recognizes only a half-methylated site. Therefore, all subsequent copies of this DNA will no longer be methylated at the designated sequence. If a given site is unmethylated after 5-azaC incorporation, a specific gene could be reactivated. Those cells in which the HPRT locus was reactivated by incorporation of 5-azaC at the necessary site grew in HAT medium. The stability of the reactivated gene loci in our experiments is also in keeping with the model.

It appears from the evaluation of human G6PD and PGK that demethylation at a specific site (or sites) does not reactivate the entire X chromosome, since G6PD and PGK were not expressed in all clones in which HPRT was reactivated. We interpret this as evidence for the inactivation of the X in discrete units. It may be that the three X markers we have evaluated, G6PD, PGK, and HPRT, are in differentially regulated segments. Such a mechanism is compatible with the existence on the human X chromosome of loci such as that for STS, which may be in a segment that is never inactivated. However, the frequency of reactivation of HPRT is at best one per thousand, whereas the expression of human G6PD or PGK was detected in 1 of 14 independent HPRT-positive clones isolated. Also, the expression of human G6PD was detectable in pooled samples of reactivated clones. Thus, coordinate reactivation of HPRT and G6PD or PGK appears to occur at a frequency greater than that expected by chance.

Our results thus provide evidence that DNA methylation plays a role in inactivation of the X chromosome and that inactivation occurs on the human X in a segmental fashion. It is well established that DNA-protein interactions are altered when DNA is methylated (*9*, *10*). Such changes could account for the inactivation of X-linked genes and heterochromatization of the X chromosome. The exact nature of this alteration must be resolved by further experimentation.

T. MOHANDAS
Division of Medical Genetics, Department of Pediatrics, Harbor-University of California Medical Center, Torrance 90509
R. S. SPARKES
Division of Medical Genetics, Departments of Medicine, Pediatrics, and Psychiatry, Center for the Health Sciences, University of California, Los Angeles 90024
L. J. SHAPIRO
Division of Medical Genetics, Department of Pediatrics, Harbor-University of California Medical Center

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Mouse Oocytes Transcribe Injected *Xenopus* 5S RNA Gene

Abstract. *Transcripts produced after injection of the* Xenopus *5S RNA gene into oocyte germinal vesicles of mice migrate electrophoretically with the 5S RNA marker, an indication that the gene is transcribed and processed with considerable accuracy. Approximately two 5S RNA molecules are transcribed per gene per hour. This system may be useful in studying DNA processing and gene regulation by the mammalian ovum and might be modified to allow permanent incorporation of specific genes into mice.*

The *Xenopus* oocyte has been used extensively to study the biological activity of macromolecules introduced by microinjection (*1–3*). The oocyte translates numerous types of injected messenger RNA (mRNA) (*2*) and transcribes several types of DNA with great fidelity (*4–5*). This system has been extraordinarily useful in studying the processing of injected mRNA and DNA by a normal living cell (*5*). Globin mRNA microinjected into the mouse oocyte or into a one-cell fertilized ovum is translated to globin protein (*6*). The translation characteristics of the mouse oocyte are different from those of the *Xenopus* oocyte, and therefore injection of the mouse ovum provides a valuable additional technique for studying mRNA processing in the intact cell. We now report that the mouse oocyte is also capable of transcribing foreign genes introduced by microinjection and can thus be used to study DNA processing by the mammalian egg cell.

Growing mouse oocytes (50 to 60 μm in diameter) were dissected from the ovaries of 14-day-old hybrid C57 × SJL females. The oocytes were collected in Brinster's medium for ovum culture (BMOC) (*7*) modified by the addition of bovine serum albumin (4 mg/ml), glucose (1 mg/ml), Eagle's essential and nonessential amino acids, and 10 percent fetal calf serum (BMOC-2-M). Mature oocytes (just prior to ovulation) were dissected from the ovaries of 6- to 8-week-old mice. The medium used for collection and maintenance was BMOC-2 (*7*). Fertilized ova were flushed from the oviduct with BMOC-2 on day 1 of pregnancy. The gene used in these experiments was for somatic *5S* RNA from *Xenopus borealis*; two repeating units were cloned in plasmid pBR322. The characteristics of the cloned sequence and gene (pXbs1) have been described (*8*). The concentration of the DNA was 1 mg/ml in a dilute salt solution (0.015*M* NaCl, 0.5 m*M* EDTA, and 5 m*M* tris at *p*H 7.8). The injection procedure was similar to that previously employed for injecting cells into blastocysts (*9*) and mRNA into one-cell fertilized ova (*6*). Growing oocytes were placed on a depression slide in BMOC-2 containing cytochalasin B (5 to 10 μg/ml) and held with a blunt pipette (*6*). The tip of the injector pipette was filled with the DNA

