Table 2. Gene frequency distributions of the Pgm^{tr} and Pgm^{ts} alleles in the samples of 6 natural populations of Culicidae. The estimate of the cumulative gene frequency of the thermosensitive alleles in each population has been obtained, assuming Hardy-Weinberg's equilibrium for the Pgm^{tr} and Pgm^{ts} alleles, in 2 ways: in the electrophoretically monomorphic species, as the square root of the frequency of the individuals with the thermosensitive phenotype; in the electrophoretically polymorphic species, as 1 minus the sum of the frequencies of all Pgm^{tr} alleles. The frequency of each of the Pgm^{tr} alleles has been in turn estimated by the general formula: $p_{Ai} = 1 - \sqrt{A_{non-i}/A_{non-i}}$, where A_i is the i^{th} electrophoretic Pgm^{tr} allele and $\sqrt{A_{non-i}/A_{non-i}}$ the frequency of individuals homozygous for any A_{non-i} allele. The frequencies of the rare alleles were calculated by direct count of heterozygotes

Species	Number of individuals tested	Alleles* Pgm^{tr} 0.90	1.00	1.10	1.15	Pgm^{ts} 0.90	1.00	1.10	1.15	Total frequency of Pgm^{ts} alleles
Ae. refiki	288	0.002	0.669	0.040	-	-	0.285	0.004	-	0.289
Ae. caspius	152	0.065	0.686	0.044	-	0.014	0.176	0.016	-	0.206
Ae. mariae	472	-	0.954	-	-	-	0.046	-	-	0.046
C. litorea	565	0.003	0.794	0.002	-	-	0.201	-	-	0.201
C. annulata	187	0.013	0.543	-	0.306	0.005	0.083	-	0.050	0.138
C. longiareolata	246	0.379	0.523	0.035	-	0.038	0.016	0.009	-	0.063

* Numbers indicate relative electrophoretic mobility.

Our data make it possible to draw some interesting conclusions. 1. All the species of Culicidae studied by combining the electrophoresis and heat denaturation studies showed notably more heterogeneity than that detectable by electrophoresis alone[5]. This is in keeping with similar observations being obtained in *Drosophila* and in other organisms by the use of refined electrophoretic techniques and electrophoretic techniques combined with other methods[6,19–23]. 2. More relevant from the point of view of evolutionary genetics, this new type of variability of the *Pgm* gene is seen in Culicidae to correlate with the environment as an adaptive hypothesis would predict, temperature being the chief factor controlling the frequencies of Pgm^{tr} and Pgm^{ts} alleles. 3. Finally, these observations represent another example of evolutionary protein changes among congeneric species. These changes are comparable to the pressure-adaptive differences observed by Siebenaller and Somero[24] in lactate dehydrogenases of congeneric fishes living at different depths. Thus, whereas a number of studies have shown that heat stability and kinetic differences exist among homologous enzymes from differently adapted species which are separated by large phylogenetic distances, these data show how some very closely-related species differ in an apparently adaptive way.

Note. After this paper was submitted for publication E. Gosling reported results in line with our own for 2 populations of a marine mussel *(Guekensia demissa)* (Nature *279*, 713 (1979)).

1 We thank Dr M. Coluzzi for providing the data on the temperature ranges for larval development of the species examined and for helpful discussions. The excellent technical assistance of Mr G. Dell'Uomo (Istituto Superiore Sanità, Roma) is also acknowledged.

