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Cloned origin of DNA replication in *c-myc* gene can function and be transmitted in transgenic mice in an episomal state

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ABSTRACT

The *c-myc* protein has recently been shown to interact with a region possessing putative origin of DNA replication and enhancer activities located 2 kb upstream of the *c-myc* gene itself. Transgenic mice were obtained by injecting constructs containing this region, termed *pmyc(H-P)*, into fertilized mouse eggs. The transgenic elements were capable of efficient replication in all mouse tissues examined and were maintained in an episomal state even in highly differentiated cells. Moreover, *pmyc(H-P)* was transmittable to the progeny throughout several generations, which suggests that the fragment derived from the region upstream of the *c-myc* gene possesses sequences necessary for partition, stability and DNA replication of the plasmid in the cells. In addition, we have shown that the plasmid might be captured only by eggs, not by sperm.

INTRODUCTION

DNA replication starts at various sites in the genome and proceeds in both directions. The isolation of replication origins (*ori*) is an important part of the study of the processes involved in DNA synthesis, since its main regulatory steps are generally believed to occur during its initiation phase (1). As estimated by genetic and electromicroscopic analyses, the number of autonomously replicating sequence (ARS) found in yeast cells is consistent with the average size of the replicon (2), which suggests that *oris* are equivalent to ARS.

Recently, we have shown that replication of a mammalian ARS obtained from mouse cells (3) relied on the availability of the *c-myc* gene product. This fact, supported by the direct cloning of an ARS from human cells as a binding sequence for the *c-myc* protein (4), indicated that the latter promotes DNA replication by interacting with specific *oris*. This behavior is similar to that previously observed in the case of Simian Virus 40 (SV40) T antigen, the factor responsible for initiation of SV40

DNA synthesis. In fact, the *c-myc* product can function as a substitute for T antigen in SV40 DNA replication systems (5). A similar observation that the SV40 *ori* dependent replication was more efficient in cells expressing *c-myc* at high level has also been reported by Classon, et al (6).

We verified the hypothesis that, like SV40 T antigen, the *c-myc* protein can bind to some region close to its own coding sequence by isolating a *c-myc* protein binding sequence from the region upstream of the *c-myc* gene. This fragment possesses the functions of enhancer as well as ARS, which suggests that the *c-myc* product may also be an enhancer-binding protein (7). Indeed, the *c-myc* protein bound the specific sequence, TCTCTTA, in the above region, and the replication origin and transcriptional enhancer of the region shared the *c-myc* protein binding sequence (8). We have also shown that the plasmid clone carrying this fragment can serve as a good template in an *in vitro* DNA replication system (9). Another group also reported that autonomously replicating activity was detected in the upstream region of the *c-myc* gene (10).

In this communication, plasmids containing the ARS sequence from the *c-myc* gene were injected into fertilized eggs to obtain transgenic mice. The injected plasmids were replicated in all mouse tissues and were transmitted to the progeny in an episomal state.

MATERIALS AND METHODS

Plasmid

pmyc(H-P) is pUC19 containing the upstream region of the human *c-myc* gene as described in a previous report (7) and summarized in Fig. 1.

Establishment of Transgenic Mice

To obtain transgenic mice, a procedure described previously (11) was employed. One thousand copies of plasmid DNA per egg were injected into fertilized mouse eggs, B6C3F1 (F1 of C57/BL6 × C3H/He).

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Detection of Replicated Plasmid DNA in Transgenic Mice

Total DNAs were extracted from the tail or other tissues of mice and 5 μ g of the DNAs, digested or undigested with restriction enzymes as indicated, were electrophoresed in a 1.0% agarose gel and subjected to Southern blotting (12) with 32 P-labelled pUC19 or HindIII-PstI fragment of *c-myc* gene as a probe. Hybridization of the blotted filter with labelled probe was carried out as described (13).

Fractionation of nuclei and cytoplasm

Brain and liver were obtained from F2-3 mouse and nuclei and cytoplasm were separated by the usual method using NP-40. DNAs were extracted from both fractions and subjected to Southern blotting.

RESULTS**Plasmids containing ori replicate in transgenic mice**

A region found 2 kb upstream of the *c-myc* gene has been characterized as a binding sequence for either the *c-myc* product itself or for protein(s) complexed with *c-myc* protein. This region has been shown to possess ARS as well as enhancer activity (7). pUC derived plasmids containing this region, named *pmyc*(H-P)

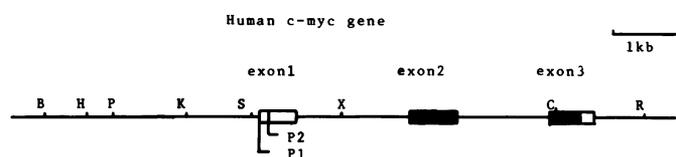


Fig. 1. Structure of human *c-myc* gene. The open and closed boxes represent non-coding and coding exon, respectively. B, BamHI site; C, ClaI site; E, EcoRI site; H, HindIII site; K, KpnI site; P, PstI site; S, SmaI site; X, XbaI site. P1, P2, promoters. A pUC19 containing the region from the HindIII site to the PstI site was designated as *pmyc*(H-P) and used for microinjection.

