

Parental Alleles of an Imprinted Mouse Transgene Replicate Synchronously

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ABSTRACT Molecular features of imprinted genes include differences in expression, methylation, and the timing of DNA replication between parental alleles. Whereas methylation differences always seem to be associated with differences in expression, differences in the timing of replication between parental homologs are not always seen at imprinted loci. These observations raise the possibility that differences in replication timing may not be an essential feature underlying genomic imprinting. In this study, we examined the timing of replication of the two alleles of the imprinted RSVlgmyc transgene in individual embryonic cells using fluorescence in situ hybridization (FISH). The *cis*-acting signals for RSVlgmyc imprinting are within RSVlgmyc itself. Thus, allele-specific differences in replication, if they indeed govern RSVlgmyc imprinting, should be found in RSVlgmyc sequences. We found that the parental alleles of RSVlgmyc, which exhibit differences in methylation, replicated at the same time. Synchronous replication was also seen in embryonic cells containing a modified version of RSVlgmyc that exhibited parental allele differences in both methylation and expression. These findings indicate that maintenance of expression and methylation differences between alleles does not require a difference in replication timing. The differences in replication timing of endogenous imprinted alleles detected by FISH might therefore reflect structural differences between the two alleles that could be a consequence of imprinting or, alternatively, could be unrelated to imprinting. *Dev. Genet.* 23:000–000, 1998. © 1998 Wiley-Liss, Inc.

Key words: Transgene; DNA replication; FISH; genomic imprinting

INTRODUCTION

In addition to differences in DNA methylation and gene expression between parental alleles of imprinted genes, the timing of replication is thought to differ between parental homologs in the same cell (replication asynchrony) [Izumikawa *et al.*, 1991; Kitsberg *et al.*, 1993; Knoll *et al.*, 1994; Gunaratne *et al.*, 1995; Kawame

et al., 1995; LaSalle and Lalande 1995]. DNA replication asynchrony is also found in genes subject to X-inactivation; the inactive allele of certain X-linked genes replicates later in the cell cycle than the expressed allele [Schmidt and Migeon, 1990; Webb, 1992; Hansen *et al.*, 1993]. Because of the frequently observed correlation between replication asynchrony and other allelic differences, it has been proposed that the differential timing of replication may imprint the locus, producing the allele-specific difference in expression that is the *sine qua non* of an imprinted gene. However, examples in which replication of the two parental homologs of an imprinted gene occurs synchronously raises the possibility that a replication timing difference between parental alleles is not required for genomic imprinting [Kawame *et al.*, 1995; Windham and Jones, 1997]. Such observations suggest that replication asynchrony, when found in association with imprinted genes, may be a consequence of the expression and/or methylation differences between the alleles, or may even be unrelated to these other imprinting features.

Difference in replication timing between parental alleles of imprinted genes has been examined by two experimental methods. The BrdU method determines the time of DNA replication by measuring incorporation of the thymidine analog, 5-bromo-deoxyuridine (BrdU), into DNA at different stages of the cell cycle [Hansen *et al.*, 1993]. Cells are grown for a short time in BrdU,

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sorted into different cell-cycle stages, and the stage at which a particular DNA sequence is replicated is determined by a PCR assay that amplifies the BrdU-containing DNA. Because short DNA sequences (100–600 bp) are amplified, the timing of replication within a portion of a gene is measured by the BrdU method. The fluorescence in situ hybridization (FISH) method assays the relative time of DNA replication of homologous parental alleles in a cell by determining the number of chromatids of each parental allele [Selig *et al.*, 1992; Kitsberg *et al.*, 1993]. In S-phase cells, the presence of two fluorescence signals indicates that DNA replication of either allele has not occurred; three signals indicate that replication of only one of the alleles has occurred; and four signals indicate that replication of both parental alleles has occurred. One difference between the two methods is that the BrdU method measures replication timing over short distances, whereas the FISH method measures replication timing within large genomic domains, determined by the size of the FISH probe. Using FISH, Kitsberg *et al.* [1993] found that the alleles of the imprinted *Igf2* and *H19* genes replicate at different times, with the paternal allele replicating first. In contrast, using the BrdU method, Kawame *et al.* [1995] and Windham and Jones [1997] found no differences in the timing of replication of the linked *Igf2* and *H19* genes. Such discordant results cannot be explained by the lack of *Igf2* and *H19* gene expression in the cells used in the BrdU assays, as the genes were expressed in an allele-specific manner in the cells used for both the BrdU and the FISH studies [Kitsberg *et al.*, 1993; Windham and Jones, 1997]. Moreover, differential timing of replication was measured by both methods at the imprinted *SNRPN* gene, which is within the imprinted cluster of genes associated with the human Prader-Willi and Angelman syndromes [Gunaratne *et al.*, 1995; Kawame *et al.*, 1995]. The *SNRPN* gene replicated asynchronously when it was expressed, but when expression was greatly downregulated, the timing of replication of both alleles was synchronous. The different results between studies of the *Igf2/H19* genes using FISH and BrdU methods are not easy to explain. The concordant results for the two methods applied to the *SNRPN* gene, however, suggest that both methods can accurately measure replication timing.