2 J.B. Johnson, in: Molecular Evolution, p. 46. Ed. F.J. Ayala. Sinauer Ass. Inc., Sunderland 1976.
3 J.A. Rioux, Les Culicides du 'Midi' Méditerranéen, Encyclopedie Entomologique, vol. XXXV. Lechevalier, Paris 1958.
4 R. Scozzari, G. Trippa, V. Petrarca and R. Cicchetti, Genetics *92*, in press (1979).
5 L. Bullini and M. Coluzzi, Parassitologia *15*, 221 (1973).
6 G. Trippa, A. Loverre and A. Catamo, Nature *260*, 42 (1976).
7 N. Spencer, D.A. Hopkinson and H. Harris, Nature *204*, 742 (1964).
8 R.K. Koehn and D.I. Rasmussen, Biochem. Genet. *1*, 131 (1967).
9 G.C. Williams, R.K. Koehn and J.B. Mitton, Evolution *27*, 192 (1973).
10 J.B. Mitton and R.K. Koehn, Genetics *79*, 97 (1975).
11 E. Nevo, T. Shimong and M. Libni, Nature *267*, 699 (1977).
12 S.H. Saul, M.J. Sinsko, P.R. Grimstad and G.B. Craig, Jr, Am. Nat. *112*, 333 (1978).
13 R.K. Koehn and G.C. Williams, Evolution *37*, 624 (1978).
14 R.K. Koehn, Science *163*, 943 (1969).
15 R.B. Merrit, Am. Nat. *106*, 173 (1972).
16 G.L. Bush, R.W. Neck and G.B. Kitto, Science *193*, 491 (1976).
17 G.B. Johnson, Biochem. Genet. *14*, 403 (1976).
18 S. Alahiotis, S. Miller and E. Berger, Nature *269*, 144 (1977).
19 G. Cobbs, Genetics *82*, 53 (1976).
20 B.J. Cochrane, Nature *263*, 131 (1976).
21 R.S. Singh, R.C. Lewontin and A.A. Felton, Genetics *84*, 609 (1976).
22 J.A. Coyne and A.A. Felton, Genetics *87*, 285 (1977).
23 G. Modiano, G. Battistuzzi, G.J. Esan, U. Testa and L. Luzzatto, Proc. natl Acad. Sci. USA *76*, 852 (1979).
24 J. Siebenaller and G.N. Somero, Science *201*, 255 (1978).

Nucleolus organizer region location and 'ring' chromosomes in the bharal

L.M. Henderson and A.N. Bruère

Veterinary Clinical Sciences Department, Massey University, Palmerston North (New Zealand), 7 May 1979

Summary. Silver-staining has been used to identify the nucleolus organizer regions (NORs) in the bharal. These show homology with sheep, goat, cattle and aoudad. The association of the NORs on both telomeres of chromosome 3 results in a 'ring' chromosome.

Nucleolus organizer regions (NORs) can be identified by silver-staining techniques[1,2]. These have been applied to sheep[3] and goat, cattle and aoudad[4] to demonstrate that each of these species has 5 NOR-bearing chromosome pairs, and that these are chromosomes with homologous G-banding patterns. This indicates that the number and location of NORs have been conserved during evolution. The members of the other 2 genera of the Capridae, the

Chromosomes which have undergone more than 1 centric fusion event during evolution. The homologous chromosomes have been determined from published G-band karyotypes of goat, sheep and cattle[8] and bharal[5]

Homologous goat chromosome number	Species in which fusion has occurred
1	Cattle ($1/_{29}$); domestic sheep (1q); bharal (2q)
3	Domestic sheep (1p); bharal (3q)
4	Domestic sheep (3q); bharal (1q)
8	Domestic sheep (Massey t_2); snow sheep (4q)[9]
25	Bharal (3p); domestic sheep (Massey t_3)
26	Cattle ($1/_{29}$); domestic sheep (Massey t_1)

tahr, *Hemitragus jemlahicus,* and the bharal, *Pseudois nayaur,* had not been studied by silver-staining. The tahr (2n = 48) as yet remains unstudied by silver-staining or G-banding. This paper describes the locations of the NORs on the chromosomes of the bharal. This species has 54 chromosomes, with 1 metacentric chromosome pair, 2 submetacentric pairs, 23 acrocentric pairs, a large acrocentric X and a small metacentric Y (figure 1). Each chromosome arm of the bharal complement corresponds to a goat acrocentric chromosome as shown by G-band homology[5]. The combinations of chromosome arms involved in the biarmed chromosomes differ from those in sheep and have been reported by Bunch et al. to be 4 and 13, 1 and 27, and 3 and 29[5]. Thus the long arms of the bharal chromosomes correspond to 3 arms of the metacentric chromosomes of domestic sheep, *Ovis aries.*