Table 1. Summary of transmission of *pmyc*(H-P) in transgenic mice.

generation	mating		progeny		presence of <i>pmyc</i> (H-P) in tail cells
	male	female	sex	no.	
F0	—	—	m	1	+
F1	F0	F0	f	1	+
			m	2	+
F2	F1	F1	m	15	+
			f	13	+
F3	F2	F2	m	15	+
			f	15	+
F4	F3	F3	m	12	+
			f	12	+
F0	F1	m	m	8	+
			f	8	+
F0	C3H/He*	m	m	6	—
			f	4	—
F1	C3H/He*	m	m	6	—
			f	6	—
C3H/He*	F1	m	m	9	+
			f	4	+
Balb/C*	F1	m	m	1	+
			f	2	+
KS*	F2	m	m	3	+
			f	3	+

*non-transgenic normal mouse

P) (See Fig. 1), were injected into fertilized mouse eggs. A male and a female mouse were obtained, and subsequently inbred as shown in Table 1. In parallel experiments we also bred transgenic mice with non-transgenic mice of different strains, the results of which will be discussed later. As control experiments, we injected vector pUC19 alone into fertilized eggs.

The total DNA was extracted from their tails and analyzed for the presence of plasmid elements by means of Southern blotting. In Fig. 2, we show the blotted patterns of the DNA extracted from male Fo-1 and female Fo-2 mice using pUC19 probe. The size of the hybridized material within the total DNA extract from female Fo-2 corresponds to that of the input construct *pmyc*(H-P), while that from male Fo-1 was a little larger. From the intensity of the hybridizable band, the copy number was estimated as about 2,000 copies of the plasmid in male Fo-1 and 20–50 copies in female Fo-2 per mouse tail cell. The same results were obtained by using a probe specific to the *myc*(H-P) region (Fig. 3). Moreover, a quantitative transformation of competent *E. coli* C600 cells with the tail cells' total DNA yielded a number of ampicillin-resistant colonies compatible with the evaluated plasmid copy number. The identity of the plasmid DNA recovered from the transformed bacteria was subsequently examined by restriction and sequence analyses. The results confirm that the observed plasmids derived from female Fo-2 mouse are identical to the input construct *pmyc*(H-P) and that

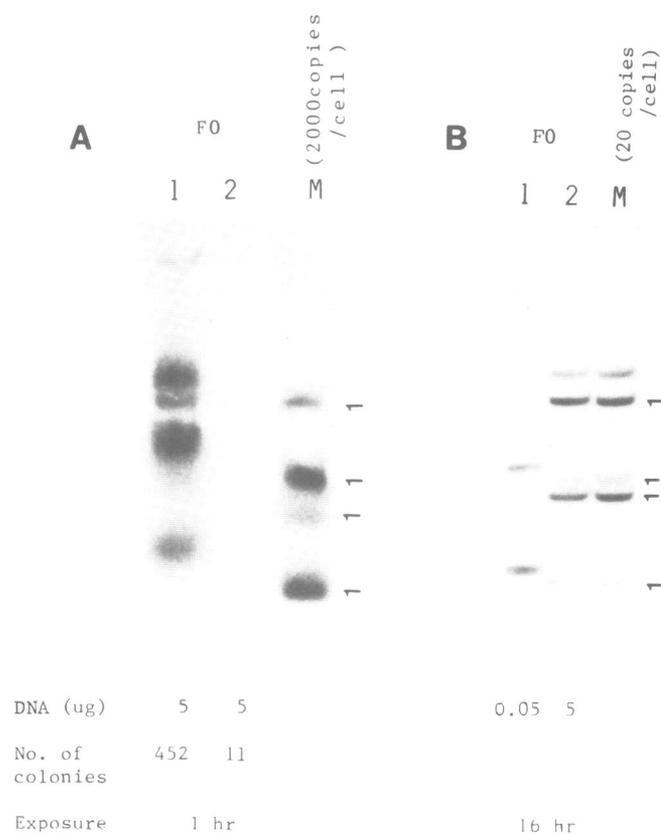


Fig. 2. Replication of *pmyc*(H-P) in F0 mice. The total DNA was extracted from the tail of F0 mice, blotted onto nitrocellulose filter (12), and hybridized with 32 P-labelled pUC19 probe. A is the result of 1 hr exposure, and B is of 16 hrs exposure and of 1/100 dilution of Fo-1 mouse DNA. The number of ampicillin-resistant colonies obtained after transformation of *E. coli* C600 with 5 μ g of total mouse DNA is shown in Fig. 2A.

those from male Fo-1 mouse contains 511 extra base pairs inserted between the PstI and the EcoRI sites in the portion of the construct derived from the pUC19 vector. These 511 extra base pairs share no homology with the sequences around the *c-myc* gene and have no ARS activity in transfected cells when separately cloned in pUC19. Further studies on these extra sequences are on the way and will be discussed elsewhere.

