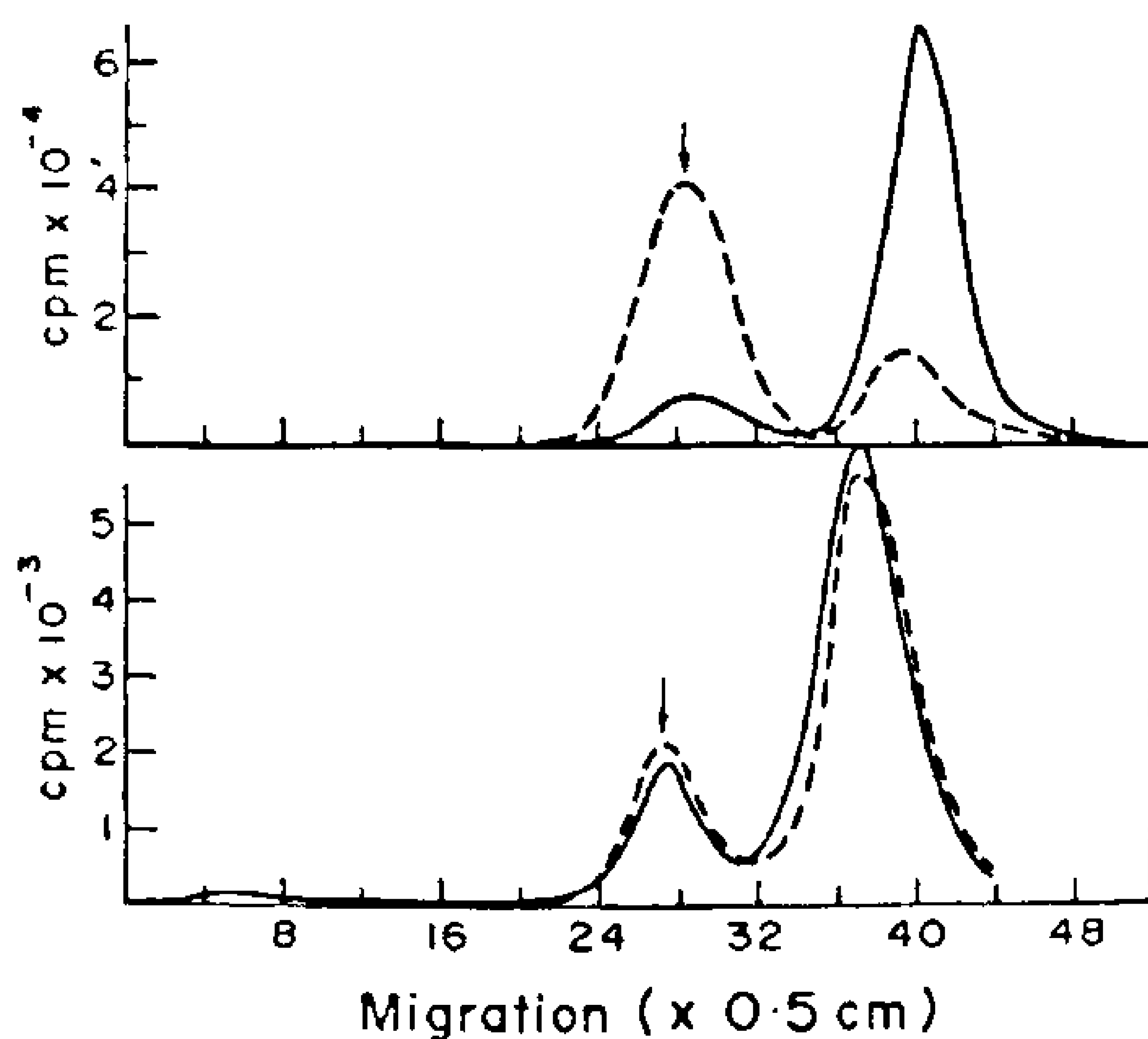

Figure 4. Paper-chromatographic analysis of abortive-initiation reaction for the synthesis of dinucleotide tetraphosphate pppGpC from T7A2 (top) and T7A2* (bottom) promoters in the absence of rifampicin (——) and in the presence of 50 μg ml^{-1} rifampicin (– – –). In both cases, fraction 1 represents origin, and the first peak from the left corresponds to the product and the second peak represents unincorporated nucleotide. The concentration of GTP was 400 μM, [α-^{32}P]CTP was 3 μM (1 μCi/nmol), DNA was 0·4 mM DNA phosphorus, with 0·16 μM RNA polymerase.

