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]

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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

Distribution of ^{3}H-methyl label in DNA of chick fibroblast chromatin fractions

	cpm/μg DNA
A) Chromatin DNA fractions from unsynchronized cells	
Unfractionated chromatin	3.2 ± 0.4
S2 (active)	6.0 ± 0.4
P1 (inactive)	
P2 (inactive)	2.4 ± 0.8
B) Chromatin DNA fractions from synchronized cells	
Early S-phase	
S2	21.1 ± 5.5
P1	4.1 ± 1.4
Late S-phase	
S2	30.6
P1	19.3
C) Reassociated DNA fractions	
S2 (Cot < 10, repeated)	4.7
S2 (Cot > 10, single-copy)	3.4
P1 (Cot < 10, repeated)	3.1
P1 (Cot > 10, single-copy)	2.2

The procedures for isolating DNA from the unfractionated chick chromatin and the chromatin fractions as well as the measurement of DNA methylation are described in the text.
The reassociation kinetics of the DNA from S2 and P1 chromatin fractions were determined by hydroxyapatite column chromatography. All the DNA sequences forming duplexes by Cot < 10 were considered to be repetitive while those which remained single-stranded at Cot > 10 were assumed to be as single-copy or nonrepetitive.

the incorporation of ^{3}H-5-uridine after a 5 min pulse in vivo. The buoyant density of the S2 DNA in neutral CsCl is similar to that of total embryonic DNA (1.700 g/cm^3), and the Tm (83.5 °C) is 2 °C below that of the total DNA.
To measure DNA methylation of the chromatin fractions, exponentially growing fibroblasts were given ^{3}H-methyl-methionine (10–25 μCi/ml) and 20 mM Na formate for 4 h after which the cells were harvested, chromatin was extracted and fractionated, and the DNA was purified and hydrolyzed. The table, A, shows that the DNA methylation of fraction S2 is twice that of the P1 and P2 fractions of total unfractionated chromatin. Such enrichment is consistent with the finding of Adams et al.[18] of a 2-fold enrichment of DNA methylation in CHO cell chromatin initially digested by DNAase I.
DNA methylation in early S-phase and later S-phase fractions was also determined. After cells were grown to confluency, they were maintained for 24 h in the presence of 0.1% serum to ensure their blockage in the G1-phase. Cells were subsequently stimulated by a very brief trypsinization, and replenishment of serum. The time of DNA synthesis was determined by ^{3}H-thymidine incorporation into DNA. Early S-phase and mid-late S-phase are defined as occurring between 3.0–6.5 h and 9–12.5 h, respectively, after trypsinization and serum stimulation, at which time the ^{3}H-methyl-methionine label was added. Early in the S-phase, the Mg^{++}-soluble, transcriptionally active (S2) DNA fraction is 4–5 times more methylated than the inactive P1 fraction (table, B). In the late S-phase, a significant difference in the degree of DNA methylation between the fractions is not observed (table, B). The increase in the degree of methylation we observed at the beginning of the S-phase is particularly interesting. It has been previously shown that DNA methylase activity can be high during the early S-phase[19], and it is believed that early replicating DNA may be enriched in active genes[19]. Our findings may indicate that there is a subset of transcriptionally active (S2) DNA which replicates early and is highly methylated.
In order to assess whether methylated bases are preferentially localized in different types of DNA sequences, DNA reassociation experiments were performed on fragments with an average size of 200–300 bases[13]. In both the active S2 and inactive P1 fractions the specific activity of the ^{3}H-methyl-labeled, repeated DNA (Cot < 10) was about 1.5 times that of the simple sequence (Cot > 10) DNA (table, C), indicating no large difference in the nature of the methylcytosine distribution in active and inactive sequences. The data of Singer et al.[13] for the DNA of mammalian cells also indicate a slight enrichment for methylation in repeated sequences.
Although transcriptionally-active DNA may be preferentially methylated, the relationship of methylation to gene activity is not obvious. This is especially apparent in the light of several recent studies[7,8,13,20]. Bird and Southern[20] propose that the absence of methylation in an otherwise heavily methylated sequence may be most significant for the binding of regulatory proteins to DNA. During terminal differentiation in Friend leukemia cells, a correlation can be made between hypomethylation[21] and the occurrence of single-stranded breaks[22] in the DNA. We feel that it is possible that eukaryotic DNA methylation is directly related to the ligation of DNA single-stranded breaks and thereby to the maintenance of the DNA supercoil. Methylation, as well as undermethylation, may thus affect the state of relaxation of the DNA supercoil which, in turn, affects the capacity of the DNA to act as a template for transcription[23,24]. This hypothesis is presently under investigation.

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2 J. Doskocil and F. Sorm, Biochim. biophys. Acta *55*, 953 (1962).
3 R. Adams, Biochim. biophys. Acta *254*, 205 (1971).
4 A. Riggs, Cytogenet. Cell Genet. *14*, 9 (1975).
5 R. Holliday and J. Pugh, Science *187*, 226 (1975).
6 R. Volpe and T. Eremenko, FEBS Lett. *44*, 121 (1974).
7 C. Waalwijk and R. Flavel, Nucleic Acids Res. *5*, 4631 (1978).
8 D. Dickinson and R. Baker, Proc. natl Acad. Sci. USA *75*, 5627 (1978).
9 E. Winocour, A. Kaye, and V. Stollar, Virology *27*, 156 (1965).
10 J. Gottesfeld, W. Garrad, G. Bagi, R. Wilson and B. Bonner, Proc. natl Acad. Sci. USA *71*, 2193 (1974).
11 K. Harbers, B. Harkeis, and J. Spencer, Biochem. biophys. Res. Commun. *66*, 738 (1975).
12 Z. Dische, in: The Nucleic Acids, vol. 1, p. 285. Ed. E. Chargaff and J. Davidson 1955.
13 J. Singer, R. Stellwagen, J. Roberts, and A. Riggs, J. biol. Chem. *252*, 5509 (1977).
14 H. Lowry, N. Rosebrough, A. Farr, and J. Randall, J. biol. Chem. *193*, 265 (1951).
15 M. Mandel, C. Schildkraut and J. Marmur, in: Methods in Enzymology, vol. *12B*, p. 184. Ed. L. Grossman and K. Moldave. 1968.
16 A. Garel, M. Zolan, R. Axel, Proc. natl Acad. Sci. USA *74*, 4867 (1977).
17 Unpublished observation.
18 R. Adams, E. McKay, J. Douglas, and R. Burdon, Nucleic Acids Res. *4*, 3097 (1977).
19 D. Comings, Exp. Cell Res. *74*, 383 (1972).
20 A. Bird and E. Southern, J. molec Biol. *118*, 27 (1978).
21 J. Christman, P. Price, L. Pedrinan, and G. Acs, Eur. J. Biochem. *81*, 53 (1977).
22 W. Scher, D. Tsuei, S. Sassa, P. Price, N. Gabelman and C. Friend, Proc. natl Acad. Sci. USA *75*, 3851 (1978).
23 J. Champoux, A. Rev. Biochem. *47*, 449 (1978).
24 A. Graessmann, M. Graessmann and C. Mueller, Proc. natl Acad. Sci. USA *74*, 4831 (1977).