Materials and methods. Fixed cells from a leucocyte culture from a male bharal kept at the Henry Doorly Zoo, Omaha, Nebraska were kindly provided by Dr T.D. Bunch, International Sheep and Goat Institute, Utah State University. The details of this specimen have been described previously[5]. This animal, the only surviving bharal in North America, has since died and other samples are not obtainable at present. Unfortunately, due to a delay in the mail, fixed cells were kept at room temperature for 4 months and arrived in poor condition. Flame-dried slides made from this preparation were stained by the Ag-I technique[2].

Results. 34 cells were analyzed and NORs were observed on the long arms of the metacentric chromosome pair[1], both ends of a submetacentric chromosome pair[3] and at least 1 large acrocentric pair[4] (figure 2). In 1 metaphase there appeared to be 3 large acrocentrics staining but this metaphase was unfortunately fairly indistinct. In some metaphases Ag-stained NORs on both arms of the submetacentric chromosome were seen to be in association, forming 'ring chromosomes' (figure 3).

Discussion. The NORs in domestic sheep are located on chromosomes 1p, 2q, 3q, 4 and 25[3,4]. The chromosome arms in the bharal which have homologous G-banding patterns to these chromosomes are 1q (sheep 3q), 3p (sheep 25), 3q (sheep 1p), 4 (sheep 2q) and 5 (sheep 4). Thus in this study at least 4 of the NORs in the bharal (1q, 3p, 3q and a large acrocentric) are homologous in location to the NORs in sheep, goat, cattle and aoudad.

A drawback of the Ag-staining technique in the identification of NORs is that it is believed to only stain regions which were active in rRNA transcription in the preceding interphase[6] so that not all NORs may be stained with silver. Therefore it is necessary to pool the data from several animals to be sure all NOR sites have been observed. This was not possible in the present case. 2 large acrocentric chromosomes were found to have NORs, but it is impossible to determine on the basis of arm-length whether these belong to either one pair or two. Either a duplicate