product) is the same in the two cases. This was further confirmed by treating the product with alkaline phosphatase and verifying comigration of the dephosphorylated product with commercial GpC on a polyethylenimine plate. Thus it is evident that the mutation in the −10 box did not alter the recognition of the initiation site by RNA polymerase. We have noticed recently[16] that rifampicin, which is a classical inhibitor of initiation of transcription in *E. coli*[17], stimulates the abortive synthesis of dinucleotide tetraphosphate at the T7A2 promoter (Figure 4). It has been observed that the product pppGpC forms a stable ternary complex at A2, resulting in poor turning over of the dinucleotide. However, in the presence of rifampicin, the complex is destabilized, and as a consequence rapid abortive synthesis continues and the product accumulates[16]. The mechanism of this effect will be discussed elsewhere. The interesting point to note here is that, even with T7A2*, rifampicin causes enhancement of the abortive synthesis of GpC, although to a much smaller extent. We have observed this stimulation repeatedly and therefore consider it real and not an experimental artifact. Interestingly, at T7A2* the stability of the ternary complex is lower than that at T7A2. It is this that has resulted (Figure 4, bottom) in the turning over and accumulation of pppGpC to a significantly greater extent with T7A2* that with T7A2.

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Microinjection of rat growth-hormone gene into zebrafish egg and production of transgenic zebrafish

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A plasmid containing rat growth-hormone gene was microinjected into fertilized zebrafish eggs prior to first cleavage; survival of the embryos one day after the injection averaged 46%. Genomic DNA extracted from presumptive transgenic fish was analysed by slot-blot and Southern-blot hybridization using labelled plasmid as probe. Patterns of hybridization indicated genomic, extrachromosomal, as well as mosaic integration. The concentration of persisting extrachromosomal DNA progressively decreased in F_1 and F_2 generations. The growth rate of transgenic fish was higher in F_0 and F_1 generations, but lower in F_2, which may be due to transient expression of extrachromosomal DNA carrying the growth-hormone gene in F_0 and F_1 generations.

GENE-transfer technology has become a powerful tool

for the study of the fate and expression of genes in surrogate hosts. Attempts to produce transgenic fish began in 1985. Since then many investigators claim to have successfully introduced foreign DNA into fish egg[1-5]. Genomic integration of foreign DNA has been achieved in common carp[6] and trout[7]. In Atlantic salmon, the injected DNA sequences were shown to persist extrachromosomally and be transmitted to the next generation[4]. Mosaic integration was found in zebrafish[3] and medaka[2]. Though expression of the injected growth-hormone gene was detected in salmon[4] and trout[7] at different developmental stages, growth enhancement has not been reported. These studies were done in fishes whose generation time is long, and it takes a long time to know whether the injected gene is transmitted to the next generation. To circumvent this problem, we have chosen to work on zebrafish, which has the following advantages: (i) the generation time is short, only 3–4 months; (ii) mature females lay over 400 eggs roughly at weekly intervals; (iii) the eggs are transparent, and hatch within 3 days; and (iv) the eggs can easily be dechorionated, rendering microinjection relatively more effective. The biological effect of mammalian growth-hormone gene in fish has already been demonstrated earlier[1,4]. Here we report microinjection of rat growth-hormone gene into zebrafish egg, and integration of the foreign gene and its transmission to F_1 and F_2 generations.

Methods

Collection of gametes

Ova and milt were collected from adult zebrafish (*Brachydanio rerio*) by gentle abdominal pressure and inseminated artificially. Prior to microinjection, the chorions of the eggs were removed mechanically with fine forceps. Injected and control embryos were incubated in 15% Hank's salt solution[8] for 3 days and thereafter in a recirculating system containing water maintained at 27 ± 1.5°C.