A central feature of the process of genomic imprinting is that the two alleles are marked in the parental germ lines by different epigenetic imprints. These imprints are subsequently maintained in *cis* throughout postfertilization development, such that allelic differences are evident in the adult [Solter, 1988; Ainscough and Surani, 1996]. This epigenetic inheritance process or genomic imprinting requires *cis*-acting imprinting sequences which must be within or closely linked to imprinted genes [Efstratiadis, 1994]. If replication asynchrony is responsible for genomic imprinting, then different timing of replication must be found in the *cis*-acting imprinting sequences on the two parental

alleles. In this regard, *cis*-acting sequences for the imprinting of endogenous genes have been difficult to identify [Ainscough and Surani, 1996]. Small transgenes containing sequences from imprinted genes are infrequently imprinted at ectopic integration sites, suggesting that some or all of the gene's *cis*-acting imprinting sequences may be at a distance far enough from the gene to be excluded from the transgene construct [Lee *et al.*, 1993; Bartolomei *et al.*, 1993]. Recently, large transgenes made from YAC clones of imprinted regions, but situated at ectopic chromosomal locations, have been reported to be imprinted, suggesting that the *cis*-acting imprinting sequences are located within the YAC transgenes [Ainscough *et al.*, 1997; Wutz *et al.*, 1997]. However, given the size of the YAC transgenes (130–300 kb), the *cis*-acting imprinting sequences may be distant from the imprinted genes themselves, and not necessarily contained within the FISH probes used to study the replication timing [Kitsberg *et al.*, 1993]. In contrast, the 18-kb RSVIgm_{yc} transgene is imprinted at all sites of random genomic integration. For all independently derived transgenic RSVIgm_{yc} lines, the maternal allele is highly methylated and transcriptionally silent, whereas the paternal allele is relatively undermethylated and expressed [Swain *et al.*, 1987; Chaillet *et al.*, 1991]. These observations indicate that RSVIgm_{yc} contains all *cis*-acting signals necessary for its imprinting [Chaillet *et al.*, 1991, 1995]. A FISH probe directed to the entire transgene will measure replication timing of the *cis*-acting imprinting sequences. Thus, to clarify the role of replication asynchrony in the process of genomic imprinting, we examined the replication features of the imprinted RSVIgm_{yc} transgene locus using the entire transgene as the FISH probe.

MATERIALS AND METHODS

Transgene Constructs and Transgenic Mice

Two transgene constructs were studied. RSVIgm_{yc} is a transgene of approximately 18 kb in length which is expressed only in the heart; its sequence composition has been described in detail [Chaillet *et al.*, 1995]. RSVpgk_{myc} is a transgene construct of approximately 17 kb which is expressed in many cells and tissues, including primary fibroblasts of embryonic origin. RSVpgk_{myc} is derived from RSVIgm_{yc} by replacing a portion of the immunoglobulin sequences with the mouse *pgk-1* promoter [Adra *et al.*, 1987], and introducing sequences from the *Aequorea victoria* green fluorescence protein (GFP) [Heim *et al.*, 1995] into the coding region of RSVIgm_{yc}'s *c-myc* gene at a unique *EcoRV* site. Specifically, the promoter region of RSVIgm_{yc} (a portion of the *IgA* sequences) is replaced with the mouse *pgk-1* promoter, removing the *IgA/c-myc* breakpoint between the *XbaI* sites described in the schematic of RSVIgm_{yc} (Fig. 3A). The GFP gene containing an S65T mutation is inserted into exon 2 of *c-myc*. The

pgk-1 promoter was PCR amplified from the pPNT plasmid using primers GAATTCTAGAGGCTAGGGGA and CAGGTCTAGAGGCCCGGAG, both with *Xba*I restriction sites. The GFP gene was inserted in-frame with the transgene's *c-myc* gene at a unique *EcoRV* site in the second exon of *c-myc*. GFP was amplified from plasmid pS65T-C1 (Clontech, Palo Alto, CA), using the primers CATGGATATCGGAGAAGAAGACTTTTCA and TGTAGATATCATCCATGCCATGTGTA, both containing *EcoRV* restriction sites.

Transgenic lines of mice were generated with either RSVIgmyc or RSVpgkmyc in the inbred FVB/N strain background. The RSVIgmyc transgenic line used in this study is designated TG.AAJ and contains 15 copies of the RSVIgmyc construct [Weichman and Chaillet, 1997]. Two different transgenic lines were generated with the RSVpgkmyc construct, and they are designated TG.HQJ (5–10 copies) and TG.HRJ (15–20 copies).

Mouse Embryonic Cell Lines

Primary embryonic fibroblasts were generated from the transgenic lines of mice. For each transgenic line, three types of matings were established: (1) a hemizygous transgenic male and a hemizygous transgenic female, (2) a hemizygous transgenic male and a wild-type FVB/N female, and (3) a wild-type FVB/N male and a hemizygous transgenic female. Embryos from each type of mating were collected at 14.5 days of gestation. To establish primary embryonic fibroblast lines, the body wall of each embryo was isolated, minced, treated with trypsin, and plated in DMEM with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD). Individual homozygous transgenic fibroblast lines from the first type of mating were identified by metaphase FISH, using the entire transgene as the FISH probe. For embryos from the other two types of matings, hemizygous transgenic embryos were identified by Southern blot analysis of the derived embryonic fibroblast lines [Chaillet *et al.*, 1995].

Cytogenetic Harvest

Unsynchronized mouse embryonic cell lines were treated with 10^{-4} M BrdU for 1 hour prior to harvest. Under dim yellow light, the cells were washed, removed from the flasks with trypsin, treated with 0.075 M hypotonic KCl solution for 25 minutes at 37°C, then fixed with cold 3:1 methanol: acetic acid fixative. Cells were washed three times in this fixative, then stored in the dark at -20°C until use.

