CpG methylation of an X-linked transgene is determined by somatic events postfertilization and not germline imprinting

ANDREW COLLICK*, WOLK REIK, SHEILA C. BARTON and M. AZIM H. SURANI

Summary

The process of X-inactivation in mammals requires at least two events, the initiation of inactivation and the maintenance of the inactive state. One possible mechanism of control is by methylation of DNA at CpG dinucleotides to maintain the inactive state. Furthermore, the paternal X-chromosome is frequently inactivated in the extraembryonic membranes. The relationship between the parental origin of the chromosome, nonrandom inactivation and DNA methylation is not clear. In this paper, we report on the CpG methylation of an X-linked transgene, CAT-32. The levels of methylation in embryonic, extraembryonic and germline cells indicates that the modifications of the transgene are broadly similar to those reported for endogenous X-linked genes. Interestingly, the methylation of CAT-32 transgene in extraembryonic tissues displays patterns that could be linked to the germline origin of each allele. Hence, the maternally derived copy of CAT-32 was relatively undermethylated when compared to the paternal one. The changes in DNA methylation were attributed to de novo methylation occurring after fertilization, most probably during differentiation of extraembryonic tissues. In order to determine whether or not the patterns of DNA methylation reflected the germline origin of the X-chromosome, we constructed triploid embryos specifically to introduce two maternal X-chromosomes in the same embryo. In some of these triploid conceptuses, methylation patterns characteristic of the paternally derived transgene were observed. This observation indicates that the methylation patterns are not necessarily dependent on the parental origin of the X-chromosome, but could be changed by somatic events after fertilization. One of the more likely mechanisms is methylation of the transgene following inactivation of the X-chromosome in extraembryonic tissues.

Key words: X-chromosome inactivation, DNA methylation, germline imprinting, transgene, fertilization, mouse embryo.

Introduction

Female mammals possess two X-chromosomes, one of which is inactive in almost every cell of the embryonic and extraembryonic lineages, and all tissues in the adult organism. X-chromosome inactivation occurs at different times in different lineages in the female conceptus (Epstein et al. 1978; Monk & Harper, 1979; Rastan et al. 1980; Monk, 1981; Takagi et al. 1982). The molecular mechanisms for X-inactivation and the maintenance of this state are unknown. However, the methylation of CpG dinucleotides has been proposed as one possible mechanism controlling X-inactivation (Riggs, 1975; Holliday & Pugh, 1975).

X-inactivation is thought to be initiated near the genetically defined X-inactivation centre Xce (Cattanach et al. 1970) but also depends on other controlling sequences necessary for the coherent regulation of the whole chromosome (Monk, 1981, 1986). The methylation status of these sequences is unknown. However, there are regions of mouse and human genes that are methylated only when present on the inactive X-chromosome. These include the CpG-rich islands of the housekeeping genes, hypoxanthine phosphoribosyl transferase (HPRT) (Wolf et al. 1984b; Yen et al. 1984), glucose-6-phosphate dehydrogenase (Wolf et al. 1984a; Toniole et al. 1984) and...
phosphoglycerate kinase (Keith et al. 1986). Methylation of these sequences may contribute to maintaining the chromosome in an inactive state. This process appears to be restricted to somatic tissues of eutherian mammals, where X-chromosomes of either parental origin are inactivated in a random fashion. In other tissues, X-chromosome inactivation is not accompanied by methylation. Hence CpG-rich regions on the inactive X-chromosome are undermethylated in sperm (Venolia & Gartler, 1983), in the extra-embryonic tissues of eutherian mammals where the paternal chromosome is inactive (Kratzer et al. 1983) and in the somatic tissues of marsupials (Kaslow & Migeon, 1987).

Transgenic DNA integrated on the mouse X-chromosome has previously been used to study the process of inactivation and methylation. In the case of an alphafetoprotein transgene (AFP), inactivation was observed in the liver but not yolk sac (Krumlauf et al. 1986). In another transgenic strain, a chicken transferrin gene escaped inactivation even when present on the inactive X-chromosome (Goldman et al. 1987). In both instances, differences in methylation between the allele on the active and the inactive X-chromosome could not be detected.