Plasmid DNA

The plasmid pMGH (obtained from R. Palmiter, University of Washington, USA) was used in the present study without any modification[9]. Plasmid pMGH consists of the mouse metallothionein promoter fused to rat growth-hormone gene (Figure 1). Plasmid DNA purified by cesium chloride centrifugation and linearized by digestion with *Eco*RI was used for microinjection.

Microinjection

Microinjection was carried out with the aid of a micromanipulator (Leitz, Germany) and an inverted microscope. The egg was fixed to a holding pipette. The microneedle (tip diameter 5 μm) loaded with DNA (15 ng/μl) was mounted on a manipulator. In all cases, eggs that had not yet reached the first-cleavage stage were used and the desired volume of DNA (200 pl) was injected by adjusting the flow of DNA by hydraulic pressure.

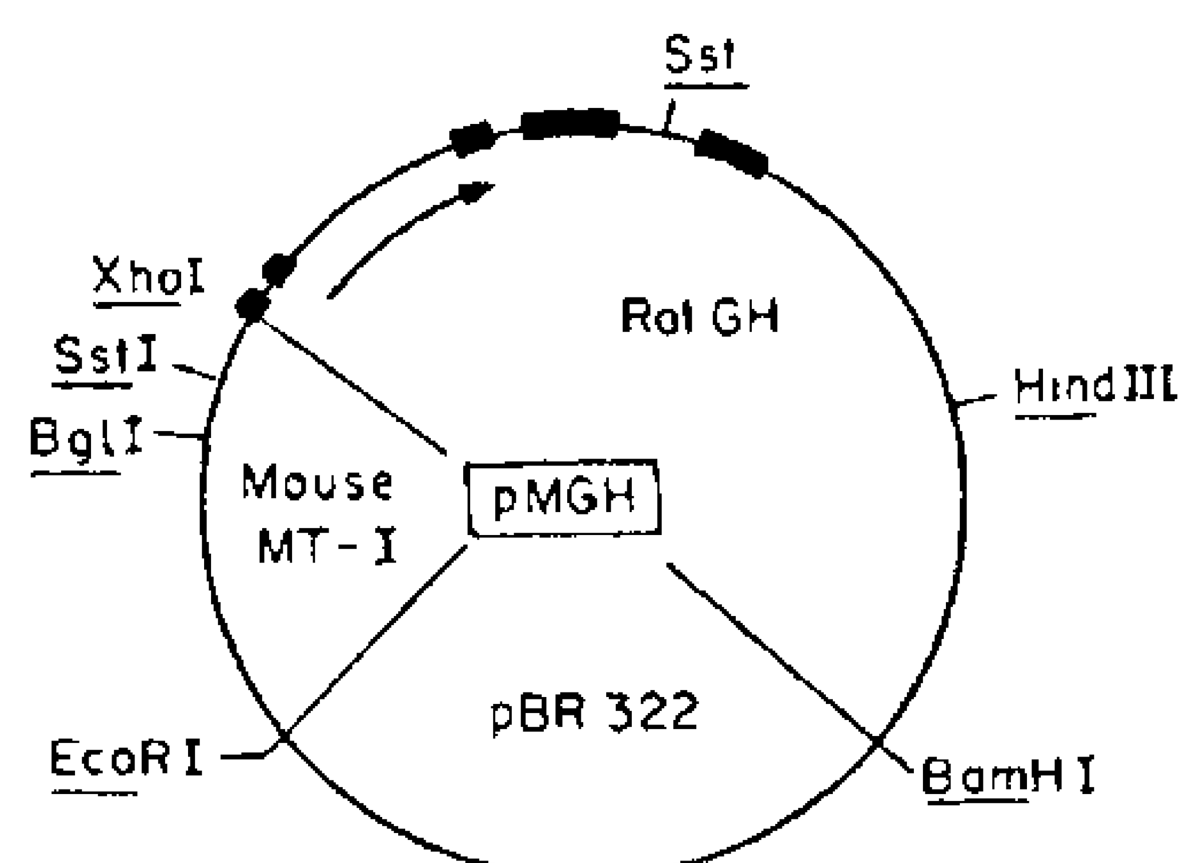

Figure 1. Restriction map of plasmid pMGH which carries the mouse metallothionein promoter MT-I fused to the rat growth-hormone gene (from Palmiter *et al.*[9]).

