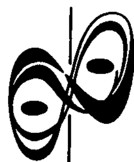
POSTERS

GENOMIC IMPRINTING

https://doi.org/10.1017/S0001566000001318 Published online by Cambridge University Press



Acta Genet Med Gemellol 45: 207-212 (1996)
© 1996 by The Mendel Institute

Asynchronous Replication Patterns of Imprinted Genes in Triploid Cells

A. Argyriou-Tirita[1], K. Romanakis[2], P. Kroisel[3], O. A. Haas[1]

[1]CCRI, St. Anna Children's Hospital, Vienna, Austria; [2]Department of Human Biology and Human Genetics, University of Kaiserslautern, Germany; [3]Institute of Human Biology and Genetics, Graz, Austria

INTRODUCTION

Several unique features distinguish imprinted from nonimprinted genes, including the unusual replication behavior the unequal methylation and the differential expression of imprinted alleles [1]. The replication timing in S phase of the two homologous alleles of a normal, nonimprinted gene is highly synchronous [2, 3]. Housekeeping genes replicate early, constitutive heterochromatic regions replicate late and tissue-specific genes replicate earlier when they are expressed than when they are not [2-4]. In contrast, imprinted genes which, by definition, display allele-specific expression replicate asynchronously [2-5].

The relative order of replication of homologous alleles as well as that of different loci can be elegantly compared with fluorescence in situ hybridization (FISH) on interphase nuclei [2-5]. Unreplicated DNA segments give singlet hybridization signals in normal diploid cells, while replicated loci are characterized by doublets. The distribution of these two patterns can be used to determine the S phase replication time of any DNA sequence. Moreover, determination of the singlet/doublet ratio allows a good estimation of the degree of replication asynchrony of two homologous alleles [2-5].

Using cell lines with deletions, disomies or associated FISH-detectable centromeric satellite polymorphisms, Kitsberg et al. [4] found that the paternal allele was the early replicating one in all the imprinted genes which they had analyzed. Subsequently, however, Knoll et al. [5] detected genes in the imprinted Prader-Willi region on chromosome 15, which also displayed other patterns. Therefore, it seems necessary to specify the relative timing of maternally and paternally derived alleles for each individual asynchronously replicating gene. Unfortunately, this is so far only feasible with a very restricted number of sequences.

Based on the fact that incomplete hydatidiform moles and triploid fetuses are com-

https://doi.org/10.1017/S0001566000001318 Published online by Cambridge University Press

posed of either two complete paternal and one maternal or two maternal and one paternal haploid chromosome set [6, 7], we have devised a method which should allow the unequivocal assignment of the parental origin to the early and late replicating allele.

Cells, cell lines and tissues

For evaluation and determination of the degree of asynchrony of the employed probes, we used diploid, phytohemagglutinin (PHA) stimulated peripheral blood (PB) cells (PB+PHA). To be able to detect the DNA-synthesizing cells (positive cell fraction: PB+BRDU; negative cell fraction: PB-BRDU), BRDU incorporation was used in some experiments. Identification of the S phase cells was accomplished with an indirect immunofluorescence technique using an anti-BRDU antibody together with the FISH assay. The detailed method has been described previously [2, 4]. For the demonstration of the parental origin of the early and late replicating alleles of probes located in the Prader-Willi syndrome (PWS) region, we also used PHA-stimulated PB cells from a patient with PWS who had a deletion 15(q11-13) (PB-PW). Unstimulated isolated mononuclear PB cells served as a control for G1 phase cells and were therefore expected to display only singlet signals (SS).

Asynchronous probes were evaluated on triploid tissues from three different sources: an Epstein-Barr-virus transformed B cell line from a triploid girl who had survived for 6 months, cells from a partial hydatidiform mole and cells from an aborted triploid fetus. Since we were unable to obtain parental material from the respective individuals and tissues, we had to deduce the parental composition of the haploid chromosome sets from the phenotypes of the tissues. This is feasible, because it is known that partial moles are exclusively composed of two paternal and one maternal set of chromosomes [6, 7]. Moreover, depending on the parental origin of the additional chromosome set, two distinct types of embryonic/fetal phenotype and placental development can be distinguished. A paternal extra haploid set correlates with a relatively normal fetal growth and a large cystic placenta, whereas an additional maternal set is associated with intrauterine growth retardation, relative macrocephaly and a small noncystic placenta [6, 7]. According to these criteria, we presumed the B cell line and the triploid fetus to be composed of two maternal and one paternal haploid chromosome sets (designated as MMP-B and MMP-F, respectively). The incomplete hydatidiform mole, on the other hand, was presumed to consist of two paternal and one maternal haploid chromosome sets (MPP- M).

Probes

For FISH analysis we used probes whose replication behavior had already been established. Altogether there were six probes from the PWS region, five of which (GABRB3, ZNF127, D15S9, D15S11 and D15S13) are known to replicate asynchronously, with the paternal allele replicating early. One probe (D15S17) replicates synchronously late and was used as a control.