Fluorescence In Situ Hybridization

Slides were prepared from fixed cell pellets and were used within 48 hours for FISH. The RSVIgmyc transgene construct was nick translated in the presence of biotin-16-UTP (Boehringer Mannheim, Indianapolis, IN) following standard methods [Lichter and Ried, 1994] or directly labeled with fluorescein-12-dUTP using a Prime-It Fluor Fluorescence labeling kit (Strata-

gene, La Jolla, CA). The RSVIgmyc probe was used to evaluate replication features of both RSVIgmyc and RSVpgkmyc, even though RSVpgkmyc has sequences not found in RSVIgmyc. These sequences (*pgk-1* promoter and GFP coding sequence) are not required for transgene imprinting, and cannot be *cis*-acting sequences for imprinting. The probe was precipitated in the presence of mouse Cot-1 DNA (Gibco BRL, Gaithersburg, MD), then resuspended in 50% formamide/2× SSC. Approximately 100 ng of probe was then used in a 10 μl hybridization in 50% formamide/2× SSC/10% dextran sulfate. Hybridization was carried out overnight under a 22 × 22 mm coverslip at 37°C. Posthybridization washes were carried out for 3 × 3 minutes in 50% formamide/2× SSC at 45°C and 3 × 3 minutes in 2× SSC at 37°C. Detection of the biotin-labeled probe was carried out with FITC-avidin (Vector, Burlingame, CA), as described by Lichter and Ried [1994]. Detection of the BrdU was carried out with a monoclonal mouse anti-BrdU antibody (1:100, 45 minutes, 37°C; Boehringer Mannheim) and a Texas Red-conjugated horse anti-mouse secondary antibody (1:100, 45 minutes, 37°C; Vector, Burlingame, CA). Slides were counterstained with DAPI (Oncor, Inc., Gaithersburg, MD), and stored in the dark at -20°C until scoring.

Slides were analyzed on an Olympus BH2 fluorescence microscope, and images were captured using a CytoVision Ultra (Applied Imaging, Santa Clara, CA). Only interphase nuclei that were positively stained with the anti-BrdU antibody were evaluated. Additionally, due to the presence of polyploid (mostly tetraploid) subpopulations of cells present in some cultures, only diploid cells were analyzed for replication timing. The transgene signals in BrdU positive cells were carefully evaluated to determine whether each signal consisted of a singlet (unreplicated) or a doublet (replicated). Signals with a dumbbell shaped appearance were scored as doublets (replicated). Cells with two singlets are designated SS. Cells with two doublets are designated DD. Cells with a singlet and a doublet are designated SD. SD cells are ones in which one allele of the transgene has replicated, and the opposite allele has not. The percentage of nonpolyploid, BrdU-positive cells with each hybridization pattern was determined from total numbers of cells between approximately 150 and 400.

RESULTS

Transgene Alleles With Methylation Differences, but not Expression Differences, Replicate Synchronously

Different parent-specific methylation patterns of the RSVIgmyc transgene are apparently maintained in all tissues of the adult and postimplantation embryo [Swain *et al.*, 1987; Chaillet *et al.*, 1995]. Despite this ubiquitous imprinting feature, RSVIgmyc is only expressed in the heart, and only expressed from the paternal allele

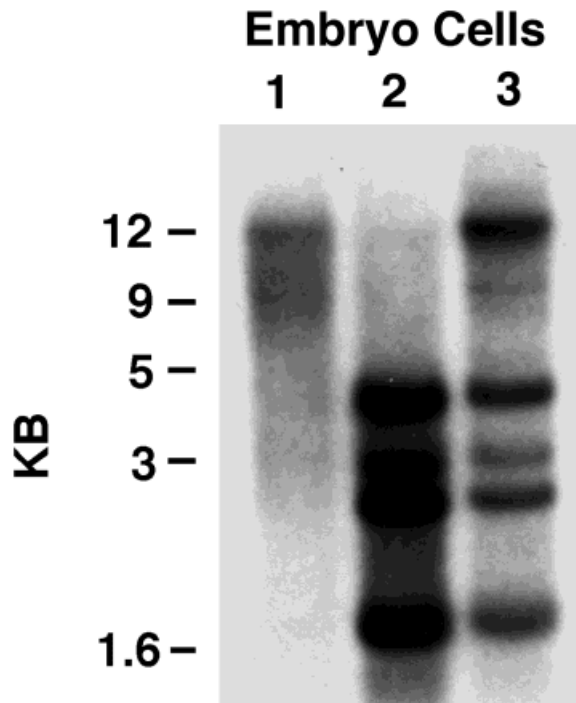


Fig. 1. Analysis of RSVIgmcy methylation in TG.AAJ transgenic carriers and in embryonic cells derived from the TG.AAJ transgenic mouse line. Lanes 1–3 are Southern blots of *HpaII* digests of genomic DNA from E14.5 embryonic fibroblasts from TG.AAJ carriers. Lane 1 is from an embryo with a maternal RSVIgmcy allele. Lane 2 is from an embryo with a paternal RSVIgmcy allele. Lane 3 is from an embryo with both parental alleles.

[Swain *et al.*, 1987]. It was not unexpected, therefore, that the transgene was not expressed in embryonic fibroblasts from TG.AAJ mice (data not shown). Characteristic parent-specific methylation patterns, however, are present in these cell lines (Fig. 1). The methylation pattern of RSVIgmcy in a cell line that inherited RSVIgmcy from the female parent is highly methylated (Fig. 1, lane 1), whereas the methylation pattern in a line that inherited the transgene from the male parent is undermethylated (Fig. 1, lane 2). These patterns are indistinguishable from the maternal and paternal patterns, respectively, found in adult tissues of transgenic carriers (data not shown). Moreover, each homozygous transgenic embryonic fibroblast line has the same methylation pattern (Fig. 1, lane 3) as found in tissues of homozygous adults [Swain *et al.*, 1987]. This homozygous pattern is consistent with the coexistence of a paternal and a maternal methylation pattern in the same cell. Therefore, the parental alleles of the imprinted RSVIgmcy transgene in embryonic fibroblast cell lines from TG.AAJ mice are differentially methylated but not differentially expressed.