DNA extraction and hybridization

DNA was isolated from embryos, fry, and adults and their progenies at selected ages and analysed by slot-/Southern-blot hybridizations using ^{32}P-labelled plasmid (pMGH) as probe according to standard protocols[10].

Confirmation of successful DNA transfer

We first determined that 15 ng μl^{-1} was the optimum concentration of DNA since this gave maximum survival of injected eggs (Table 1). We therefore used this concentration throughout the experiment. As many as 1266 eggs were injected with pMGH DNA and the hatched fry were reared individually. Survival of the injected eggs until feeding stage averaged 46%, but ranged between 16 and 72% (Table 2). Samples from the injected fish were subjected to slot-blot analysis. It is clear from Table 2 that the percentage of integration

Table 1. Effect of DNA concentration on survival of microinjected zebrafish.

DNA concentration (ng μl^{-1})	Survival (%)			
	Cleavage	One day after injection	Hatching	Feeding
5	55 ± 2.8	50 ± 6.4	47 ± 7.8	41 ± 7.4
10	74 ± 2.0	75 ± 4.3	63 ± 4.7	41 ± 3.7
20	76 ± 22.5	53 ± 7.2	46 ± 0.5	42 ± 3.3
50	28 ± 18.2	21 ± 8.5	20 ± 13.5	13
100	38 ± 16.5	7 ± 15.1	10 ± 3.0	0
500	35 ± 8.4	19 ± 11.0	0	0
1000	9 ± 5.1	0	0	0

Volume injected was maintained at 200 pl in all cases.

Table 2. DNA-hybridization analysis to confirm integration of injected DNA in zebrafish of F_0 and F_1 generations.

Batch number	Number of eggs injected	Survival (%)			Integration (%)
		One day after injection	Hatching	Feeding	
F_0 generation					
1	51	53	39	35	0
2	61	30	25	16	0
3	34	41	35	29	0
4	42	74	62	42	0
5	37	65	65	62	31*
6	28	29	78	65	27*
7	29	66	58	52	100*
8	61	61	45	28	8
9	31	90	36	38	12*
10	51	57	54	39	0
11	39	77	76	72	37
12	52	87	75	42	0
13	31	77	61	48	52*
14	43	74	65	51	0
15	29	69	62	48	0
16	34	76	58	29	—
17	39	82	66	59	100*
18	37	92	81	40	0
19	40	95	72	52	—
20	21	95	85	71	—
21	35	18	62	45	0
22	43	86	72	55	—
23	33	91	58	48	—
F_1 generation					
Parent batch number	Number of eggs produced				
5	287	—	—	31	—
5	176	—	—	40	22†
7	297	—	—	23	100†
8	240	—	—	33	0
9	218	—	—	18	0
11	410	—	—	49	0
17	311	—	—	37	76†
F_2 generation					
	465	—	—	40	0
	360	—	—	41	0
	386	—	—	81	60
	414	—	—	53	0
	373	—	—	45	25