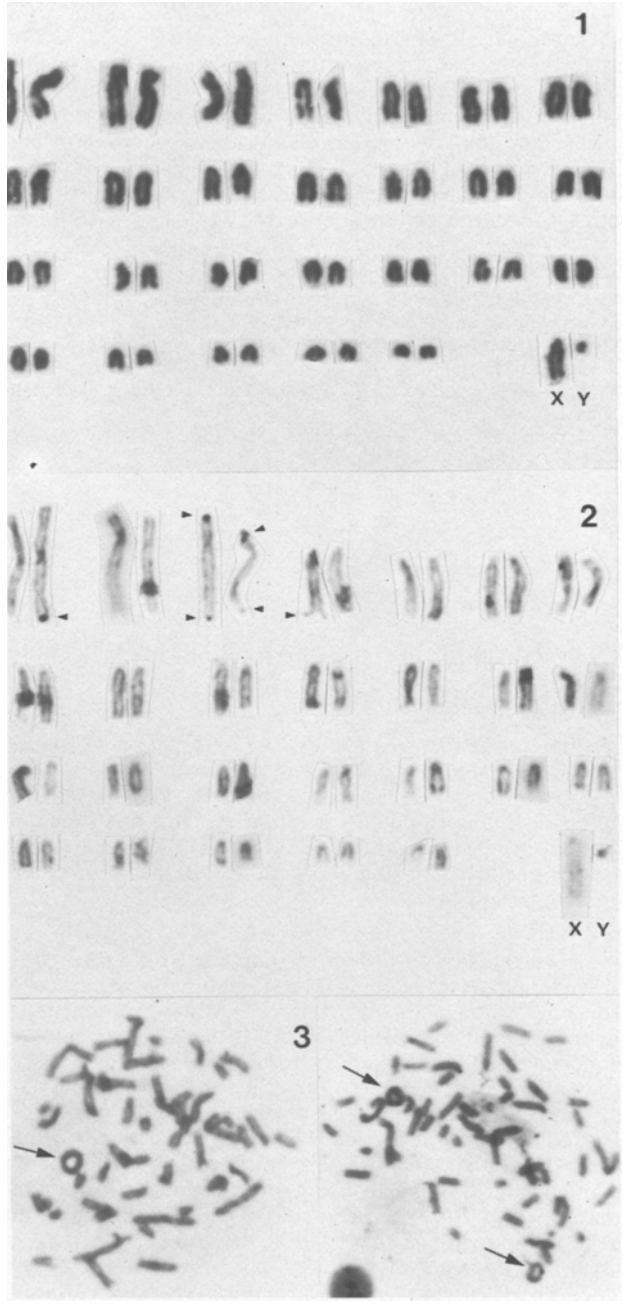

Fig. 1. Karyotype of male bharal, *Pseudois nayaur.*
Fig. 2. Ag-stained bharal karyotype showing Ag-stained NORs on 1q, both 3p and 3q arms, and a large acrocentric.
Fig. 3. Prometaphase spreads from a bharal showing 'ring' (3) chromosomes due to NOR association.

G-banding and silver-staining study or a study of other specimens which may have a different silver-staining pattern might elucidate this. Unfortunately neither of these alternatives is possible at present. It is however likely, as shown by the results of this study, that the location and number of the NORs in the bharal show the same conservation during evolution that has previously been demonstrated in sheep, goat, aoudad and cattle[4].

The 'ring' chromosome observed in the bharal as a result of association of NORs on the same chromosome is a previously unreported phenomenon in mammalian cells. A similar 'ring' has been observed in *Drosophila*[10]. True ring

chromosomes result from deletion of both telomeres of a chromosome and a rejoining of the ends.

Of further interest in this study is the demonstration that the short arm of the bharal chromosome 3 is homologous to the short arm of a centric fusion chromosome, the Massey t_3 chromosome[7], which occurs in domestic sheep. The G-banded karyotype of the bharal[5] indicated a possible homology of these chromosomes. Identification of the small acrocentric chromosomes by G-banding is difficult. However the homology is now almost certain because of the presence of NORs on both these chromosomes. This shows that at least twice during evolution within the family this chromosome has undergone centric fusion events. Other chromosomes in the Capridae and cattle have also been involved in centric fusion events more than once during evolution (table) suggesting there may be a predisposition of some chromosomes to undergo or to tolerate centric fusions.

1 C. Goodpasture and S.E. Bloom, Chromosoma *53*, 37 (1975).
2 S.E. Bloom and C. Goodpasture, Hum. Genet. *34*, 199 (1976).
3 L.M. Henderson and A.N. Bruère, Cytogenet. Cell Genet. *19*, 326 (1977).
4 L.M. Henderson and A.N. Bruère, Can. J. Genet. Cytol. *21*, 1 (1979).
5 T.D. Bunch, C.F. Nadler and L. Simmons, J. Hered. *69*, 316 (1978).
6 D.A. Miller, V.G. Dev, R. Tantravahi and O.J. Miller, Exp. Cell Res. *101*, 235 (1976).
7 A.N. Bruère, D.L. Zartman and H.M. Chapman, Cytogenet. Cell Genet. *13*, 479 (1974).
8 H.J. Evans, R.A. Buckland and A.T. Sumner, Chromosoma *42*, 383 (1973).
9 T.D. Bunch, J. Hered. *69*, 77 (1978).
10 H. Beck and Ž. Srdić, Genetica *50*, 1 (1979).

DNA methylation in chromatin fractions of chick embryo cells[1]

A. Remington and P.K. Ranjekar

Département de Biologie, Université Laval, Quebec (Canada, G1K 7P4), and Biochemistry Department, National Chemical Laboratory, Poona 411 008 (India), 10 May 1979

Summary. Transcriptionally active chromatin was prepared from cultured fibroblasts of chick embryos by fractionation after partial digestion with DNAase II. The degree of DNA methylation in the active chromatin fraction is twice that of inactive or unfractionated chromatin in unsynchronized cells and 4 to 5 times greater at the beginning of the S-phase in synchronized cells.