The next step was to verify whether the recovered plasmid DNA actually had replicated in the mouse cells. For this purpose, the total DNAs were digested with DpnI (which cleaves all non-replicated material) or with MboI (which cleaves all replicated material) and subsequently processed by Southern blotting. As shown in Fig. 4, the plasmid DNA extracted from the female Fo-2 mouse was DpnI resistant and MboI sensitive. Moreover, the intensity of the bands hybridizable with pUC19 probe was exactly the same before and after the DpnI digestion, which indicates that not only a part but all of the recovered material had replicated within the mouse cells.

Another essential point to be tested was whether the plasmid was replicated in an autonomous, episomal state or as a chromosomal integrate (or possibly as a re-excised integrate). A first important observation was that no hybridizable bands were detectable in the position of chromosomal DNA (see Figs. 2, 3 and 4), which renders unlikely the hypothesis of an integration of the plasmid into mouse chromosomes. Secondly, the plasmid was stably transmitted to the progeny (see below) and not according to Mendelian rules as one would expect from integrated, chromosomal elements. Thirdly, we compared the restriction patterns of the recovered plasmids and of the input construct previously digested with several 'one cut' restriction enzymes (i.e. which linearize the original *pmyc*(H-P), such as EcoRI and HindIII) and 'no cut' enzyme (i.e. which *do not* cut the original *pmyc*(H-P), such as BglII and XhoI). As shown in

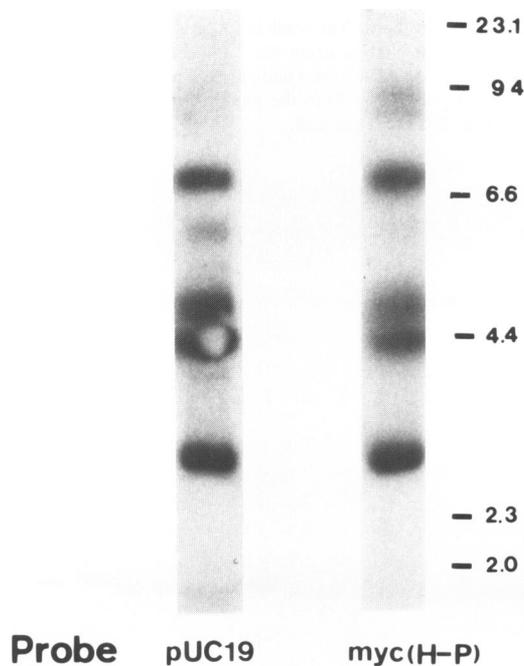


Fig. 3. Replication of *pmyc*(H-P) in F0 mice. The total DNA from the tail of Fo-2 was subjected to Southern blotting with ³²P-labelled pUC19 or *pmyc*(H-P) probe. The numbers on the right of the figure show the size (kb) of lambda phage DNA fragments digested with HindIII.

Fig. 4, the recovered plasmids behaved the same way as the input construct: when the DNA was digested with EcoRI or HindIII, a single band is observed equal in molecular weight to linearized *pmyc*(H-P). This suggests the visualized bands were not due to digests of *pmyc*(H-P) integrated into host chromosomal DNA. The hybridizable bands remained in open and closed circular forms after digestion with XhoI or BglII, enzymes which do not cleave *pmyc*(H-P). These results suggest a qualitative identity of the elements. Moreover, the mixing experiment was carried out using the Fo-2 mouse DNA and pUC19X prepared in dam⁺ *E. coli* (Fig. 5). After DpnI digestion, only pUC19X was digested while the DNA derived from Fo-2 mouse remained undigested. In the case of MboI digestion, on the contrary, the Fo-2 -derived DNA was digested but pUC19X was not digested. After BglII digestion, neither of the DNAs was digested. pUC19X, where SmaI site in polylinker sites has been changed into XhoI site, was linearized by XhoI, but the Fo-2 DNA remained undigested. These results also suggest that the total DNA extracted from Fo-2 mouse tail contained the *pmyc*(H-P) extrachromosomally replicated in mouse cells. Although further confirmation remains desirable, these data strongly indicate that *pmyc*(H-P) replicates extrachromosomally in transgenic mice.