Fifty per cent of the surviving fish were analysed by DNA hybridization

*These were bred to F_1 to confirm integration

†These were subsequently bred to F_2 to confirm germline integration

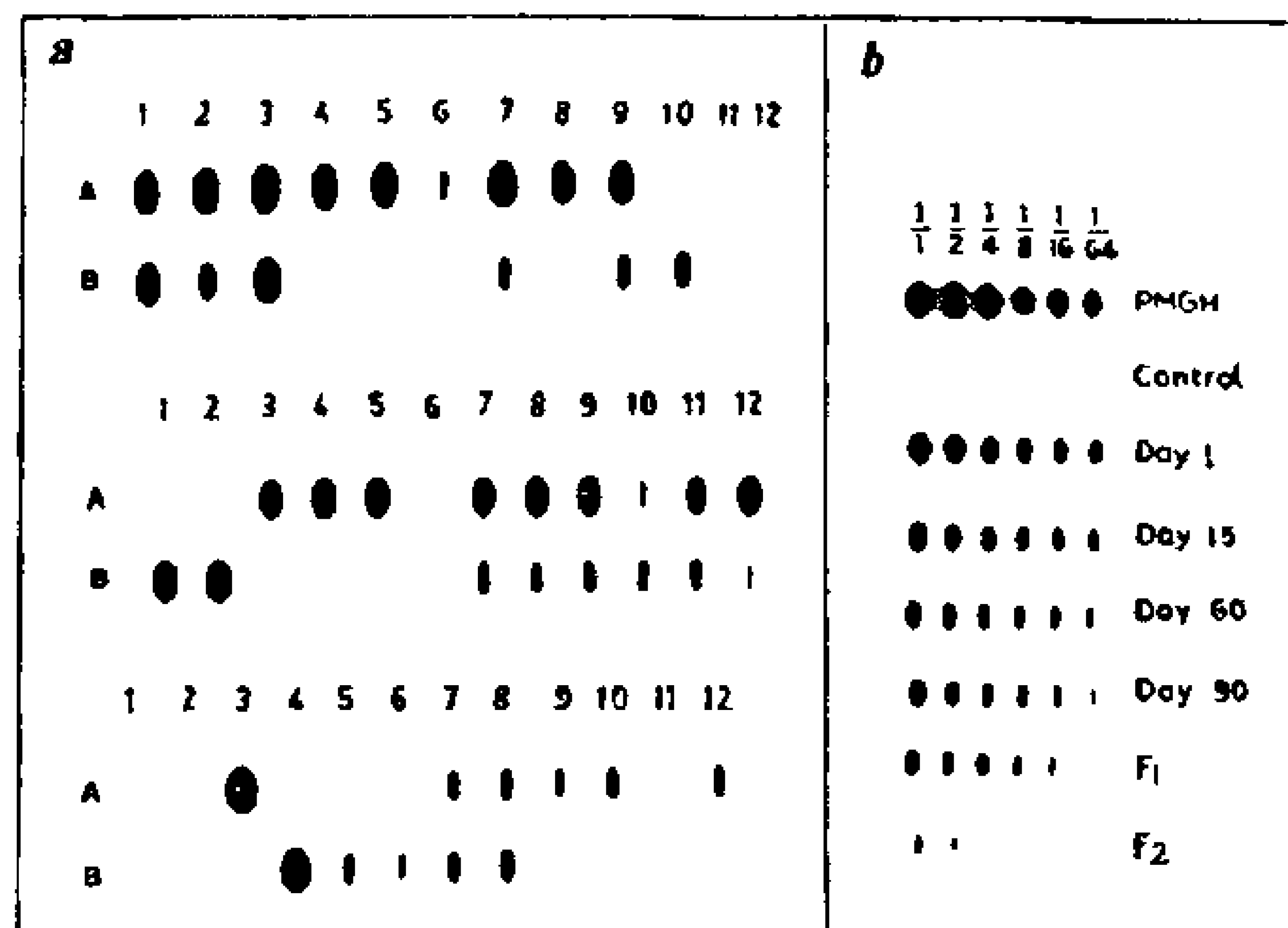

Figure 2. Slot-blot analysis of DNA extracted from randomly selected embryos, fry and adults of zebrafish which received pMGH DNA at 1-cell stage (F_0), as well as their progenies belonging to F_1 and F_2 generations. Panel *b* shows hybridization in serially diluted samples.

varied from 0 to 100 in the tested groups. Among the batches subjected to slot-blot analysis, 26 out of 38, 11 out of 18, and 8 out of 11 samples belonging to F_0, F_1 and F_2 generations respectively showed hybridization with the probe (Figure 2,*a*), i.e. the injected DNA persisted in 69, 61 and 73% of individuals belonging to F_0, F_1 and F_2 generations respectively. From a careful analysis of the data obtained from the slot-blot analysis of 72 samples, it was noted that strong hybridization occurred only in 50, 17 and 9% samples from F_0, F_1 and F_2 generations respectively. The foreign DNA persisted in significantly higher quantity in the 'transgenic' individuals of F_0 generation but progressively decreased in those of F_1 and F_2 generations. To confirm these results, DNA samples from 1-day-old embryos, fry of different ages, and adults of F_0, F_1 and F_2 were tested in serially diluted slot blots. The intensity of the hybridization signal was proportional to the quantity of DNA loaded (Figure 2,*b*).