Representative fluorescence images of the biotin-labeled RSVIgmcy probe hybridized to homozygous TG.AAJ fibroblasts are presented in Figure 2. An

intense fluorescent signal from the integrated transgene array localizes near the centromeric end of a chromosome (Fig. 2A). The transgene signals are easily observable in interphase nuclei (Fig. 2B). Synchronous replication of the two alleles (SS or DD pattern) was observed in 87–90% of the cells (Table 1, Fig. 2B,C). Replication asynchrony (SD pattern) was observed in only 4–13% of the cells (Table 1, Fig. 2D). This value is comparable to the observed percentages of cells with three FISH signals from nonimprinted loci, which are believed to replicate synchronously [Kitsberg *et al.*, 1993; Knoll *et al.*, 1994; Gunaratne *et al.*, 1995]. As well, we observed a similar level (7–13%) of replication asynchrony at a nonimprinted control locus (*Ccnd1*; Table 1). The low levels of apparent asynchronous replication in the TG.AAJ fibroblasts are, therefore, consistent with a synchronously replicating transgene locus. Similar levels of asynchronous replication were observed in other independent fibroblast lines derived from homozygous TG.AAJ embryos, despite the different apparent average stage in S-phase of the population (as determined by the ratio of cells within SS pattern to ones with a DD pattern). This may indicate that results obtained by the FISH method are not significantly influenced by the average amount of DNA replication that has been completed in the TG.AAJ fibroblasts examined from asynchronously growing cultures.

Transgene Alleles With Both Methylation and Expression Differences Replicate Synchronously

The results of interphase FISH studies on TG.AAJ fibroblast cell lines are consistent with synchronous replication of the two parental transgene alleles. Because these lines do not express the RSVIgmcy transgene, we were unable to address the relationship between replication timing and another important aspect of RSVIgmcy imprinting, namely parent-specific expression differences. This is a relevant issue because two studies, one using FISH and the other using the BrdU method, indicate that replication asynchrony of the *SNRPN* gene may be seen only in cells expressing *SNRPN*, and not in cells with both alleles of *SNRPN* silent [Kawame *et al.*, 1995; Gunaratne *et al.*, 1995]. These observations may indicate that replication timing differences between parental alleles may be necessary for differences in parental allele expression. We were therefore interested in knowing whether the RSVIgmcy transgene replicated asynchronously in cells in which it was expressed. RSVIgmcy is known only to be expressed in cardiac cells, which are not easily amenable to cell culture growth and FISH analysis. Therefore, we modified RSVIgmcy for expression in embryonic fibroblast cells, and then examined the integrated, modified transgene for the timing of its replication.

RSVIgmcy was modified to RSVpgkmyc by replacing a portion of RSVIgmcy with the mouse *pgk-1* promoter and by introducing coding sequences of the GFP gene

