only when its adenine is methylated in both strands, and *Mbo*I, which cleaves the same sequence only when the adenine is not methylated. By utilizing these enzymes one can reveal changes in the methylation pattern which in turn will suggest a replication event. To faithfully map the pattern of methylation of pSV5neo, the plasmid was grown in wild type and dam$^-$ (deficient in adenine methylase) strains of *E. coli*. Indeed when the plasmid is methylated at all of its adenine residues in the sequence GATC it is sensitive to *Dpn*I and resistant to *Mbo*I digestion, while the opposite pattern of digestion was obtained with the unmethylated plasmid (fig.4).

To examine whether the prokaryotic methylation pattern of the plasmid has been modified during its persistence in the animal tissues, DNA from liver and spleen were digested by *Dpn*I or *Mbo*I. To ascertain a complete digestion of the DNA, a pSV5neo-derived DNA fragment was mixed, with a small sample of the genomic DNA and digested in parallel. The added fragment was either methylated (digestion with *Dpn*I) or unmethylated (digestion with *Mbo*I). Fluorescent analysis of ethidium bromide stained gel revealed the expected corresponding fragments in each case (fig.5A). Southern blot analysis of the genomic DNA has been performed using the polyoma origin of replication sequence as a probe. Plasmid DNA in the liver, was partially demethylated as shown by

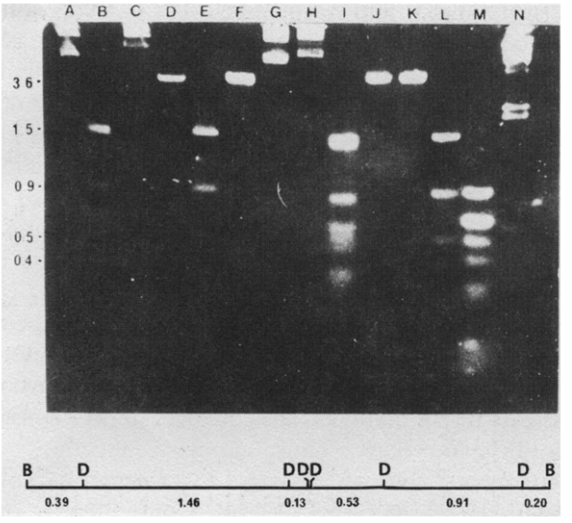

Fig.4. Identification of the methylation pattern of the plasmid pSV5neo. pSV5neo was grown in wild type or GM48 (dam$^-$) *E. coli*. GM48 is deficient in dam enzyme, a methylase of adenine in the sequence GATC. Size separation of plasmids extracted from wild type (A–F) and dam$^-$ (G–L) bacteria, before and after cleavage with methyl-sensitive enzymes, is shown: uncleaved plasmids (A,G) and plasmids cleaved by *Dpn*I (which cleaves the sequence GATC only if adenine is methylated) (B,H) and by *Mbo*I (which cleaves this sequence only when adenine is unmethylated) (C,I). In addition, the isolated 3.6 kb *Bam*HI fragment (containing the polyoma origin of replication) (D,J), cleaved with *Dpn*I (E,K) and *Mbo*I (F,L) is shown. Size markers in kb were obtained by using *Alu*I cleaved pBR322 (M) and *Hin*dIII cleaved λ phage (N). A schematic map of the GATC sites (D) in the 3.6 kb *Bam*HI (B) fragment, containing the polyoma origin of replication [12], is given. The size of each *Dpn*I/*Mbo*I-cleaved fragment in kb is indicated below the scheme.

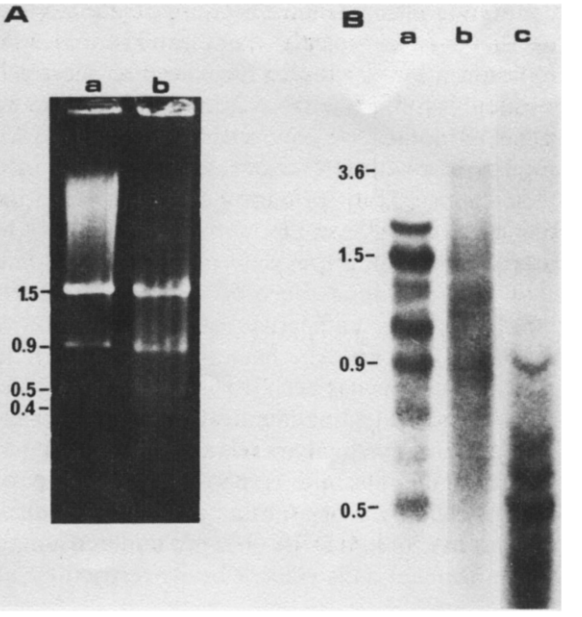

Fig.5. Methylation patterns of the plasmid in mouse tissues. DNA was extracted from the liver and spleen 2 days after injection of methylated pSV5neo (where adenine residues in the sequence GATC are methylated) (see fig.4). To follow changes in the methylation pattern of the injected plasmid, 30 μg of the DNA were first cleaved overnight with *Bam*HI and then with *Dpn*I (which cleaves GATC only when adenine is methylated) or with *Mbo*I (which cleaves only when adenine in the sequence GATC is unmethylated). To assess complete cleavage of genomic DNA by the methyl-sensitive enzymes, we have added to one tenth of the genomic DNA a 3.6 kb *Bam*HI fragment of pSV5neo which was either methylated for digestion with *Dpn*I (Aa) or unmethylated for digestion with *Mbo*I (Ab). Digestion of these samples was complete as revealed by ethidium bromide staining of the plasmid sequences, on a background of genomic DNA (A). Note that the genomic DNA is not digested by *Dpn*I (requiring methylation of the sequence) but is digested by *Mbo*I (as it is not methylated in the GATC sequence). Southern hybridization analysis of genomic DNA from liver (Ba,Bb) and spleen (Bc), digested with *Bam*HI + *Dpn*I (Ba) and with *Bam*HI + *Mbo*I (Bb,c) and probed with a sequence containing the polyoma origin of replication. The size of the fragments is given in kb. As a reference for the expected bands see the *Dpn*I/*Mbo*I restriction map given in fig.4.

the partial digests of both *Dpn*I and *Mbo*I (fig.5B). A more progressive demethylation was revealed in the plasmid DNA isolated from the spleen as indicated by the existence of small sized fragments after digestion with *Mbo*I (fig.5B). The extent of demethylation varied among animals, yet, it seems that demethylation results from replication. Thus, when the plasmid pSV2neo, which is identical to pSV5neo but lacks the polyoma origin of replication [12], has likewise been introduced to animals, no change in the prokaryotic methylation pattern has been observed (not shown). Although the results indicate that the foreign DNA is replicating

its survival time is limited. Thus, degradation of the DNA is apparently more pronounced than replication. Similar results have been implicated in cultured myoblasts [14] and in DNA directly injected into tissues [3]. Nevertheless, as the DNA, which is directly introduced into newborn mice, undergoes replication, it appears that this method may enable identification in vivo of replication events in foreign DNA.

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