Southern-blot analysis was performed to detect the persistence and/or integration of the injected DNA. A careful analysis of the results leads to the following conclusions: (i) In most 'transgenic' individuals the injected foreign DNA persisted extrachromosomally (Figure 3,*a*). (ii) In a few 'transgenic' individuals the injected foreign DNA was integrated into the genome of the fish but did not persist extrachromosomally (Figure 3,*b*). (iii) In some 'transgenic' individuals the injected DNA was integrated genomically and persisted extrachromosomally as well (Figure 3,*c*).

A strong hybridization in the positive control and in the DNA of 'transgenic' individuals at the same position on the gel (8.9 kb) implies the extra-chromosomal persistence of the injected plasmid DNA in F_0, F_1 and F_2 generations. The concentration of extrachromosomal DNA progressively decreased from 3-day-old individuals to 120-day-old individuals, as well as in the F_1 and F_2 generations.

Figure 3,*b* shows the pattern of Southern hybridization of the DNA from F_0, F_1 and F_2 individuals. These 'transgenic' individuals showed hybridization to a high-molecular-weight DNA band larger than 8.9 kb. It should be noted that the parents of F_1 were from 'transgenic' F_0 and of F_2 from 'transgenic' F_1 (Table 2). Since the restriction pattern is similar in F_0, F_1 and F_2, the injected DNA is obviously integrated in the genome in F_0 and has been passed on to the subsequent