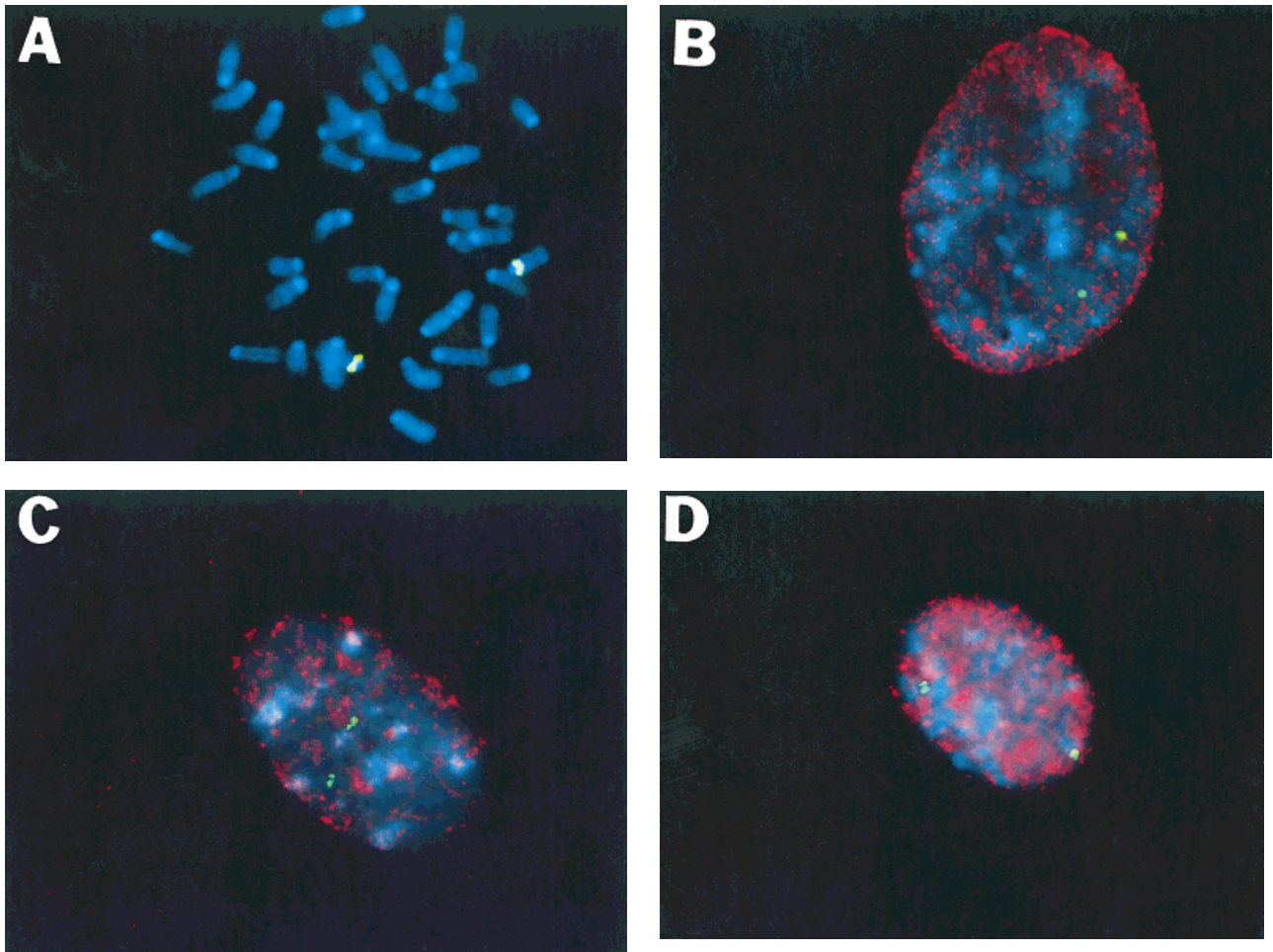


Fig. 2. Metaphase and interphase FISH analysis of embryonic cells derived from the TG.AAJ transgenic line. **A:** Metaphase spread of DAPI staining of TG.AAJ embryonic fibroblasts showing location of integrated transgene array. **B:** Two FISH signals (SS pattern) in an

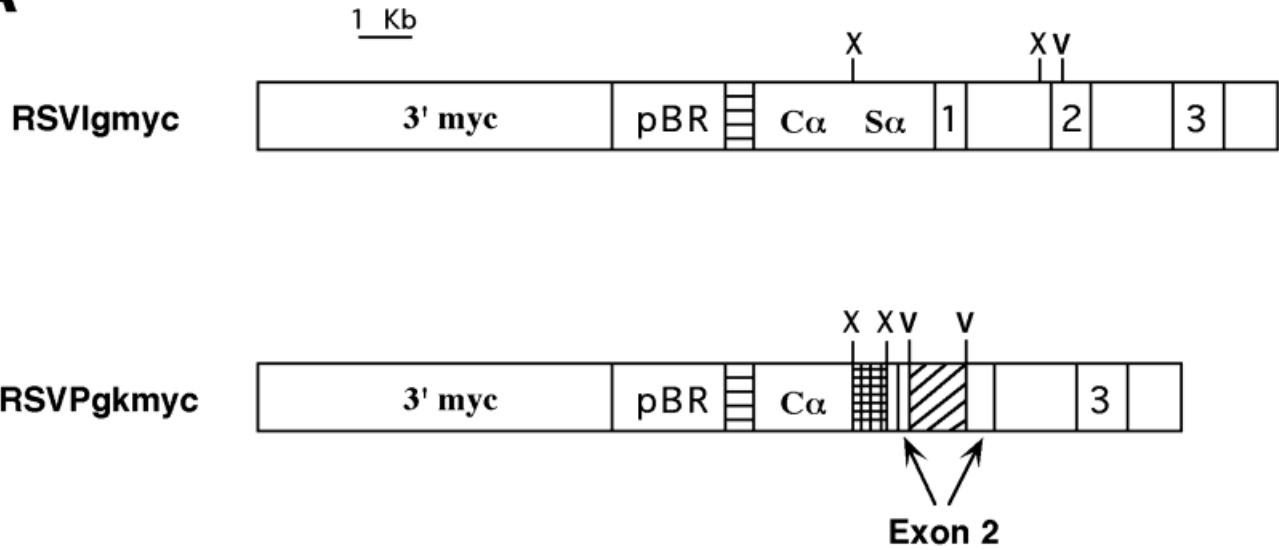
interphase nucleus. **C:** Four FISH signals (DD pattern) in an interphase nucleus. **D:** Three FISH signals (SD pattern) in interphase an nucleus with asynchronously replicating transgene alleles.

into the coding region of the second exon of the *c-myc* gene (Fig. 3A and Materials and Methods). Two independent transgenic mouse lines (TG.HRJ and TG.HQJ) were generated with RSVPgkmyc and examined for parent-specific methylation and expression differences. The TG.HRJ line showed allelic differences in both transgene methylation and expression (Fig. 3). Specifically, in cell lines derived from TG.HRJ embryos as well as DNA from TG.HRJ adult carriers, the RSVPgkmyc transgene is methylated differently on the maternal and paternal alleles (Fig. 3B). The maternal allele in the embryo cells is highly methylated (Fig. 3, lane 1) and not different from the adult (data not shown), whereas the paternal allele from both sources is relatively undermethylated (Fig. 3, lane 2 and data not shown). The methylation pattern in cells containing both parental alleles appears to be the sum of the maternal and paternal patterns (Fig. 3, lane 3). Moreover, embryo cells containing only a maternal RSVPgk-

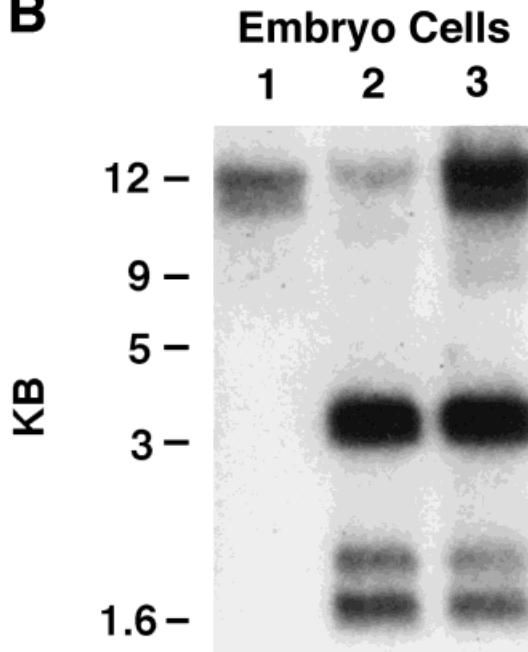
myc allele do not express the transgene, whereas cells containing a paternal allele do express it (Fig. 3C). From this analysis of DNA methylation and transgene expression, we conclude that RSVPgkmyc in the TG.HRJ line is imprinted, both in terms of methylation differences and expression differences.