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solution and inserted into the oocyte nucleus (germinal vesicle). The volume injected was determined by measuring the germinal vesicle before and after injection. The average increase in volume was 2 picoliters. Injections into the germinal vesicle of mature oocytes and the pronucleus (male) of fertilized ova were accomplished in the same manner. The survival rate was approximately 75 percent for growing oocytes, 25 percent for mature oocytes, and 50 percent for fertilized ova. The reason for the difference in survival is not known but may be related to the speed with which the punctured membranes can be repaired. After injection, the oocytes or ova were incubated in the appropriate medium (see above) containing [^{3}H]guanosine (4 μCi/μl; specific activity, 28 Ci/mmole; Amersham/Searle) for 24 hours. After the oocytes were labeled, they were washed three times in medium and placed in a tube (6 by 60 mm) containing 20 μg of 4*S* RNA from *Escherichia coli* as carrier and 20 μg of proteinase K in 10 μl of 50 m*M* tris (*p*H 7.5), 10 m*M* EDTA, and 0.5 percent sodium dodecyl sulfate (*10*). The tubes were stored at −70°C. Immediately before electrophoresis, the tubes containing the oocytes and proteinase K were incubated for 2 hours at 37°C to liberate RNA. (Extraction with a mixture of phenol and chloroform did not improve RNA separation.) Nonradioactive 5*S* RNA (25 μg) from *E. coli* (Boehringer Mannheim) was added to each tube as a marker, and the samples were subjected to electrophoresis on 8 percent acrylamide slab gels containing 0.2 percent sodium dodecyl sulfate by the tris-acetate system (*p*H 7.2) (*11*).

Growing oocytes serving as controls and growing oocytes injected with 5*S* DNA from *X. borealis* both show considerable radioactive 18*S* and 28*S* ribosomal RNA at the top of the gel (Fig. 1). There is a band of labeled RNA migrating with the 4*S* carrier and a radioactive band migrating with the dye front. The latter band has not been identified, but all of the bands disappear with ribonuclease treatment of the lysed oocyte. The only difference between separations in the control and injected oocyte samples is a clearly visible radioactive band migrating with the 5*S* marker RNA in the DNA-injected oocyte sample. We interpret this finding to indicate that 5*S* RNA is synthesized from the *Xenopus* 5*S* DNA gene present in the plasmid. There is no evidence of plasmid DNA transcription. Synthesis of 5*S* RNA from 5*S* DNA also occurs when pXbs1 is injected into the germinal vesicle of the mature oocyte. The plasmid alone does not stimulate transcription from endogenous 5*S* genes, since no new bands appear when pBR322 without the 5*S* gene is injected (Fig. 2). However, if the plasmid containing a 150-nucleotide adenovirus-associated RNA gene is injected simultaneously with pXbs1, a new RNA band appears above the 5*S* band (Fig. 2). This band is in the location expected for an RNA transcribed from a 150-nucleotide gene. The band is less dense than the 5*S* band, perhaps because pAd123 has one cloned viral gene sequence and pXbs1 has two repeating units of the cloned 5*S* gene.

The location of the 5*S* band, which migrates with the 5*S* marker, suggests that the gene is transcribed with some accuracy. If small pieces of plasmid DNA were transcribed concomitantly with the gene, one would not expect to see exact comigration. In fact, transcription of 5*S* genes by *E. coli* or *Xenopus* polymerases in vitro does not produce significant quantities of 5*S* transcripts (*4*). Most of the transcripts obtained in vitro are incorrect. The location of the adenovirus RNA on the gel also suggests accurate transcription by the oocyte. The accuracy of transcription in the *Xenopus* and mouse oocyte systems is an important characteristic of the technique.

We have not yet detected 5*S* RNA synthesis after injection of the plasmid into one-cell fertilized ova. Since the volume of plasmid solution injected (2 picoliters) is the same in the one-cell fertilized ovum as in the growing oocyte, the absence of detectable 5*S* RNA (or any other new RNA bands) must result from the conditions that are responsible for the low levels of RNA synthesis in one-cell fertilized ova and cleaving embryos (*12*). It is not known why detectable 5*S* RNA synthesis is absent in control oocytes and fertilized ova, even though 18*S* and 28*S* RNA are synthesized in considerable amounts. However, the synthesis of 5*S* RNA does not always parallel synthesis of 18*S* and 28*S* RNA, and their rates of production vary independently in the *Xenopus* oocyte and early embryo (*4*, *13*). These relationships have not been determined for mouse oocytes and fertilized ova.