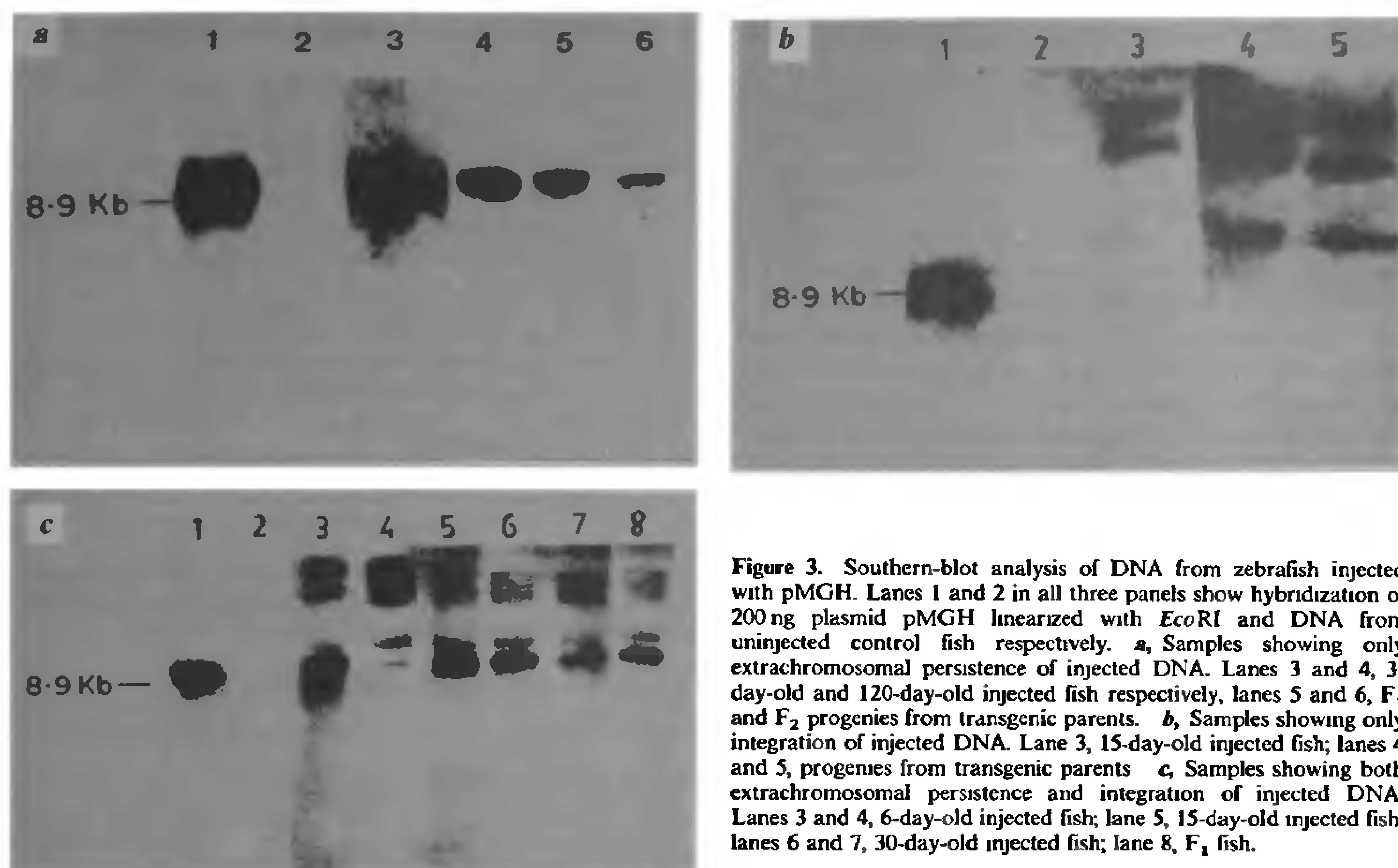

Figure 3. Southern-blot analysis of DNA from zebrafish injected with pMGH. Lanes 1 and 2 in all three panels show hybridization of 200 ng plasmid pMGH linearized with *Eco*RI and DNA from uninjected control fish respectively. ***a***, Samples showing only extrachromosomal persistence of injected DNA. Lanes 3 and 4, 3-day-old and 120-day-old injected fish respectively, lanes 5 and 6, F_1 and F_2 progenies from transgenic parents. ***b***, Samples showing only integration of injected DNA. Lane 3, 15-day-old injected fish; lanes 4 and 5, progenies from transgenic parents ***c***, Samples showing both extrachromosomal persistence and integration of injected DNA. Lanes 3 and 4, 6-day-old injected fish; lane 5, 15-day-old injected fish; lanes 6 and 7, 30-day-old injected fish; lane 8, F_1 fish.

generations unaltered; the presence of more than one band suggests integration at multiple sites. Detection of the injected plasmid as high-molecular-weight DNA also provides circumstantial evidence for genomic integration of the foreign DNA in the fish. The absence of a hybridization signal at the position corresponding to that of the positive control confirms that the injected DNA did not persist extrachromosomally.

The pattern of hybridization shown in Figure 3,*c* is a combination of Figures 3,*a* and 3,*b*. The DNA extracted from injected fish of different ages and generations showed complex but identical restriction patterns; in all these individuals the injected DNA is integrated genomically and persists extrachromosomally as well. The similar restriction pattern and hybridization in F_0 and F_1 can be taken as convincing evidence for germline integration of the injected DNA.

Our results demonstrate that mammalian growth-hormone gene transferred into zebrafish egg by microinjection is integrated in the fish genome and transmitted to F_1 and F_2 generations. Extrachromosomal persistence of the injected plasmid DNA and its subsequent degradation in the zebrafish are similar to observations in mouse[11] and a nematode[12], in which the injected gene sequences were not only maintained extrachromosomally but also passed on to one or two subsequent generations. In 'transgenic' zebrafish[3] and salmon[4] also injected sequences were found genomically as well as extrachromosomally. Mosaic animals do transmit foreign DNA to subsequent generations[3]. In our study, loss of extrachromosomal foreign DNA was observed consistently through developmental stage and generation; hence the persisting extrachromosomal DNA sequences do not get amplified during development. Stuart *et al.*[3] noted that the plasmid pSV-hydro was amplified until 5 h of development in zebrafish but was subsequently degraded totally. In our experiments the presence of more than one band hybridizing with the probe suggests the likelihood of plasmid integration at multiple sites. Similar observations have been reported in salmon[4] and carp[6].

Table 3. Growth of control and 'transgenic' zebrafish.