In this paper, we investigate the methylation of an X-linked transgene, CAT-32. Differences in the methylation status of the transgene in the inactive and active X-chromosome were observed in extraembryonic tissues. Since there is preferential inactivation of the paternal X-chromosome, these observations could be attributed to germline-specific imprinting of the transgene locus. Such a phenomenon has recently been demonstrated for a number of autosomal transgenic loci (Reik et al. 1987b; Sapienza et al. 1987; Swain et al. 1987). However, we could not rule out the possibility that the difference was associated only with the inactive X-chromosome in the extraembryonic tissue. Hence we used nuclear transplantation techniques to distinguish between these two explanations. Digynic triploid zygotes with two maternal X-chromosomes were constructed and, in these, it was hoped to force inactivation of a normally active maternally derived X-chromosome in the extraembryonic tissues bearing the transgene, CAT-32. This approach was based on previous observations that showed that, at least in some instances, maternal X-chromosomes can be inactive in parthenogenetic and in digynic triploid conceptuses (Kaufman et al. 1978; Jacobs et al. 1979 and Rastan et al. 1980). Hence, methylation analysis in triploid embryos could potentially distinguish between strictly germ-cell-specific patterns of DNA methylation, and patterns arising from events after fertilization.

### Materials and methods

**Origin of strain CAT-32**

Strain CAT-32 was one of a number of transgenic strains described by Reik et al. (1987a). Briefly, it contains five to six copies of a chloramphenicol acetyl transferase gene (CAT) linked to an immunoglobulin heavy chain enhancer sequence (IgH-E), details of which are shown in Fig. 1. The CAT-32 transgenic locus is not expressed in lymphoid cells, or in other tissues (Reik et al. 1987a).

**Embryo manipulation**

For all matings involving wild type (C57BL6 × CBA)F1 and CAT-32 mice, the day of the vaginal plug was taken as the first day of gestation, the day when fertilized eggs were recovered (Barton et al. 1984). To generate digynic triploid embryos, a female pronucleus was transferred from the wild-type (F1 × F1) egg to a (F1 × CAT-32) egg, as described previously (McGrath & Solter, 1984; Barton et al. 1984). Diploid and the reconstituted triploid eggs were cultured overnight in M16 plus 0.4% BSA at 37°C in 5% CO2 in air (Whittingham, 1971). On the next day, two-cell embryos were transferred to the oviduct of day-1 pseudopregnant wild-type females, of F1 × F1 genotype. Conceptuses resulting from normal and reconstituted eggs were recovered from recipients on day 10 or day 14 of gestation. The conceptuses were dissected to separate the embryo, yolk sac and trophoblast. DNA was then prepared from each component tissue as described below.

**Analysis of CpG methylation**

Embryonic tissues were homogenized in PBS, and cells resuspended in 100 mM-NaCl, 25 mM-EDTA pH 8.0, 10 mM-

---

![Fig. 1](image-url)  
**Fig. 1.** The construct used in the preparation of strain CAT-32 (Reik et al. 1987a). In brief, it contains a promoter and poly(A) addition site from SV40, flanking a chloramphenicol acetyl transferase gene (CAT). This is linked to an immunoglobulin heavy chain enhancer sequence (IgH-E). The four internal HpaII sites all lie within the CAT sequence, and are marked H1, H2, H3 and H4. Complete digestion at these sites will give restriction fragments of sizes 0.05, 0.15 and 0.40 kb. Note that the 0.55 kb fragment discussed in main text and subsequent figures will arise as a result of digestion at the HpaII sites H3 and H4, and not H2. Solid bar: SV40 promotor; open bar: CAT sequences and SV40 poly(A) addition site; stippled bar: Ig heavy chain enhancer. B, E and HIII represent recognition sites for the restriction enzymes BamHI, EcoRI and HindIII, respectively.
was digested with restriction enzymes BamHI and HpaII. Precipitation and dissolved in TE (10 mM-Tris-HCl pH 8.0, 1 mM-EDTA pH 8.0), chloroform and the DNA was isolated by isopropanol precipitation and dissolved in TE (10 mM-Tris-HCl pH 8.0 and 1% SDS; 200 µg/ml 1 Proteinase K recommended by the suppliers (NBL, NEB). The DNA digestions. After initial autoradiographic exposures, those fragments were stripped with 0.4M-NaOH at 42°C and 40 min, and reprobed with probe pY353/8 specific for the X-chromosome (Bishop et al. (1976)).