The size of the growing oocyte (50 to 60 μm) and the number of oocytes that can be injected in each experiment (50 to 100) make it difficult to quantify precisely the response of the cell to the injected DNA, but some estimates are possible. The radioactivity present in the 5*S* RNA band of injected oocytes is approximately 1 percent of that for 18*S* plus 28*S* synthesis. There was no indication that the synthesis of the *Xenopus* 5*S* RNA depressed the synthesis of 18*S* and 28*S* RNA in the injected oocytes in comparison to controls. The incorporation into 18*S* plus 28*S* RNA was 501 ± 60 disintegrations per minute for controls and 523 ± 38 for injected ova per hour per

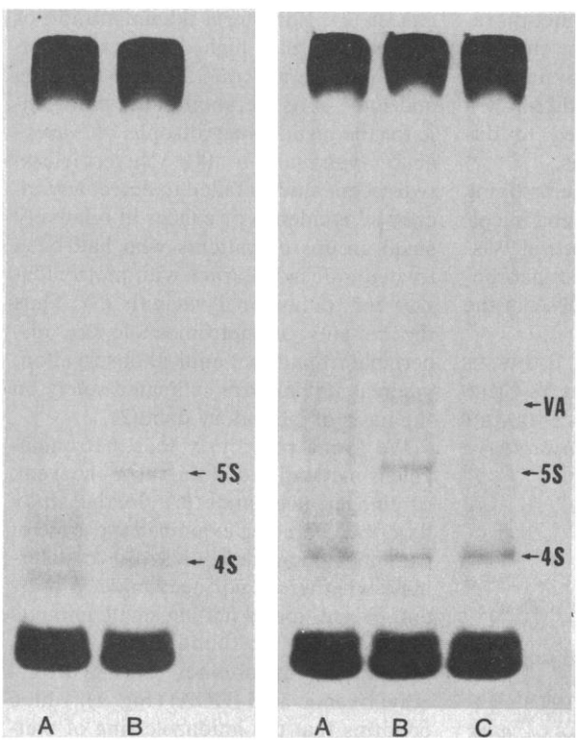

Fig. 1 (left). Fluorograph of polyacrylamide gel electrophoretic separation of ^{3}H-labeled RNA synthesized by 95 growing mouse oocytes after injection with (A) dilute salt solution and (B) dilute salt solution containing pXbs1 plasmid DNA (1 mg/ml). Each plasmid contained two repeating units of somatic 5*S* gene from *Xenopus borealis*. The positions of the nonradioactive marker 5*S* RNA and 4*S* RNA are indicated. Fig. 2 (right). Fluorograph of polyacrylamide gel electrophoretic separation of ^{3}H-labeled RNA synthesized by 120 growing mouse oocytes after injection with (A) dilute salt solution, (B) dilute salt solution containing pXbs1 (0.5 mg/ml) and pAd123 (0.5 mg/ml), and (C) dilute salt solution containing pBR322 (1 mg/ml). Plasmid pAd123 has a 1.8-kilobase insert including the type 2 adenovirus-associated RNA gene (*VA*) and flanking regions (prepared by David Bogenhagen). The adenovirus gene is approximately 150 nucleotides (5*S* and 4*S* RNA genes are approximately 120 and 90 nucleotides, respectively). All three genes are transcribed by polymerase III. Procedures and labeling are as in Fig. 1.

ovum. The 4S band and the bands that migrate just ahead of and behind the 4S RNA marker are slightly less dense in some samples from injected ova than in controls. The difference in radioactivity is not statistically significant but could represent the appearance of competition of the injected 5S gene for some component of the polymerase III transcription machinery that handles both 5S and 4S genes. On the basis of the radioactivity in the 5S band, the specific activity of guanosine, and the amount of DNA injected, it is estimated that at least two 5S RNA molecules are synthesized each hour from each *Xenopus* gene in an injected oocyte. This is similar to the transcription rate for 5S DNA injected into the germinal vesicle of the *Xenopus* oocyte (*5*, *14*).