	Growth (mg)			
		Transgenic		
Age (month)	Control	F_0	F_1	F_2
1	85 ± 11	86 ± 32	89 ± 10	—
2	272 ± 6	222 ± 75	—	252 ± 61
3	506 ± 103	540 ± 133	610 ± 168	480 ± 108
4	632 ± 117	726 ± 82	694 ± 113	—
5	621 ± 68	846 ± 158	789 ± 132	—
7	712 ± 84	918 ± 164	—	—
9	800 ± 135	1018 ± 202	—	—
12	935 ± 177	1120 ± 215	-	—

Each value is the average (± SD) for 40–50 individuals.

Growth rates

Data on growth rate (Table 3) show that growth of 'transgenic' fish was faster in F_0 and F_1 generations but slower in F_2 generation than that of control fish. Secondly, there were indications that the accelerated growth observed in 'transgenic' fish of the F_0 and F_1 generations was at the cost of reproductive growth. It appears that the accelerated growth observed in the F_0 and F_1 generations may be correlated to the presence of extrachromosomal DNA in these individuals. Since it has already been demonstrated that extrachromosomal DNA is involved in transient expression[4], a similar effect may explain the accelerated growth rate.

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Imparting hydrogen-recycling capability to *Cicer*-rhizobial strains by plasmid pIJ1008 transfer

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Conjugal transfer of plasmid pIJ1008, which carries determinants for hydrogen uptake (Hup) activity, from *Rhizobium leguminosarum* to two Hup⁻ *Cicer*-rhizobial strains G 36–84 and BG4 conferred Hup activity in the free-living state as well as under symbiotic conditions. The acquired capability of the rhizobial strains to recycle hydrogen evolved by nitrogenase improved their relative efficiency of nitrogen fixation. This transfer and expression of *hup* genes suggests the possibility of improving the symbiotic energy efficiency of *Cicer-Rhizobium*, which lacks *hup* genes.

THE ATP-dependent evolution of hydrogen catalysed by the enzyme nitrogenase is a source of inefficiency in the legume-*Rhizobium* symbiotic system as nitrogen fixation is limited by the supply of photosynthate[1]. Nodule bacteroids that have a hydrogen-uptake (Hup) system can recycle this hydrogen and generate ATP[2,3] and reductant[4,5]. It has been suggested that an efficient hydrogen-recycling capability under symbiotic conditions is a desirable characteristic for *Rhizobium* strains[6]. The nitrogen-fixation efficiency of any Hup^- *Rhizobium* species may be improved if a functional Hup system can be transferred into it and be stably maintained. No strain of *Cicer-Rhizobium* that possesses Hup activity has been identified[7,8]. Presumably these strains do not contain *hup* genes[8]. In the present paper we report experiments aimed at interspecies transfer of Hup determinants located on the *Rhizobium leguminosarum* recombinant plasmid pIJ1008 into the Hup^- strains of *Cicer-Rhizobium*. Expression of Hup activity in the resulting transconjugants has been demonstrated under free-living as well as symbiotic conditions.

*For correspondence

Methods

Cicer-Rhizobium strain G 36–84 was obtained from the Division of Microbiology, IARI, New Delhi, while strain BG4 was isolated from the root nodules of chickpea in our laboratory. Plasmid pIJ1008 in *R. leguminosarum* strain B164 was kindly supplied by Dr N. J. Brewin of the John Innes Institute, UK. Cosmid pHU52 in *Escherichia coli* strain HB101 was kindly supplied by Prof. H. J. Evans of the Oregon State University, USA.

Yeast extract mannitol agar medium[9] was used for culturing *Rhizobium* strains. For bacterial matings, TY medium[10] was used. Luria–Bertani (LB) medium[11] was used for culturing *E. coli*. H_2-uptake medium as described by Maier *et al.*[12] was used to induce uptake-hydrogenase activity in rhizobial strains.

Plasmid transfer and Hup activity

Plasmid pIJ1008 was transferred from *R. leguminosarum* B164 to *Cicer-Rhizobium* strains by the biparental plate-mating system of Ditta *et al.*[13] The stability of plasmid pIJ1008 in the recipient strains was assayed by subculturing them for about 15 generations under non-selective conditions, i.e. in TY plates without kanamycin