Spermatogonia were isolated from testis cell suspensions of 10-day-old male mice by means of an overnight culture step. A suspension of cells from testis was prepared as described by Romrell et al. (1976) and cultured for 16 h in DMEM + 10% FCS at 37°C in 5% CO2 in air. During this culture period, Sertoli cells attach to the culture dish (Falcon), whilst spermatogonial cells remain in suspension. Supernatants (spermatogonial) and attached cells (Sertoli cells) were harvested separately and DNA was prepared as described in Reik et al. (1987a).

**Results**

**Methylation of embryonic CAT-32 alleles**

To determine the effect of maternal versus paternal origin on CpG methylation, we have recently carried out an analysis of various strains of transgenic mice (Reik et al. 1987b). Transgenic strain CAT-32 was initially included in this study. However, from the breeding of mice carrying the CAT-32 locus, it became apparent that this transgene was not an autosomal integral. Whilst heterozygous females bred with wild-type males gave rise to positive male and female offspring, male carriers of the locus when bred with wild-type females gave only positive female and negative male offspring (Table 1 and Fig. 2). This indicated that the transgenic locus CAT-32 was present on the X-chromosome.

CpG methylation of the transgene was determined in midgestation embryos, following mating of CAT-32 mice with wild-type animals. After both male and female derivation, the transgene was found to be methylated as judged by restriction enzyme digestion with BamHI plus HpaII (Fig. 2 lanes A–C and D–I). However, some of the embryos that contained a maternally derived transgene showed a consistent increase in the intensity of the lower molecular weight fragments, notably 0.8 kb. This indicated lower levels of CpG methylation in these cases (Fig. 2, lanes A and C). Reprobing these filters with a probe that detects sequences located on the Y-chromosome (pY353/8, Bishop et al. 1983), we were able to demonstrate that only male embryos display this slight decrease in methylation (Fig. 2, lanes A and C). In male embryos, the X-chromosome bearing the transgene is active in all cells, whereas in heterozygous female embryos because of random X-chromosome inactivation, it is only active in 50% of cells. It appears therefore that the overall level of transgene methylation in day-10 embryonic tissues is relatively high, irrespective of transmission from mother or father. Methylation of the transgene is, however, slightly decreased when it is present on the active, as compared to the inactive, X-chromosome.

**Methylation of extraembryonic CAT-32 alleles**

Comparison of the CpG methylation of male- and female-derived alleles of CAT-32 in extraembryonic tissues at the same gestational time showed an obvious difference. While overall levels of methylation were much lower than in embryonic tissues, paternal CAT-32 alleles generated roughly equal intensities of a 0.55 and a 0.4 kb fragment, whereas maternal alleles showed a 5- to 10-fold stronger signal of the 0.4 kb over the 0.55 kb fragment (Fig. 3, compare lanes A, B and F with lanes C and E). This difference in intensity shown by the 0.4- and 0.55 kb fragments arises as a consequence of differential methylation at HpaII site H3 (Fig. 1), with the paternally derived allele being relatively more methylated at this site. Change of sex of the transmitting parent resulted consistently in a change of the methylation at site H3 (followed through three generations, with transmission from ten CAT-32-positive parents, data not shown). This difference in methylation of site H3 was...
Fig. 2. DNA from day-10 embryos was analysed for CpG methylation of the CAT-32 transgenic locus. Shown are samples of DNA from embryos, having a female (lanes A–C), or male (lanes D–I) CAT-32-positive parent, digested with the restriction enzyme *BamH*I and *HpaII* and hybridized to a CAT-specific probe (see Methods). In both cases, the overall pattern is one of relatively high CpG methylation levels. The fragments corresponding to those produced by *BamH*I alone (3-2, 3-0 and 2-7 kb) remain the major ones, although minor lower molecular weight fragments are present indicating that part of the locus is unmethylated in embryonic DNA. The sex of each embryo was determined using the Y-chromosome-specific probe pY353/8 (Bishop et al. 1983). From a CAT-32-positive male parent, only CAT-32-positive female offspring are produced (see Table 1). This is demonstrated by lanes D, F, G and H containing DNA from CAT-32-positive female embryos, and lanes E and I DNA from CAT-32-negative male embryos. However, with a female CAT-32-positive parent, both male (lanes A and C) and female (lanes B) CAT-32-positive embryos are produced. The low molecular weight *HpaII* fragments, notably that of 0-8 kb, are relatively more intense in male embryos (lanes A and C) than female embryos (lane B), indicating a decrease of methylation in male embryos.