Transmission of *pmyc*(H-P) to progeny

The F0 mice were inbred to yield F1 individuals, which in turn were inbred to yield F2 animals. As shown in Fig. 6 and

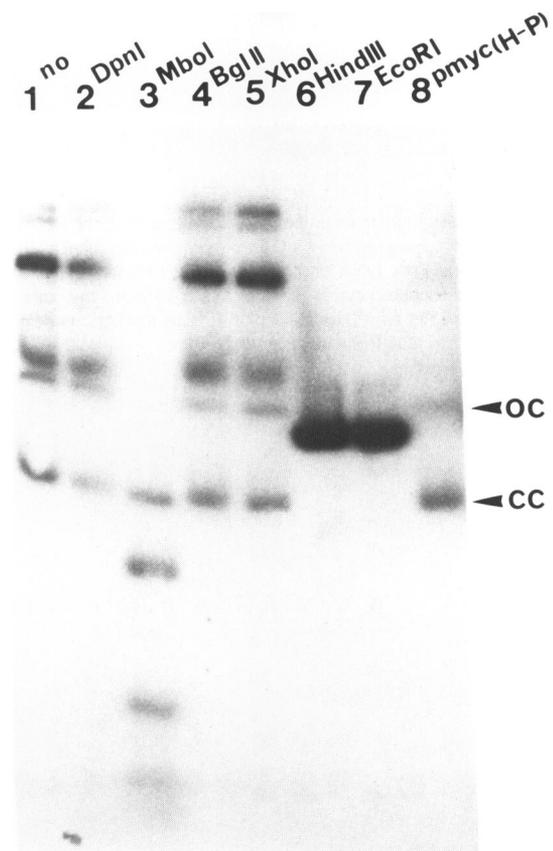


Fig. 4 Identification of *pmyc* (H-P) in tail cells of F0 (female) mouse. Total DNA was extracted from tail cells and analyzed by Southern blotting before or after digestion with the restriction enzymes indicated. The equivalent amount of 20 copies of *pmyc* (H-P) per cell is shown as a copy number marker (lane 8). oc, open circular form; cc, closed circular form of plasmid DNA.

	pmyc(H-P) marker		pUC19X					
	no	no	DpnI	MboI	BglII	XhoI	XhoI	EcoRI
sample	+	-	+	+	+	+	-	+
pUC19X	-	+	+	+	+	+	+	-
	1	2	3	4	5	6	7	8

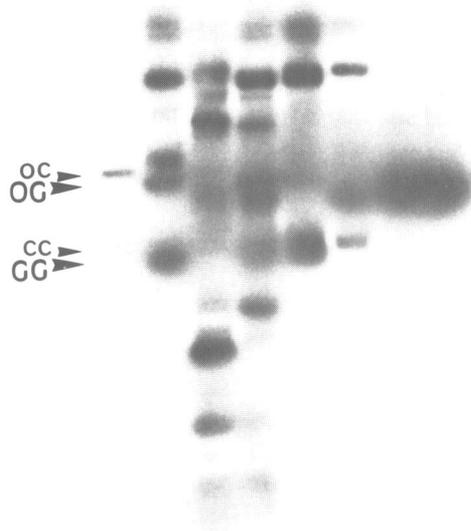


Fig. 5. Different sensitivity to restriction enzyme digestion of the DNAs. pUC19X was constructed by changing SmaI site in polylinker sites of pUC19 into XhoI site. Five μ g of the Fo-2 DNA and 10 pg of pUC19X were digested with the various kinds of restriction enzymes (lane 3–8) and subjected to Southern blotting. Lane 1 and 2 show the pmyc(H-P) and pUC19X as markers. oc, open circular form; cc, closed circular form of plasmid DNA.

7, all of the newborn animals possessed pmyc(H-P) in their tail cells, at approximately 20–50 copies per cell. These results imply that the *c-myc* upstream region contains the sequences necessary for partition, stability and replication of the plasmid in mouse cells. The DNA extracted from male F2-2 mouse was subjected to digestion by various restriction enzymes. As shown in Fig. 8, the pUC19-hybridizable band of the size identical to pmyc(H-P) was resistant to DpnI, but sensitive to MboI; undigested by BglII nor XhoI, while linearized by HindIII or EcoRI. The results suggested that pmyc(H-P) was extrachromosomally transmitted and replicated in F2 mice.

In parallel experiments, we mated F1 or F2 transgenic females bearing pmyc(H-P) with three different strains (C3H/He, KS, and Balb/c nu/nu) of normal (non-transgenic) males: all of the progeny carried pmyc(H-P) in their tail cells (Fig. 9-A; also see

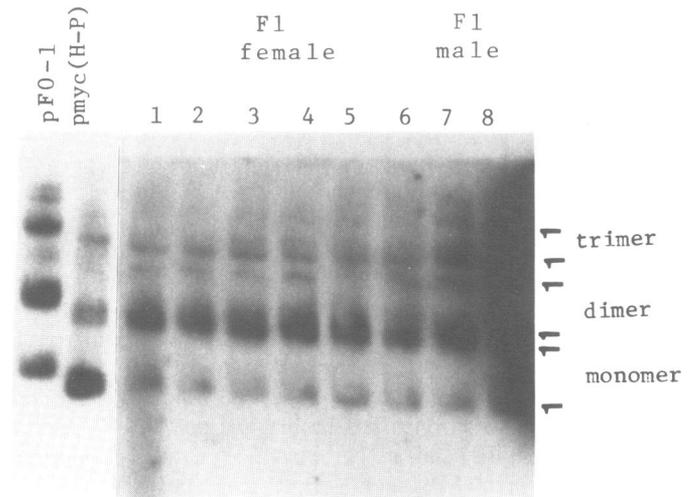


Fig. 6. Transmission of pmyc(H-P) into F1 mice. Fo-1 and Fo-2 mice were mated and six F1 mice were born. The total DNA was extracted from the tails and analyzed by Southern blotting using the 32 P-labelled *myc*(H-P) fragment as a probe. The arrows indicate the open and closed circular forms of trimer, dimer, and monomer of pmyc(H-P) from the top, respectively. Lanes of pFo-1 and pmyc(H-P) show 20 copies per cell.