The results of hybridization of the FISH probe to a single fibroblast cell line derived from a homozygous TG.HRJ embryo are summarized in Table 1. FISH images on metaphase cells indicated that the RSVPgkmyc construct integrated at a single chromosomal position, different from the TG.AAJ integration site (data not shown). When replicating cells from the fibroblast cell line were analyzed in the same manner as the TG.AAJ cell lines, the differentially expressed RSVPgkmyc alleles were found to exhibit low levels of replication asynchrony (16%). This level of replication asynchrony is slightly higher than the levels of asynchronous replication measured at the nonimprinted *Ccnd1*

A



B



C

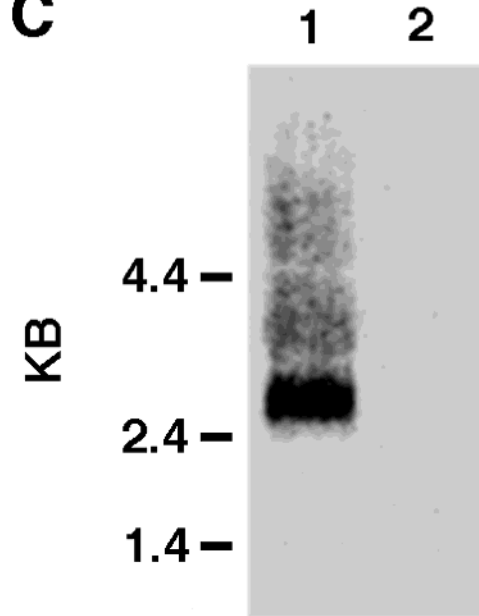


Fig. 3. Analysis of RSVPgkmyc methylation and expression in the imprinted TG.HRJ transgenic line and embryonic cells derived from TG.HRJ. **A:** Schematic of linearized versions of the RSVIgmyc plasmid and its derivative RSVPgkmyc. The composition of RSVIgmyc is described in detail in Chaillet *et al.* [1995]. The key sequence elements of the DNA transgene constructs are shown. pBR is a portion of pBR322 plasmid. The horizontal lines represent Rous sarcoma virus sequences. C α and S α represent the coding region and switch recombination region of IgA, derived from the breakpoint region of a mouse IgA/*c-myc* translocation. The boxes labeled 1, 2, and 3 represent exons 1, 2, and 3 from the *c-myc* genomic region. RSVPgkmyc is derived from RSVIgmyc by the replacement of a portion of the immunoglobulin sequences and a portion of the *c-myc* sequences of RSVIgmyc with the mouse *pgk-1* promoter (hatched box), and the introduction of GFP

sequences (diagonal lines) into the second exon of *c-myc* (see Materials and Methods). Restriction sites used for converting RSVIgmyc to RSVPgkmyc are shown. X = *Xba*I, V = *Eco*RV. **B:** Southern blots of methylation-sensitive restriction enzyme digestion of TG.HRJ mice and derived cell lines. Lanes 1–3 are Southern blots of HpaII digests of genomic DNA from E14.5 embryonic fibroblasts from TG.HRJ carriers. Lane 1 is from an embryo with a maternal RSVPgkmyc allele. Lane 2 is from an embryo with a paternal RSVPgkmyc allele. Lane 3 is from an embryo with a maternal and a paternal allele. **C:** Northern blots of TG.HRJ expression in embryonic cells from the TG.HRJ transgenic line. Lane 1 is from a cell line containing a paternal transgene allele, and lane 2 is from a cell line containing a maternal transgene allele.

TABLE 1. Analysis of Replication Timing in Embryonic Cells*

Cell line	Probe	Methylation differences	Expression differences	%SD	%SS	%DD	%0	%1	Number
AA4-2	Tg	+	-	13	59	26	0	2	330
AA4-3	Tg	+	-	9	63	24	1	3	123
AA4-1 ^a	Tg	+	-	4	68	28	0	0	433
AA4-1 ^a	<i>Ccnd1</i>	NT	NT	13	42	45	0	0	109
AA9 ^a	Tg	+	-	6	69	25	0	0	774
AA9 ^a	<i>Ccnd1</i>	NT	NT	8	48	41	0	3	107
AA14 ^a	Tg	+	-	6	72	21	0	1	279
AA14 ^a	<i>Ccnd1</i>	NT	NT	7	48	39	0	6	113
AA22	Tg	+	-	10	37	52	0	1	290
HR4	Tg	+	+	16	35	49	0	0	410
HQ5	Tg	-	-	6	24	67	0	3	251
HQ9	Tg	-	-	13	23	60	0	4	146

*AA4, AA9, AA14, and AA22 are embryo cell lines derived from four separate TG.AAJ homozygous embryos. AA4-1, AA4-2, and AA4-3 refer to three separate cultures of the AA4 cell line. The embryo cell line HR4 is from a homozygous TG.HRJ embryo, and lines HQ5 and HQ9 are from two separate homozygous TG.HQJ transgenic embryos. Methylation differences between parental alleles of a particular transgene were determined from Southern blot analysis (see Figs. 1, 3, and 4). Expression differences between the parental alleles of the TG.HRJ transgene in the HR4 cell line were determined by Northern blot analysis (see Figure 3C). Tg refers to the RSVIgmcy transgene construct. *Ccnd1* (cyclin D1) was used as a control FISH probe. + indicates the presence of methylation or expression differences; - indicates equal levels of methylation or expression; NT is not tested for that gene. %SD is the percentage of cells with three FISH hybridization signals; %SS is the percentage of cells with two FISH signals; and %DD is the percentage of cells with four FISH signals (see text). %0 and %1 are the percentage of cells with 0 or 1 FISH signals. Number is the total number of cells scored.