seen only in the trophoblast component of the extra-embryonic tissues, and not the yolk sac (data not shown). Furthermore, the difference in methylation at site H3 was stably maintained from day 10 to at least day 14 (Fig. 3). It is possible, however, that day-14 trophoblast was contaminated with cells from parietal and visceral yolk sac. Nevertheless, the differences in the 0-55 and 0-4 kb fragments is evident (Fig. 3 lanes E and F). Since the yolk sac does not generate either the 0-4 or the 0-55 kb fragment (not
CpG methylation of an X-linked transgene

Fig. 3. Extraembryonic DNA was prepared from trophoblast tissues of day-10 and day-14 conceptuses. For the latter, DNA was prepared from the ectoplacental region. Care was taken to remove as much yolk sac from the edge of this tissue during the physical dissection of the conceptus. (It is known that yolk-sac cells contain a methylated CAT-32 locus (not shown), and thus any contamination by such cells would not influence the amounts of 0-40 and 0-55 kb HpaII fragments generated.) Samples were digested with the restriction enzymes EcoRI and HpaII, electrophoresed and hybridized with a CAT-specific probe (see Methods). Lanes A and B maternally derived transgene; lane C paternally derived transgene; lane D EcoRI and MspI as a control for complete demethylation. Lane E, paternally derived transgene; lane F maternally derived transgene. Paternal transmission generates 0-55 and 0-4kb fragments, whereas maternal transmission results in the almost exclusive presence of the 0-4 kb fragment. Hence, HpaII site H3 (Fig. 1) is almost completely demethylated on maternal transmission (compare with lane D), whereas it is partially methylated on paternal transmission.

shown), the presence of some contaminating yolk sac cells within the day-14 trophoblast will not affect the result.

The CAT-32 locus contains both head-to-tail and head-to-head arrangements of the CAT IgH-E construct (not shown), with an approximate copy number of 5–6. Using restriction enzyme digests that released distinct fragments from the head-to-tail or head-to-head regions, we determined that the differential methylation of site H3 was not confined to a specific part of the transgenic locus (not shown).

In extraembryonic tissues, the maternally derived X-chromosome is preferentially active and the paternal one is inactivated (Takagi & Sasaki, 1975; West et al. 1977; Harper et al. 1979). Because the active X-chromosome is always of maternal descent and the inactive homologue of paternal descent, the methylation difference could have been caused by two processes. First, it could have been a consequence of activity versus inactivity of the transgene-bearing X-chromosome. Or second, it could have arisen as a product of germline-specific imprinting acting on maternally or paternally derived alleles of the CAT-32 locus. If so, then this difference would have been retained in the trophoblast and not the embryo, irrespective of later activity of the chromosome.

Methylation of the CAT-32 locus in conceptuses containing two maternal X-chromosomes

In order to distinguish whether germline-specific imprinting or later events after fertilization including X-chromosome activity was most important in determining the methylation of the extraembryonic CAT-32 transgene, we devised an experiment in which it was hoped that the X-chromosome carrying a maternally derived CAT-32 locus might be inactivated. The X-inactivation of maternally derived X-chromosomes has been observed in the extraembryonic cells of diploid parthenogenetic conceptuses (Kaufman et al. 1978; Rastan et al. 1980), as well as in those of human digynic triploid embryos (Jacobs et al. 1979). However, the extraembryonic development of parthenogenones is known to be poor (reviewed Surani et al. 1986). Therefore, it was decided to use digynic triploid conceptuses in order to try to force inactivation of maternally derived X-chromosomes carrying the CAT-32 locus. It was hoped that, by this approach, enough extraembryonic tissue for biochemical analysis might be obtained. If only one of the two maternal X-chromosomes remains active, then each digynic triploid cell will contain an active or an inactive X-chromosome bearing the CAT-32 locus and, on average, half the extraembryonic cells pres-
A. Collick, W. Reik, S. C. Barton and M. A. H. Surani