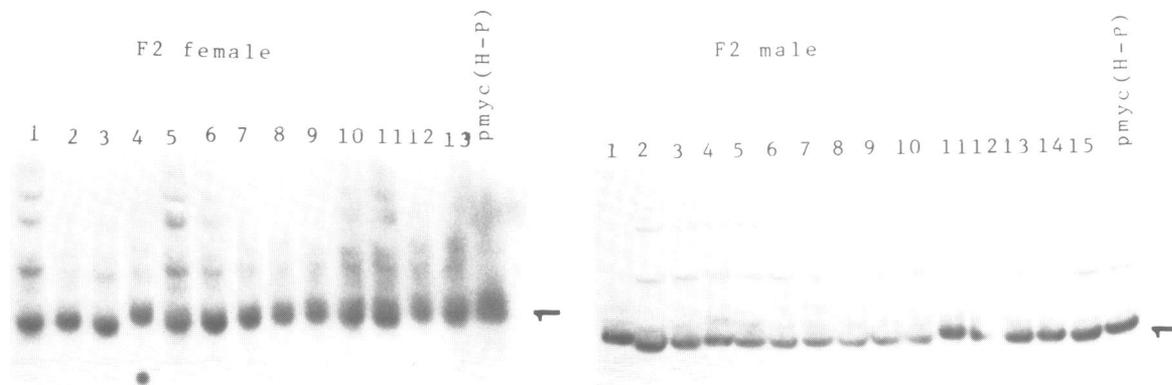


Fig. 7. Transmission of pmyc(H-P) to F2 mice. Male and female mice of F1 were mated to produce F2 mice (see Table 1). Total DNA was analyzed as described in Fig. 2 (without restriction digestion). The arrows indicate the closed (bottom) circular DNA of pmyc(H-P). The equivalent of 20 copies of pmyc(H-P) per cell is shown as a copy number marker.

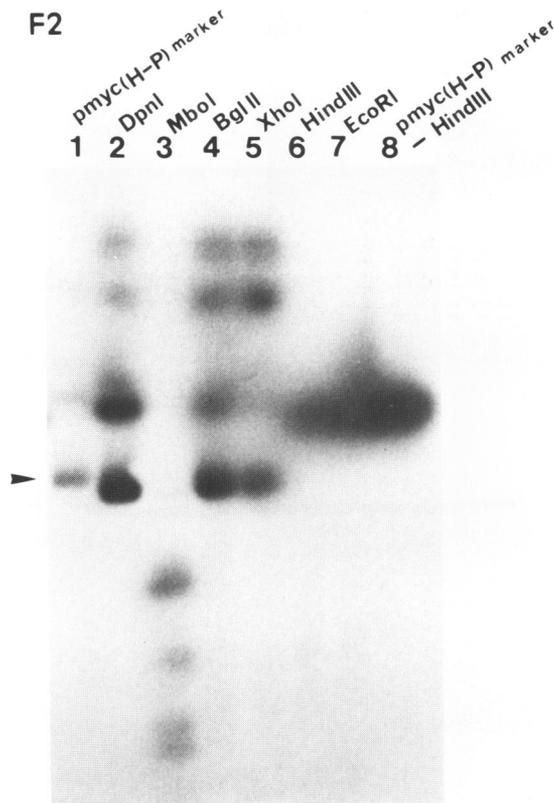


Fig. 8. Replication of *pmyc(H-P)* in F2 mouse. The DNA from one of the F2 mice was digested with various kinds of restriction enzymes and subjected to Southern blotting as described in Fig.4.

Fig. 13 and Table 1). Since in these cases the parental male mice did not contain any plasmid DNA, it is clear that the constructs had been transmitted by the transgenic parental females. Surprisingly, the mating of transgenic F0 or F1 males bearing *pmyc(H-P)* with three different strains of normal (non-transgenic) females yielded a progeny that completely lacked plasmid DNA (Fig. 9-B,C; see Fig. 13 and Table 1).