The significant increase in 5S RNA synthesis by the mouse oocyte after injection of the plasmid containing the *Xenopus 5S* gene and the very precise migration of the radioactive material with the nonradioactive 5S RNA marker provide evidence that the gene has been accurately transcribed by the oocyte nucleus.

Mammalian oocytes and fertilized ova may respond to injected DNA in exactly the same way as *Xenopus* does. However, while mouse and *Xenopus* oocytes have at least a similar capability for response, there may be differences in transcription ability, as has been demonstrated for mRNA translation (*6*). It is possible that our technique can be extended or modified to allow incorporation of the injected gene into the chromosomes of the ovum and thereby into the mouse, an extension that would allow a wide range of studies related to differentiation and carcinogenesis.

Note added in proof: By employing the techniques described here and in collaboration with Dr. Carlo Croce of Wistar Institute, we have obtained incorporation of injected genes into DNA of the mouse.

RALPH L. BRINSTER
HOWARD Y. CHEN
MYRNA E. TRUMBAUER
Laboratory of Reproductive Physiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia 19104

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A Metronidazole Metabolite in Human Urine and Its Risk

Abstract. *Metronidazole is a drug used for the treatment of trichomonal vaginitis, amebiasis, giardiasis, and certain anaerobic bacterial infections in humans. Acetamide and* N-*(2-hydroxyethyl)oxamic acid are metabolites of metronidazole in the rat, and we find small amounts of both metabolites in the urine of human patients taking the drug. Although acetamide is carcinogenic for rats, we do not believe that our finding further defines metronidazole's risk for humans. That risk can only be estimated from surveillance of people previously exposed to the drug.*

Metronidazole is useful in the treatment of trichomonal vaginitis, amebiasis, giardiasis, and certain anaerobic bacterial infections in humans. Either alone or in combination with other antibiotics, metronidazole can decrease the incidence of bacterial infections after intestinal and gynecological surgery (*1*). Acute toxicity is rarely of significance at doses necessary for these indications, but concern has been expressed that metronidazole may pose a risk of human cancer (*2*). Long-term administration of metronidazole at high doses causes tumors in mice and rats (*3*), and the drug and some of its metabolites are mutagenic for the histidine auxotrophs of *Salmonella typhimurium* (*4*). Nevertheless, two recent studies failed to detect any increased incidence of cancer in relatively small groups of patients who had been treated a decade earlier with metronidazole for trichomonal vaginitis (*5*). Thus the benefits of metronidazole are discernible from direct clinical observation, whereas its risks are estimated solely on the basis of laboratory data (*2*).

We found previously that metronidazole is metabolized in the rat to the weak carcinogen acetamide (*6*). In the study described here, we examined the urine of patients taking metronidazole to determine whether or not acetamide is present. In addition to finding small amounts of acetamide we found small amounts of another metabolite, *N*-(2-hydroxyethyl)oxamic acid (HOA) (Fig. 1), which confirms that the imidazole ring of metronidazole is cleaved in the human as it is in the rat (*6*, *7*).

For this study we used metronidazole (melting point, 158° to 160°C) and [1′,2′-$^{14}C_2$]metronidazole (11.7 mCi/mmole) (gifts from G. D. Searle and Co.); [1-^{14}C]acetamide (3.0 mCi/mmole) (California Bionuclear Corporation); [1,2-$^{14}C_2$]ethanolamine hydrochloride (2.44 mCi/mmole) (New England Nuclear); and *N*-[1,2-$^{14}C_2$-(2-hydroxyethyl)]oxamic acid (11.7 mCi/mmole) which was synthesized as described previously (*7*). All other chemicals were purchased from Fisher Scientific Company unless otherwise specified.

Under a protocol approved by the Human Studies Committees at Harvard Medical School, the Peter Bent Brigham Hospital, and the Beth Israel Hospital, urine was collected for a 24-hour period from five hospitalized patients who received metronidazole (Flagyl, Searle) at a dose of 750 mg per 24 hours on their physician's orders, and from six normal subjects (two males, ages 26 and 32 years; four females, ages 20, 28, 30, and 54 years). Urine was kept at 0° to 4°C during collection and was then stored at − 15°C until analyzed. Patient characteristics are listed in Table 1.

The HOA and acetamide in urine were separated by cationic exchange chromatography. The HOA was further purified by anionic exchange chromatography and then hydrolyzed and quantified as the dinitrophenyl derivative of ethanolamine by high-pressure liquid chromatography. Acetamide was quantified by gas-liquid chromatography and its identity confirmed by mass spectroscopy. The analysis was performed on 125 to 150 ml

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