Fig. 4. Analysis of CpG methylation of maternally derived CAT-32 loci present in six digynic triploid embryos made by pronuclear transfer. Each experimental lane (D–I) contains the total DNA obtained from one conceptus digested with EcoRI and HpaII, subjected to electrophoresis on minigels and hybridized to the CAT probe. In each case, the recovered DNA was less than 0.2 μg in total. Subsequent autoradiography was for a period of 10–14 days. Control lanes show paternally derived (C) and maternally derived (A and B) copies of the CAT-32 locus from normal extraembryonic tissues. The pattern of low molecular weight fragments seen after electrophoresis through minigels is that the male-derived CAT-32 control generates visible 0.4 and 0.55 kb fragments, but in the female control the 0.4 kb is the only band present in this region. This is a pattern consistently observed with such minigel analysis of male- and female-derived extraembryonic CAT-32 loci.

ent will have the CAT-32 locus on the inactive X-chromosome. If germline imprinting and the maternal origin of the X-chromosome were responsible for the observed methylation pattern upon maternal transmission, the pattern would not change with a change of activity of the chromosome. If, however, a change of methylation were observed, imprinting could essentially be ruled out as a cause.

However, the inactivation status of the two female-derived X-chromosomes in triploid conceptuses on day 10 is not known. Endo et al. (1982) examined mouse digynic triploids on day 7 or 8 of gestation. They were unable, using histological methods, to detect the extraembryonic inactivation of maternally derived X-chromosomes. This contrasts with significant inactivation of maternal X-chromosomes in the extraembryonic cells of parthenogenetic conceptuses (Kaufman et al. 1978; Rastan et al. 1980). Furthermore, Jacobs et al. (1979) examined human triploid conceptuses and were able to detect the extraembryonic inactivation of maternal X-chromosomes. While in this study the actual inactivation status of the X-chromosome would not be known, a change of female-derived extraembryonic transgene methylation pattern would still be indicative of a postfertilization event, whereas the observation of no change would be suggestive of the influence of germline imprinting.

Accordingly, in embryos of genotype (CAT-32 × F1), a female pronucleus extracted from wildtype eggs (F1 × F1) was added. With this procedure, embryos of genotype XmCAT-32, Xp, Xm or XmCAT-32, Y, Xm were produced, trophoblasts of which were expected to be a mixture of XmCAT-32, Xm and XmCAT-32, Xm genotypes with respect to the activity of the two maternal X-chromosomes. Of 94 digynic triploid embryos transferred to recipient mice, ten conceptuses were recovered on day 10 of gestation. These showed embryonic malformation characteristic of digynic triploid embryos (S.C.B. and A.C. unpublished; Surani & Barton, 1983). DNA analysis using the Y-specific probe pY353/8 (Bishop et al. 1983) showed eight out of ten to be of XXY genotype (not shown).

Seven out of these ten digynic triploid conceptuses carried the CAT-32 transgene and the DNA from six of them was subjected to methylation analysis via restriction enzyme digestion with EcoRI and HpaII. In three of these conceptuses the 0.55 kb fragment was clearly seen to be present in addition to the 0.4 kb fragment (Fig. 4, lanes D, E and H). One triploid conceptus showed only the 0.4 kb band (F) and in the
CpG methylation of an X-linked transgene

A B C D E F

Fig. 5. Methylation of CAT-32 locus in male germ cells. Spermatogonia and Sertoli cells from transgenic carriers were separated by an overnight culture step as described in Methods, and DNA prepared from each. DNA was digested with BamHI plus HpaII with subsequent hybridization to the CAT-specific probe (see Methods). Lane A: BamHI and MspI digest of CAT-32 DNA as a control for complete demethylation; lane B: BamHI digest of CAT-32 DNA; lane C: BamHI and HpaII digest of spermatogonial DNA; lane D and E: two different Sertoli cell preparations from transgene carriers, digested with BamHI and HpaII; lane F: DNA from mature sperm digested with BamHI and HpaII. Note that most of the DNA from Sertoli cells is methylated (D and E) as observed for other somatic tissues (compare with Fig. 2).

remaining two the total amount of DNA recovered was too low to be informative (lanes G and I). It appears from this analysis that processes occurring in the digynic triploid, X-inactivation or other, can lead to an increase in methylation at the HpaII site H3 (Fig. 1). This result is consistent with the hypothesis that it is the state of activity of the CAT-32-bearing X-chromosome, and not germline-specific imprinting, that determines extraembryonic methylation patterns at this transgenic locus.