We also mated male Fo-1 harboring the larger plasmid with extra sequences and female F1-5 carrying the intact *pmyc(H-P)*. As shown in Fig. 10, all the progeny of Fo-1 × F1-5, both male and female, contained the plasmid of the size identical to *pmyc(H-P)*; none of them harbored the larger plasmid as the paternal Fo-1 mouse. These observations seem to indicate that once *pmyc(H-P)* is introduced into a mouse, it replicates and is transmitted to the progeny through the ovulum and not through the sperm. One may assume that the replication and transmission of *pmyc(H-P)* requires some oocyte specific feature (see 'Discussion'), yet one cannot exclude that this effect may be due to 'dilution' events related to the considerable difference in size of oocytes and spermatocytes. To clarify this matter, further experiments are being performed and will be discussed elsewhere. Another possibility to be considered is that *pmyc(H-P)* was transmitted through cytoplasm where maternal transmission of mitochondrial DNA occurs. The DNAs, therefore, were extracted from cytoplasm or nucleus fraction of two tissues and subjected to Southern blotting (Fig. 11). The results showed that *pmyc(H-P)* was observed only in nucleus, but not in cytoplasm fraction.

Replication of *pmyc(H-P)* in different mouse tissues

Up to this point, we have considered the presence of *pmyc(H-P)* only in the tail cells of the obtained transgenic mice. We next

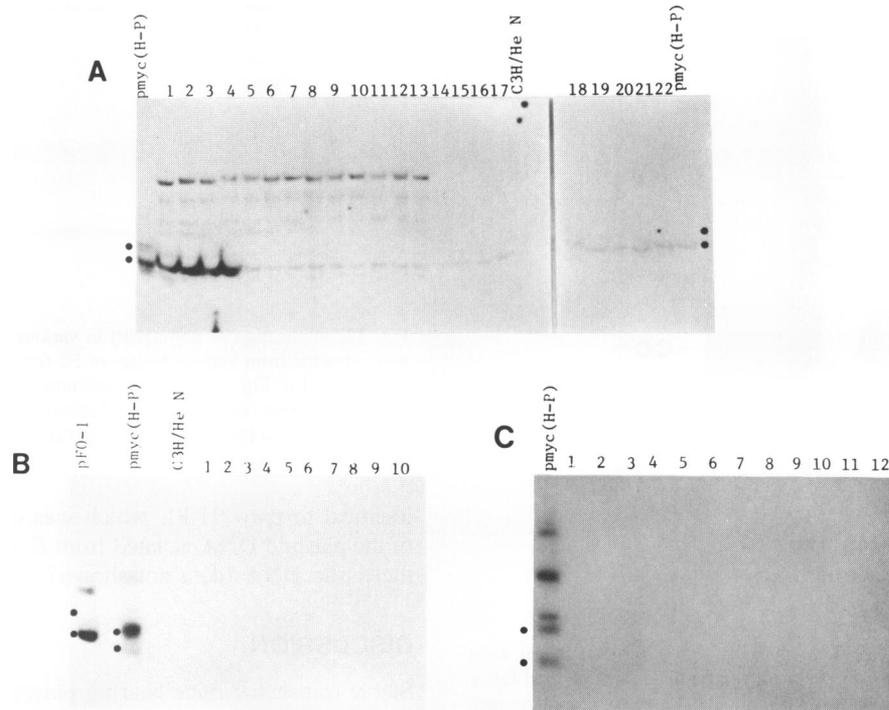


Fig. 9. Transmission of plasmid DNA through egg. The F1-3 female, the F1-2 female and the F2-1 female mice were mated with non-transgenic C3H/He male, Balb/c nu/nu male, and KS male mice, respectively, while the Fo-1 male and the F1-8 male mice were with non-transgenic C3H/He female mice (See Fig. 13 and Table 1). The tail DNA of the progeny and of normal mice were analyzed by Southern blotting as described in Fig. 2. Dots show the positions of the open and the closed circular form of monomer *pmyc(H-P)* from the top, respectively. **A**, the female transgenic mice mated with normal male mice. Twenty copies of pFO-1 or *pmyc(H-P)* per cell were shown as a copy number marker. **B**, the Fo-1 male transgenic mouse mated with normal female mice. One copy of pFO-1 or *pmyc(H-P)* per cell was shown as a copy number marker. **C**, the F1-8 male transgenic mouse mated with normal female mice. One copy of *pmyc(H-P)* per cell was shown as a copy number marker.

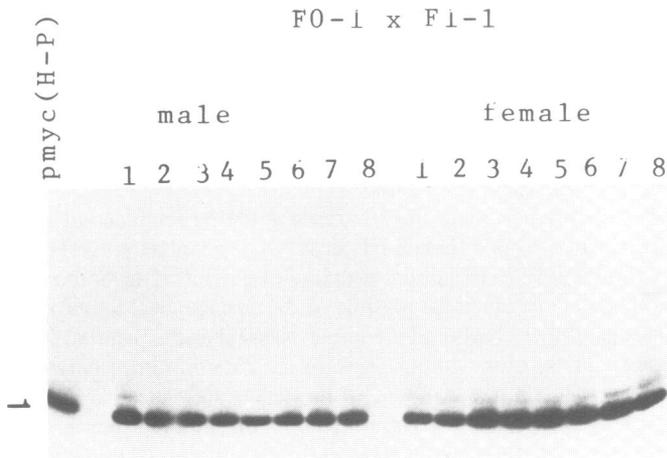


Fig. 10. Transmission of *pmyc(H-P)* through egg. The Fo-1 male mouse harboring the larger plasmid than *pmyc(H-P)* was mated with the F1-5 female mouse carrying *pmyc(H-P)*. The total DNA from the tail of their progeny was analyzed by Southern blotting as described in Fig. 2. The arrow indicates the position of closed circular form of *pmyc(H-P)*.