In addition, we analyzed several probes from three different gene regions on three different chromosomes. The insulin-like growth factor receptor (IGF2R, clone SBV) is

https://doi.org/10.1017/S0001566000001318 Published online by Cambridge University Press

located on chromosome 6q27. Three different cosmids (cos 4, cos ca-9-1 and cos 18) were available from the *ABL* oncogene on chromosome 9q34 and a YAC clone (D107F9) as well as a cosmid (cos bridge 2) were available from the *BCR* gene on chromosome 22q11. All these regions are known to replicate asynchronously; however, it has not yet been established which allele replicates early and which late.

The probes GABRB3 and D15S11 were obtained from Oncor (Gaithersburg, M., USA). All other probes from the Prader-Willi region (ZNF127, D15S9, D15S13 and D15S17) were a kind gift from Rob Nicholls (Department of Genetics, Case Western Reserve University, Cleveland, Ohio, USA). The ABL and BCR clones were kindly provided by Bruce Roe (Department of Chemistry and Biochemistry, University of Oklahoma, Norman, Okla., USA) and Christoph Lengauer (currently at the Johns Hopkins Oncology Center, Molecular Genetic Laboratory, Baltimore, M.D., USA) and the SBV clone of the IGF2R gene was a kind gift from Denise P. Barlow (Research Institute of Molecular Pathology, Vienna, Austria).

Results and Discussion

The results of our investigations are summarized in Tables 1-3.

The employed FISH assay is an easy and efficient method for studying synchronous and asynchronous replication patterns of homologous alleles. In diploid cells, the asynchronous probes clearly showed the expected pattern. The asynchrony was very pronounced within the S phase fraction. However, it should be noted that a small fraction of the BRDU-negative cells also displayed this pattern. Even more intriguing is the high proportion of nonlabeled cells with two doublets. It is unlikely that this high level is due to a technical, artifact, but it is similarily unlikely that this cell fraction contains such a high number of G2 cells for which this pattern would be typical. This finding challenges the current opinion that this pattern exclusively indicates the replication status of the respective gene sequence. Perhaps this pattern rather reflects the expression status of the respective gene, since a strict correlation has been demonstrated between the expression and relative time of replication [8]. The notion that the

Table 1 - Percentages of S phases in diploid and triploid cells as determined with BRDU incorporation for various time periods

Cells	Hours +BRDU	%
PHA-PB	1	20
MMP-B	1	5
	2	18
MMP-F	4	15
MPP-M	5	12

https://doi.org/10.1017/S0001566000001318 Published online by Cambridge University Press

Table 2 - Replication pattern analysis of asynchronous-replicating probes on PHA-stimulated PB cells of normal controls and one PWS patient with a deletion of region 15(q11-13)

Probes	PB-PHA (BRDU)			PB+BRDU			PB-PW	
	SS	*SD*	DD	SS	*SD*	DD	*S*	D
Asynchronous								
IGF2R[a]	42	*35*	23					
ABL ca-9-1				37	*41*	22		
BCR D107F9	34	*13*	44	4	*36*	60		
Asynchronous, paternal early								
GABRB3	38	*21*	41	28	*64*	8	*92*	8
D15S11				44	*33*	23	*78*	22

The asynchronous pattern of normal cells and the early replication pattern of the nondeleted, paternally derived allele from the PWS patient are shown in italics.

S = Singlet; D = doublet; SS = singlets (synchronous late); SD = singlet + doublet (asynchronous); D = 2 doublets (synchronous early).

[a] IGF2R was not used on BRDU-labeled cell material.

SS, SD and DD patterns obtained with the FISH assay may also reflect mono and/or biallelic expression and, therefore, indirectly gene activity, is further supported by the fact that with probes from the ABL and BCR genes, a considerable fraction of granulocytes, which are definitely in G1 phase seem to display a DD pattern. Unstimulated, isolated mononuclear blood cells, on the other hand, show a SS pattern (data not shown).

Our analysis is based on the assumption that the determination of the paternal and maternal origin of the three haploid chromosome sets in triploid conceptions should be possible with a single probe, if one takes advantage of probes from defined asynchronously replicating regions, such as the PW/Angelman region on chromosome 15. This could even be accomplished without comparing with parental polymorphisms. In addition to being simple and fast, this analysis could be performed on interphase nuclei of any tissue. Moreover, once the distribution of the haploid sets in a particular tissue is established, the paternal and maternal origin of early and late replicating alleles of any asynchronously replicating gene can easily be determined with such cells.