Methylation in the male germline

The analysis of methylation of the CAT-32 transgene in the male germline was carried out for two reasons. First, determining the levels of methylation in sperm might allow an assessment to be made as to whether the observed extraembryonic patterns of modification reflected de novo methylation of the male-derived locus, or whether they were more likely to be due to demethylation on maternal transmission. The presence in BamHI plus HpaII digests of fragments of sizes 0.4 and 0.8 kb, but not 0.55 kb indicates that there is virtually no CpG methylation of the CAT-32 locus in mature sperm (Fig. 5 lane F). Thus HpaII site H3 is not modified in sperm DNA, but becomes so in the extraembryonic tissues inheriting the male-derived locus. This must reflect de novo methylation, which is presumably linked to the inactivation of the male-derived X-chromosome in the extraembryonic lineage.

Second, comparison of the methylation of the CAT-32 locus was made between spermatogonial and mature sperm cells. The X-chromosome is known to become genetically inactive during spermatogenesis (Monesi, 1971), and this is an event that does not result in an increase in CpG methylation of endogenous genes such as HPRT (Venolia & Gartler, 1983). This is despite evidence for autosomal de novo methylation in the male germline (Groudine & Conkin, 1985; Monk et al. 1987). The patterns of CAT-32 methylation in immature spermatogonial cells were virtually identical to those in mature sperm (Fig. 5, compare lane C with lane F), indicating that HpaII site H3 is probably unmethylated from very early stages of germ cell development and remains so during spermatogenesis (compare with Fig. 2). This observation further supports the notion that it is lineage-specific de novo methylation that results in the methylation of HpaII site H3 on the paternal allele of CAT-32 in the extraembryonic lineage.

Discussion

The preferential inactivation of the male-derived X-chromosome in female extraembryonic cells of the mouse has been suggested to be the direct result of imprinting placed upon this chromosome during its passage through the male germline (reviewed Monk, 1986). Alternatively, it has been proposed that it is the female-derived X-chromosome which is imprinted to remain active in the extraembryonic tissues (Lyon & Rastan, 1984). CpG methylation is one possible mechanism of germline imprinting of the X-chromosome (Gartler & Riggs, 1983). The observation that the transgenic locus CAT-32 displayed parent-specific patterns of this modification in the extraembryonic tissue was therefore of interest. The relatively higher levels of methylation of paternally inherited CAT-32 locus could be ascribed either to its paternal germline origin or to the preferential paternal X-chromosome inactivation in the extraembryonic tissues. A possible way of distinguishing between these two alternatives is to force inactivation of maternal X-chromosomes in the extraembryonic tissues. It is not possible to achieve this in normal embryos.

Previous studies on midgestation parthenogenetic embryos have clearly demonstrated that, in the absence of a paternal X-chromosome, one of the maternal X-chromosomes can become inactivated (Kaufman et al. 1978; Rastan et al. 1980). There is also some evidence for inactivation of two
X-chromosomes in triploid conceptuses (Jacobs et al. 1979) even though this is not observed consistently in all embryos, at least in the very early postimplantation embryos (Endo et al. 1982). We chose digenic triploid for these studies because such conceptuses usually yield just about sufficient extraembryonic tissues for the DNA analyses. Parthenogenetic concepts have far less extraembryonic tissue because of the absence of the paternal genome (Barton et al. 1984, reviewed Surani et al. 1986). Even so, there is insufficient tissue from each triploid conceptus to carry out both DNA and X-inactivation analyses. Nevertheless, we propose that any deviation in the methylation pattern in digenic triploids from female- to male-specific pattern could be attributed to the inactivation of the female X-chromosome, although failure to observe such a change would be inconclusive. However, our results show conclusively that in three out of the four informative analyses of digenic triploid embryos, there was a shift from the female- to male-specific methylation patterns. There are no valid reasons to believe that such changes should be attributed to the fact that triploids develop abnormally. Abnormal development of parthenogenetic embryos is clearly consistent with inactivation of the maternal X-chromosomes (Kaufman et al. 1978; Rastan et al. 1980).