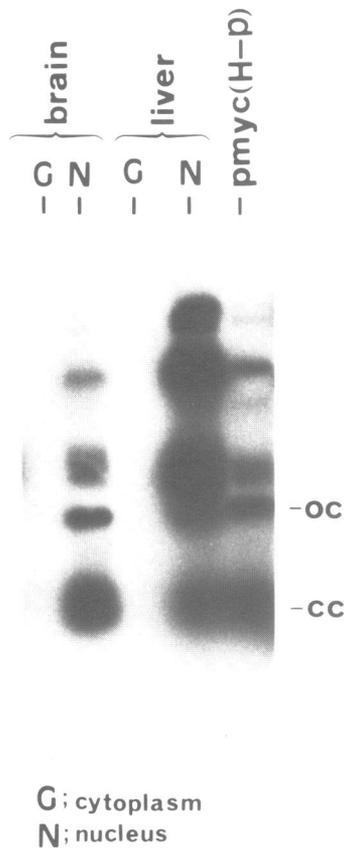


Fig. 11. Presence of *pmyc(H-P)* in the nucleus. Cytoplasm and nuclei were prepared from the brain or the liver of one of the F2 mice, and the total DNA was analyzed by Southern blotting. oc, open circular form; cc, closed circular form of *pmyc(H-P)*.

extracted total DNAs from various tissues of an F2 male and an F2 female and examined them for the presence of *pmyc(H-P)*. As shown in Fig. 12, a similar copy number of plasmid was detected in all the tissues tested. The contained plasmids were

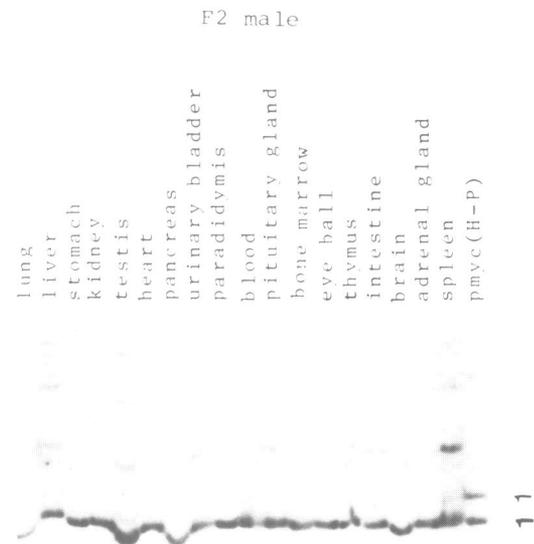


Fig. 12. Replication of *pmyc(H-P)* in various tissues of F2 mice. Total DNA was extracted from various tissues of F2 female and male mice, and analyzed as described in Fig. 2 (without restriction digestion). The arrows indicate the positions of open (top) and closed (bottom) circular DNA of *pmyc(H-P)*. The equivalent of 20 copies of *pmyc(H-P)* per cell is shown as a copy number marker.

identical to *pmyc(H-P)*, which was confirmed by the analyses of the plasmid DNA isolated from *E. coli* cells transformed with the tissue DNA (data not shown).

DISCUSSION

Stable transgenic mice bearing *pmyc(H-P)* in an episomal state were obtained by microinjection of the plasmid DNA into fertilized eggs. *pmyc(H-P)* carries the region present upstream of the *c-myc* gene, and this region contains both an *ori* of DNA replication and an enhancer of transcription (7). In this report we have shown that *pmyc(H-P)* is present in all tissues examined, and that it is retained in cells even after differentiation. The transgenic elements have the characteristic of persisting at