The nuclear DNA of differentiating, eukaryotic cells contains 5-methyl cytosine (5-MeC) predominately in the sequence 5-MeCpG[2]. Although DNA methylation is a general phenomenon which occurs at the DNA level after replication[3], its function is unknown. There has been some speculation and evidence to the effect that DNA methylation may be directly involved in regulating gene transcription in developing organisms[4–8]. We have fractionated the chromatin DNA of embryonic chick cells into transcriptionally active and inactive components, and have found a small but significant enrichment of DNA methylation in the transcriptionally active fraction.

Materials and methods. Cultured skin fibroblasts of 8-day (stage 33-34) embryos were used. Fibroblasts were cultured in roller bottles using McCoy's 5a medium (modified) with 16% fetal calf serum. When radioactive methionine was administered, the methionine concentration of the medium was reduced to 1/10 of the normal level, and 20 mM sodium formate was added to reduce the incorporation of methyl label through the 1 carbon pool[9]. ^{3}H-methylmethionine (70–85 Ci/mmole), ^{3}H-thymidine (6.7 Ci/mmole) and ^{3}H-5-uridine (6 Ci/mmole) were obtained from New England Nuclear. Nuclei were isolated by homogenization of cells in 0.25 M sucrose, 0.004 M $MgCl_2$, 0.01 M Tris-HCl, pH 7.4 in the presence of 0.2% Triton X-100. The homogenate was passed through cheesecloth and the strained homogenate was centrifuged at 1000 × g for 10–20 min to give a crude nuclear preparation.

The nuclear pellet was washed at least twice with Tris-HCl (0.01 M, pH 8.0) buffer. The Tris-extracted nuclei were lysed, the chromatin was ultracentrifuged across 1.7 M sucrose and was dialyzed in 0.025 M Na acetate buffer (pH 6.6). The dialyzed chromatin was digested with DNAase II (Worthington, RNAase-free) for 2 or 5 min at 24 °C and was fractionated according to the technique of Gottesfeld et al.[10] to yield a 2 mM Mg^{++}-soluble, transcriptionally-active fraction (S2), and inactive fractions designated as P1 or P2. P1 is the chromatin which is not initially solubilized by DNAase II. P2 is DNAase-soluble, but Mg^{++} precipitable. After proteinase K and RNAase A digestion, DNA was isolated from the fractions by phenol and chloroform-isoamyl alcohol extractions. The re-extracted and reprecipitated DNA was hydrolyzed twice for 6 h in 0.3 M KOH to remove any residual RNA fragments. The released radioactivity from ^{3}H-methyl-methionine labeled DNA, after acid hydrolysis, was used as a measure of DNA methylation. Hydrolysis of labeled DNA by formic acid and separation of the bases by 2-dimensional, thin-layer chromatography[11] revealed that over 90% of the methylmethionine label was localized in the 5-methylcytosine residue of the DNA. Standard methods were used to measure the amounts of nucleic acids[12], DNA reassociation[13], and protein[14], as well as DNA melting temperature[15] and buoyant density in neutral CsCl[15].

Results and discussion. The S2 fraction comprises 1–8% of the chromatin DNA depending upon the length of DNAase digestion. For methylation experiments, 5 ± 1% of the chromatin was obtained after 5 min digestion. Evidence from a number of laboratories has demonstrated that limited digestion of chromatin, either by DNAase I or DNAase II, preferentially solubilizes transcriptionally-active chromatin[10,16]. We also found significant differences between the S2 chromatin and other fractions.[17]. The S2 fraction has 3 times more acidic protein per µg of DNA than that of total chromatin, twice the total RNA, and more than 10 times the nascent, labeled RNA-specific activity as measured by