The analysis of digenic triploid conceptuses containing a maternally derived CAT-32 locus indicates that the male-derived patterns of modification were not only the specific product of a germline passage but could also result from other events, including perhaps X-inactivation. Hence, the results indicate that the observation cannot be attributed to the germline imprinting acting directly upon the CAT-32 locus in such a way as to modify CpG methylation. Indeed, it appears likely that the methylation patterns could be altered by somatic events following fertilization. It seems highly probable, even though we are unable to prove it, that the methylation of the transgene is correlated with the inactivation of the X-chromosomes.

The difference in methylation between the extraembryonic CAT-32 alleles present on the active and inactive X-chromosomes can be compared with the fate of endogenous genes. Loci, such as HPRT, show low methylation of the inactive allele in extraembryonic tissues (Kratzer et al. 1983; Lock et al. 1987). Hence, it is significant that we have been able to detect de novo methylation of the CAT-32 allele on the inactive extraembryonic X-chromosome. CpG methylation may act both in the inactivation and maintenance of the inactive X-chromosome (reviewed Monk, 1986). This de novo methylation observed at the CAT-32 locus may indicate that CpG methylation of the extraembryonic X-chromosome can occur, and that this may be involved in its coherent regulation. In contrast to this, no change in the methylation of the CAT-32 transgene was observed between the stages of immature spermatogonia and mature sperm. This is despite de novo methylation activity known to be present in the male germline (Sanford et al. 1984; Groudine & Conkin, 1985; Monk et al. 1987). The CpG-rich regions of endogenous genes such as HPRT, like CAT-32, also show low levels of CpG methylation in sperm, despite inactivation during spermatogenesis (Venolia & Gartler, 1983). CAT-32, therefore, seems to be a transgenic locus which is able to accurately reflect those CpG methylation changes that affect at least some endogenous X-linked sequences. This is a fact that strengthens the validity of using X-linked transgenes to study the relationship between CpG methylation and X-inactivation.

Two other cases of X-linked transgenes have been described. Krumlauf et al. (1986) found that an alphafetoprotein (AFP) minigene could partially escape X-inactivation, remaining active in extraembryonic yolk-sac cells, but not in the adult liver. This observation was thought to reflect differences between the two tissues in the maintenance of the inactive state (Krumlauf et al. 1986). No differences were found in the CpG methylation of the transgenic AFP locus present on the inactive X-chromosomes of yolk sac and liver. In the second case, a chicken transferrin transgenic insertion of large size (187 kb) was found to completely escape X-inactivation, remaining active in the predicted tissue-specific manner (Goldman et al. 1987). Again no differences in CpG methylation were observed between this transgene when present on active or inactive homologues. It was suggested that the large size of the transgenic insertion is important in allowing it to resist X-inactivation (Goldman et al. 1987). It would be of particular interest to compare the CpG methylation levels of the endogenous sequences flanking the CAT-32 transgene with the two X-linked transgenes described previously (this study, Krumlauf et al. 1986; Goldman et al. 1987). It would also be of interest to know the chromosomal location of each transgene, especially with reference to the position of Xce, the genetically defined, X-inactivation-controlling element (Cattanach et al. 1970), and those other loci as yet undefined that are thought to be necessary for the coherent regulation of the whole X-chromosome (Monk, 1981).

We thank Drs C. E. Bishop and P. Goodfellow for making the Y-specific probe pY353/8 available to us. We thank Mike Norris, Sarah Howlett and Nick Allen for helpful discussion of the manuscript, Bob Patman for photography, Linda Notton and Sheila Mackley for typing.
During this work A.C. was in receipt of an AFRC studentship, and W.R. an EMBO Fellowship. W.R. is a Fellow of the Lister Institute of Preventive Medicine.

References


ROMRELL, L. J., BELLVE, A. R. & FAWCETT, D. W.
A. Collick, W. Reik, S. C. Barton and M. A. H. Surani


Toniolo, D., D’Urso, M., Martini, G., Persico, M.,