That this approach is most probably feasible can be deduced from our results which have been obtained with various asynchronously replicating probes on different triploid tissues (Table 3). Using asynchronously replicating probes, we were able to demonstrate an uneven distribution in all instances. Moreover, this pattern was generally identical for several different probes from the same gene region (e.g. ABL, BCR). Unfortunately, the probes from the PWS region did not always reveal the expected pattern (e.g. GABRB3 in MMP-F and MPP-M). This finding is a further indication that the asynchronous replication pattern is not such a stable feature of cells as has previously been claimed. This

https://doi.org/10.1017/S0001566000001318 Published online by Cambridge University Press

Table 3 - Replication pattern analysis of synchronous- and asynchronous-replicating probes on three different triploid tissues

Probes	MMP-B				MMP-F				MPP-M			
	SSS	SSD	SDD	DDD	SSS	SSD	SDD	DDD	SSS	SSD	SDD	DDD
Asynchronous												
IGF2R	2	16	*48*	34	1	8	*63*	28	32	*39*	13	16
ABL 18	34	*43*	9	14								
ABL 4					41	*38*	9	12				
ABL ca-9-1					39	*45*	4	12				
BCR D107F9	1	8	*58*	33	1	8	*62*	14	14	16	*46*	24
BCR bridge 2	10	13	*52*	25	10	13	*52*	25				
Asynchronous, paternal early												
GABRB3	13	11	*45*	31	10	*38*	10	42	2	4	*52*	42
ZNF127	18	13	*36*	33	2	8	56	34	10	*58*	3	28
D15S9	16	20	*36*	28	3	23	*47*	27	21	*49*	16	14
D15S11	5	20	*50*	25								
D15S13					14	*65*	13	8	28	*62*	8	2
Synchronous late												
D15S17					*90*				*98*	1	1	

The asynchronous pattern is emphasized in italics.

SSS = 3 singlets (synchronous late); DDD = 3 doublets (synchronous early); SSD = 2 singlets + 1 doublet (asynchronous; depending on the composition of the three haploid sets either 2 maternal or 2 paternal copies late replicating); SDD = 1 singlet + 2 doublets (asynchronous; depending on the composition of the three haploid sets either 2 maternal or 2 paternal copies early replicating).

discrepancy may be either due to different patterns in different tissues or to a temporary switch of the replication pattern within a particular tissue. Both possibilities are conceivable, since it has been shown that asynchronous replication is strictly correlated with gene expression [8]. Therefore, epigenetic modifications which are responsible for transcription regulation may perhaps not only alter gene expression in different tissues as well as during in vitro propagation of cells, but also influence the replication behavior of these gene sequences. On the other hand, it cannot be excluded that the additional homologue in triploid cells may also exert an unforeseeable effect. To make full use of our new method, it is undoubtedly necessary to repeat our experiments on triploid tissues whose parental composition has unequivocally been determined by molecular genetic means.

Our preliminary results obtained with this approach underline the previous finding of Smrzka et al. that the human IGF2R replicates asynchronously and, therefore, is proba-

https://doi.org/10.1017/S0001566000001318 Published online by Cambridge University Press

bly imprinted. We also confirm that the *ABL* and *BCR* genes replicate asynchronously. Owing to the uncertainty associated with the assignment of the parental origin in the triploid cells studied, it was unfortunately not possible to reach firm conclusions with regard to the parental origin of the early and late replicating alleles in these genes.

Acknowledgments: This work was made possible by grants from the österreichische Kinderkrebshilfe, the European Community (grant No. CA-CT94-1703), the Fonds zur Förderung der wissenschaftlichen Forschung (grant No. P7777) and the Jubiläumsfonds der österreichischen Nationalbank (grant No. 3838).

REFERENCES

1. Efstratiadis A: Parental imprinting of autosomal mammalian genes. Curr Opin Genet Dev 1994;4: 265-280.
2. Selig S, Okumura K, Ward DC, Cedar H: Delineation of DNA replication time zones by fluorescence *in situ* hybridization. EMBO J 1992;11: 1217-1225.
3. Brandeis M, Kafri T, Ariel M, Chaillet JR, McCarrey J, Razin A, Cedar H: The ontogeny of allele-specific methylation associated with imprinted genes in the mouse. EMBO J 199332: 3669-3677.
4. Kitsberg D, Selig S, Brandeis M, Simon I, Keshet I, Driscoll DJ, Nicholls RD, Cedar H: Allele-specific replicaton timing of imprinted gene regions. Nature 1993;364: 459-463.
5. Knoll JHM, Cheng SD, Lalande M: Allele specificity of DNA replication timing in the Angelman/Prader-Willi syndrome imprinted chromosomal region. Nat. Genet 1994; 6: 41-46.
6. McFadden DE, Kalousek DK: Two different phenotypes of fetuses with chromosomal triploidy: Correlation with paternal origin of the extra haploid set. Am J Med Genet 1991; 38: 535-538.
7. McFadden DE, Kwong LC, Yam IYL, Langlois S: Parental origin of triploidy in human fetuses: Evidence for genomic imprinting. Hum Genet 1993;2: 465-469.
8. Boggs BA, Chinault AC: Analysis of replication timing properties of human X-chromosomal loci by fluorescence in situ hybridization. Proc Natl Acad Sci USA 1994;91: 6083-6087.

Correspondance: Prof. Dr. Oskar A. Haas, Children's Cancer Research Institute (CCRI), St. Anna Children's Hospital, Kinderspitalgasse 6, A - 1090 Vienna, Austria, E-mail: o.a.haas@magnet.at

https://doi.org/10.1017/S0001566000001318 Published online by Cambridge University Press