^aRefers to hybridization with a direct labeled probe (see Materials and Methods).

locus (7–13%), but similar to the levels measured in the TG.AAJ cell lines. Moreover, the 16% level of asynchrony is significantly lower than the 30–40% level found at some endogenous imprinted loci [Kitsberg *et al.*, 1993; Knoll *et al.*, 1994; LaSalle and Lalande, 1995]. We conclude from this analysis that the parental alleles of the RSVPgkmyc transgene in the TG.HRJ line replicate at the same time.

Alleles of A Nonimprinted Transgenic Line Replicate Synchronously

The expression and methylation imprinting features of the RSVIgmcy transgene are largely independent of the site of chromosomal integration [Swain *et al.*, 1987; Chaillet *et al.*, 1991, 1995]. However, this does not appear to be the case for the modified RSVPgkmyc construct shown in Figure 3A. The TG.HRJ and the TG.HQJ transgenic lines were generated with RSVPgkmyc. TG.HRJ is imprinted in mice, as shown by allele-specific differences in methylation and expression (Fig. 3). However, the TG.HQJ line is not imprinted; both alleles are highly methylated and silent (Fig. 4 and data not shown). We have not investigated the reasons underlying the difference between TG.HRJ and TG.HQJ, although the most likely reason is that the modification of RSVIgmcy to produce RSVPgkmyc (introducing other mouse sequences [*pgk-1*], as well as foreign sequences [GFP]) resulted in a construct that is more subject to genomic influences at its site of integration. The TG.HRJ line is not noticeably influenced by its chromosomal integration location, and maintains its imprinting features (see above). In contrast, the TG.HQJ transgene has most likely integrated in a chromosomal

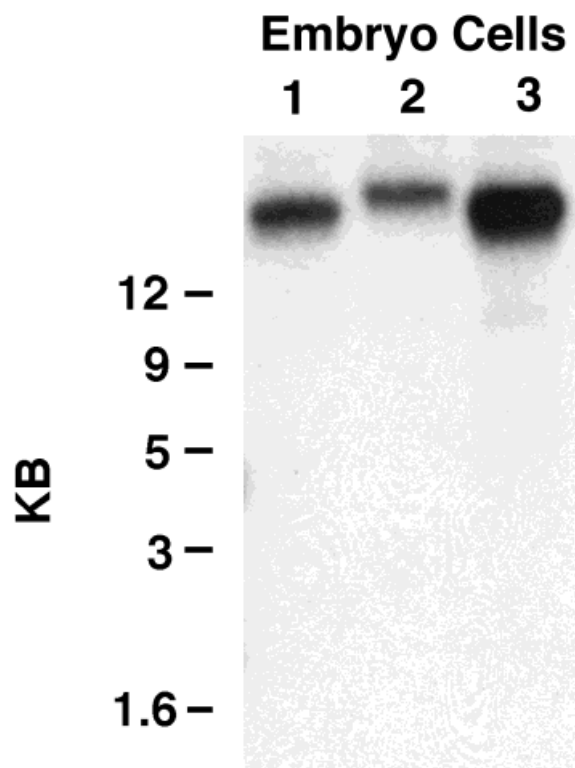


Fig. 4. Analysis of RSVPgkmyc methylation and expression in the non-imprinted TG.HQJ transgenic line and embryonic cells derived from TG.HQJ. Lanes 1–3 are Southern blots of *Hpa*II digests of genomic DNA from E14.5 embryonic fibroblasts from TG.HRJ carriers. Lane 1 is from an embryo with a maternal RSVPgkmyc allele. Lane 2 is from an embryo with a paternal RSVPgkmyc allele. Lane 3 is from an embryo with both maternal and paternal alleles if RSVPgkmyc.

location that influences its methylation, regardless of its parental origin, resulting in the same methylation pattern on both alleles. Consequently, TG.HQJ is not imprinted, as shown by identical methylation patterns on the maternal and paternal alleles (Fig. 4) and by the absence of expression from either allele (data not shown). In embryo cell lines derived from TG.HQJ carriers, the maternal allele (Fig. 4, lane 1) and paternal RSVPgkmyc allele (Fig. 4, lane 2) are methylated equally, and are identical to the pattern seen in TG.HQJ transgenic mice (data not shown). Because TG.HQJ represents a nonimprinted control transgenic line with the same RSVPgkmyc construct as the imprinted TG.HRJ line, we would expect to observe the minimal level of replication differences associated with the RSVPgkmyc transgene construct.