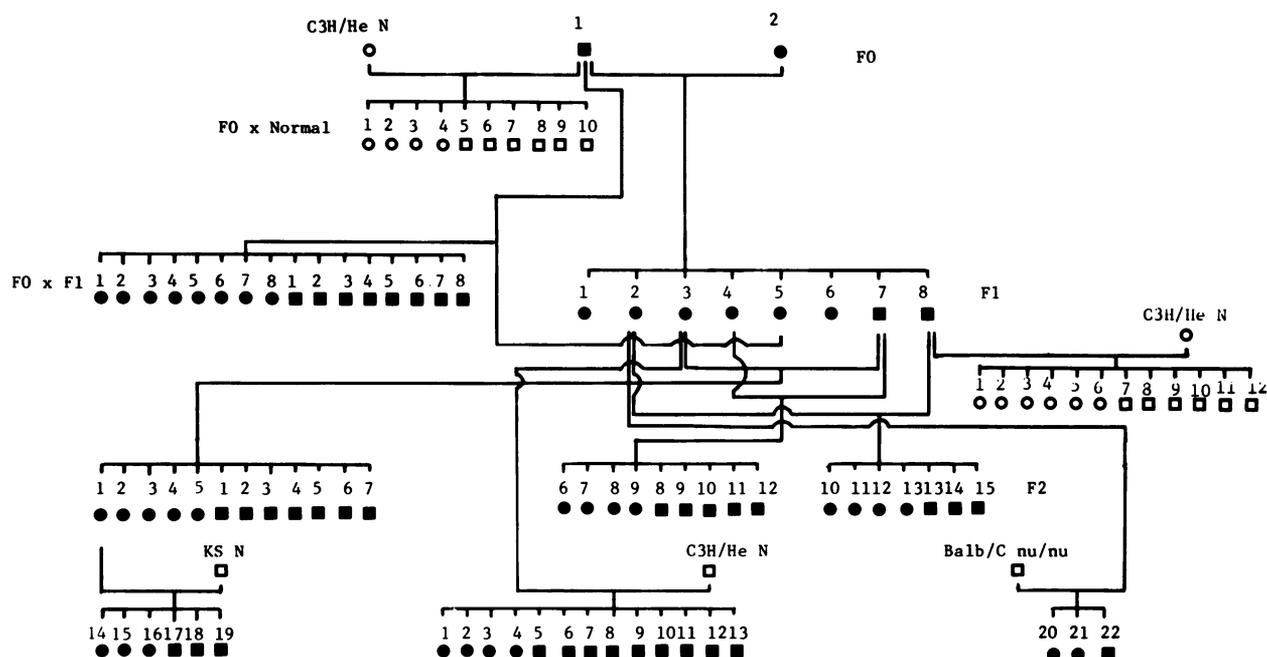


Fig. 13. Pedigree of the transgenic mice. The transgenic mice and their progeny examined in this study are schematically summarized. Circles and squares indicate the female and the male mice, respectively. Black symbols represent the mice possessing *pmyc*(H-P), hatched symbols the mice with pFo-1, and open symbols the mice carrying no plasmids. N represents 'normal', non-transgenic. The number of each mouse coincides with the lane number in the respective figures.

relatively low copy numbers in an autonomous stable state, due to the presence of the *cis*-acting *ori* sequences for autonomous replication and efficient segregation through both mitosis and meiosis. Such segregation functions are usually due to the presence of centromere (CEN) sequences (14–17). However, *pmyc*(H-P) shows no homology with those sequences so far reported. The fragment from the HindIII to the PstI site of the *c-myc* gene carried by *pmyc*(H-P) is sufficient for maintenance and segregation of our plasmid in an episomal state capable of being stably transmitted in mice through several generations. The HindIII-PstI region of the *c-myc* gene seems to contain some independent sequences necessary for CEN-like function.

The results for the transgenic mice obtained in this study are summarized in Table 1, and Fig 13. It cannot be completely excluded that *pmyc*(H-P) might be first integrated into chromosomes, transmitted, and then re-excised into an episomal state. However, if the plasmids were transmitted by being carried in chromosomal DNA, they should appear in the progeny according to Mendelian rules, i.e. 3:1 after mating transgenic and nontransgenic mice. Our results show, on the contrary, that the plasmids were detected in all the progeny between transgenic female and non-transgenic male, supporting our contention that the plasmids remained and were transmitted in an extrachromosomal state. Thus we feel it is reasonable to assume that the plasmids replicated and were transmitted in an episomal state.

Interestingly, *pmyc*(H-P) was quite possibly transmitted to progeny only through eggs, not sperm. The mechanism of meiosis in mammalian cells has not been well characterized to date. It is possible that there are different mechanisms of meiosis in oogenesis and spermatogenesis which require different DNA sequences and/or proteins, and that *pmyc*(H-P) may carry only the sequences required for meiosis in oogenesis. It has been reported that the *c-myc* mRNA is detectable in spermatogonia,

but not in spermatocytes (18, 19). Since the replication of *pmyc*(H-P) is dependent upon *c-myc* protein in cultured cells *in vitro* and in cell-free system (7), the plasmid may not be able to replicate in spermatocytes and be lost during meiosis. On the other hand, we cannot completely exclude the possibility of paternal inheritance of the plasmid, because, considering the size difference between oocytes and sperms, the failure so far to obtain progeny with the paternal plasmid may be due to dilution of the transgenic element. Progeny carrying the plasmid of paternal origin might be found if we examined a much larger number of mice. As a practical application of these findings, we propose that *pmyc*(H-P) can be used directly as an expression vector not only for producing proteins of interest in mammalian cells but also for gene therapy. Experiments in this direction are now under way.

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