Hybridization of the FISH probe to two independent TG.HQJ embryo cell lines also demonstrated very low levels of replication asynchrony between the RSVPgkmyc alleles (Table 1). The frequencies of replication asynchrony observed in the two cultures (6 and 13%) are again similar to those observed in the TG.AAJ and TG.HRJ cell lines, and similar to the values reported for non-imprinted loci [Kitsberg *et al.*, 1993; Knoll *et al.*, 1994; Gunaratne *et al.*, 1995]. We conclude that the frequencies of asynchronously replicating transgenes in imprinted transgenic mouse lines (TG.AAJ and TG.HRJ) and a nonimprinted control transgenic line (TG.HQJ) are similar. These findings are consistent with the notion that allele-specific methylation and expression differences, characteristic features of imprinted genes, can be maintained in the absence of significant differences in replication timing between the parental homologs.

DISCUSSION

Measurement of Replication Timing Using FISH

FISH measures replication within single cells by measuring the number of molecules of double-stranded DNA hybridized to a fluorescently-labeled DNA hybridization probe. To detect the hybridized DNA (alleles of genes) within single cells, long hybridization probes are used that have been prehybridized with Cot-1 DNA [Lichter and Reid, 1994]. In diploid cells, replication of the two parental alleles can be synchronous or asynchronous. In principle, the measure of synchronous replication of alleles by FISH can result from two types of effects. First, there is true synchronous replication detected along the entire length of the FISH probe. Secondly, a synchronously replicating region, as well as an asynchronously replicating region are both hybridized by the FISH probe, but the asynchronously replicating area is small and below the limit of detection of the FISH method. For both of these effects, the observed results would be cells with primarily two or four FISH signals, the ratio of cells with two signals to cells with four signals indicative of the extent of DNA replication

in the cell population. In contrast, asynchronous replication of the two homologs is measured as the occurrence of three FISH signals in an S-phase cell. In a population of cells, asynchronous replication is observed when many cells (>25%) have three signals [Kitsberg *et al.*, 1993; Boggs and Chinault, 1994; Knoll *et al.*, 1994; Torchia *et al.*, 1994]. This must occur when all or a significant portion of a chromosomal region detected by a FISH probe replicates asynchronously. Any small synchronously replicating region within the sequences hybridizing to the FISH probe would not be detected in these circumstances because of insufficient hybridization to produce a visible FISH signal.

From the description above of the FISH method as applied to the measure of replication timing, we would expect that each cell within a synchronously replicating population of cells would exhibit either two or four FISH signals. Alternatively, for an asynchronously replicating population of cells, we would expect to observe a significant proportion of cells with three FISH signals. Many genes have been examined for replication timing differences between parental homologs using the FISH method. For all such autosomal genes examined, there appears to be a minimal percentage of cells that have three FISH signals, in the range of 5–15% [Kitsberg *et al.*, 1993; Knoll *et al.*, 1994]. Because it is believed that many of these loci replicate synchronously, there appears to be a background level of three FISH signals at synchronously replicating loci. There are a number of possible explanations for this background level of three signals, including technical difficulties in observing all allelic FISH signals in individual cells, and a real, but detectable, minor degree of asynchronous replication present at virtually all loci [Kitsberg *et al.*, 1993; Knoll *et al.*, 1994]. At present, it is not possible to distinguish between these different explanations. However, FISH experiments to measure replication timing at individual autosomal loci have been interpreted as detecting asynchronous replication if the percentage of three FISH signals has been significantly greater (25–40%) than the background level [Kitsberg *et al.*, 1993; Knoll *et al.*, 1994; LaSalle and Lalande, 1995].

In light of the preceding discussion and review of published reports on the measurement of replication timing [Kitsberg *et al.*, 1993; Knoll *et al.*, 1994; LaSalle and Lalande 1995], we conclude that the three different transgenes examined in this report all replicate synchronously. In particular, the TG.HRJ line, which is imprinted both by expression and methylation differences between the maternal and paternal alleles, exhibits a nearly identical percentage of three FISH signals as the non-imprinted TG.HQJ line. If the percentage of three FISH signals in cells from the nonimprinted TG.HQJ line represent the background of three FISH signals at an absolutely synchronously replicating locus, then the imprinted TG.AAJ and TG.HRJ lines also are replicating synchronously. Therefore, replicating timing differ-

ences are not always associated with other molecular features of imprinted loci.

Allele-Specific Replication Timing Differences in Genomic Imprinting

Replication asynchrony is often associated with imprinted genes. It has been observed to coincide with methylation and/or expression differences between the parental alleles of imprinted genes, including genes with the imprinted *Igf2/H19* region and genes within the PWS/AS region [Kitsberg *et al.*, 1993; Knoll *et al.*, 1994; LaSalle and Lalande, 1995]. In particular, studies of the imprinted SNRPN gene suggest that replication asynchrony may be associated only with imprinted expression differences in lymphocytes, fibroblasts, and lymphoblastoid cells [Kawame *et al.*, 1995]. In HeLa cells that do not express *SNRPN*, but presumably exhibit allele-specific differences in DNA methylation, the parental alleles replicate synchronously [Kawame *et al.*, 1995]. To further address the importance of expression on replication asynchrony, we examined the timing of replication in both expressing and nonexpressing imprinted transgene loci in the same cell types. Moreover, the transgene FISH experiments were performed using a FISH probe containing all transgene sequences required for its imprinting. Regardless of the presence or absence of allelic differences in expression and/or methylation, we always observed replication synchrony at the three transgene loci.

The often observed association of replication asynchrony with endogenous imprinted loci suggests that there may be a *cis*-acting element of these endogenous loci that promotes replication asynchrony. Such a component does not appear to be present in the transgene. However, if a *cis*-acting element affecting the timing of replication between parental alleles could be characterized, the addition of such an element to RSVIgmyc would be expected to promote replication asynchrony while maintaining differences in methylation and expression. However, in the absence of such a defined element, we can only conclude that differences in the timing of replication between parental alleles at imprinted loci are the consequence, rather than the cause, of allelic differences in expression and methylation.

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