Proc. Natl. Acad. Sci. USA
Vol. 75, No. 12, pp. 5855–5859, December 1978
Biochemistry

Change in quantity and size distribution of small circular DNAs during development of chicken bursa

(extrachromosomal DNA/B lymphocyte ontogeny/immunoglobulins)

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Communicated by George K. Hirst, September 11, 1978

ABSTRACT **Small circular DNAs ranging in contour length from 0.06 to 3.5 μm have been isolated from bursas of 19-day chicken embryos and 4- to 5-week-old chickens. Small circular DNA is present in bursas of 19-day embryos at approximately 0.2 molecules per cell and is very heterogeneous, lacking distinct size classes; most molecules have contour lengths of less than 0.4 μm. In contrast, small circular DNA is present in bursas of 4- to 5-week-old chickens at about 4 molecules per cell, and although this DNA is still heterogeneous, it contains a major distinct class of molecules 0.8 μm in size. These small circular DNAs may be products of developmental gene rearrangements occurring in the chromosomal DNA of lymphocytes in the bursa.**

Eukaryotic cells often contain a population of small polydisperse circular (spc) DNAs with molecular weights ranging from 1×10^5 to 4×10^6. For example, such species have been identified in human (HeLa) (1), mouse (1), and monkey (BSC-1) (2, 3) cell lines, *Drosophila* eggs and cell lines (4), and human warts (5). At present, the most extensive information regarding purified spcDNA relates to the material derived from HeLa (1), *Drosophila* (4), and BSC-1 (3) cells. Specifically, HeLa spcDNA appears to be predominantly cytoplasmic, present at a level of about 100–400 circles per exponentially growing cell, of greater molecular complexity than its average molecular weight, and derived from chromosomal DNA; *Drosophila* spcDNA appears to be predominantly nuclear, present at a level of about 3–40 circles per cell, and composed of molecules with different sensitivities to restriction endonuclease digestion; BSC-1 spcDNA appears to be present at a level of about 1000–2000 circles per cell, resolvable into relatively distinct size classes by gel electrophoresis, of a greater molecular complexity (in the range of 1×10^8) than its average molecular weight (5×10^5) and derived, at least in part, from chromosomal DNA. It should be noted that no biological function has been assigned to any of these spcDNAs.

In this communication we report the isolation and characterization of covalently closed (form I) small circular DNAs from the developing chicken bursa, an organ known to contain large numbers of B lymphocytes undergoing development to a committed state for the production of particular antibodies; in brief, small circular DNAs derived from the bursas of embryonic (19-day) and 4- to 5-week-old chickens differed markedly in both quantity and size distribution. It is proposed that these molecules may represent products of immunoglobulin gene rearrangements, which are known to occur during development of B cells (6–8).

MATERIALS AND METHODS

Isolation of Circular DNAs from Chicken Organs. Chicken embryos (White Leghorns, Hubbard strain, obtained from Shamrock Poultry Farms, New Brunswick, NJ) were sacrificed at 19 days (2 days before hatching), and livers and bursas were removed, frozen on dry ice, and stored at −20°C until use. Four- to 5-week-old chickens (White Leghorns, H and N strain, obtained from Truslow) were sacrificed, and organs were removed and frozen in the same way. In general, 200 embryos yielded 100 g of liver and 7 g of bursa, while 7–10 chickens, 4–5 weeks old, yielded 50 g of liver and 10 g of bursa. To prepare small circular DNA from these tissues, 5–10 g of bursa or 30–40 g of liver was thawed on ice, minced into cold (4°C) 0.15 M EDTA at pH 8.0 (8 ml/g of tissue), and homogenized with two strokes of a Kontes Duall 25 homogenizer. Sodium dodecyl sulfate ($NaDodSO_4$), 10% wt/vol, was then added to a final concentration of 0.5% and the preparation was titrated to pH 12.10 with 1.25 M NaOH, incubated for 10 min at room temperature, and neutralized with 6 M HCl to pH 7.8, a procedure known to selectively denature linear and noncovalently closed (form II) circular DNAs (9). This lysate was then extracted with ½ vol of redistilled phenol (equilibrated with 0.15 M EDTA), and nucleic acids in the aqueous phase were precipitated overnight at −20°C by the addition of 2 vol of cold 100% ethanol. After centrifugation and ethanol washing of this flocculent precipitate (terminated by an ether wash), the pellet was resuspended at room temperature in 30 ml of 0.01 M EDTA at pH 8.0, brought to 70°C, adjusted to an RNase A concentration of 300 μg/ml by the addition of crystalline enzyme (Sigma), incubated for 10 min at this temperature, and then incubated for an additional 30 min at 37°C. The preparation was then subjected to Sephadex G-100 and nitrocellulose chromatography (to remove single-stranded DNAs) in 0.3 M NaCl/0.03 M trisodium citrate as previously described (9), flash evaporated to a final DNA concentration of 150 μg/ml, dialyzed against 0.15 M NaCl/0.015 M trisodium citrate, and centrifuged to equilibrium in CsCl/ethidium bromide in a Beckman Ti 60 rotor (40 hr, 38,000 rpm, 20°C) (10). Gradients were collected dropwise from the bottom, and the regions *below* the single intense visible band (which consists of partially renatured DNA fragments that escaped nitrocellulose removal) were pooled, passed through a 1-ml Dowex 50W-X8 (Sigma) column to remove ethidium bromide, and dialyzed against 0.2 M sodium phosphate buffer, pH 6.8. This material (in 10 ml of 0.2 M phosphate buffer) was then adjusted to 0.18 M phosphate/0.4% $NaDodSO_4$ and passed through a 5-ml column of hydroxyla-

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Abbreviations: spcDNA, small polydisperse circular DNA; $NaDodSO_4$, sodium dodecyl sulfate.

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patite (Bio-Gel HTP, Bio-Rad DNA grade) maintained at 60°C; after the column had been washed with several 1-ml aliquots of 0.18 M phosphate/0.4% $NaDodSO_4$, double-stranded DNA was eluted with 0.4 M phosphate/0.4% $NaDodSO_4$, dialyzed at room temperature against 0.15 M EDTA at pH 8.0, and centrifuged to equilibrium in CsCl/ethidium bromide in a Beckman Ti 50 rotor (36 hr, 38,000 rpm, 20°C). Fractions corresponding to form I DNA were pooled, cleared of ethidium bromide as before, dialyzed against 0.15 M NaCl/0.015 M trisodium citrate, and centrifuged to equilibrium in 5 ml of CsCl (input density 1.70 g/ml) for 36 hr at 40,000 rpm and 20°C in a Beckman SW 65 rotor. Following collection of this gradient with an ISCO model 185 density gradient fractionator equipped with a model UA-2 monitor, fractions corresponding to double-stranded DNA (identified by the sharp peak of mitochondrial DNA) were pooled, dialyzed against 0.03 M NaCl/0.003 M trisodium citrate, and concentrated to a volume of 0.2 ml by flash evaporation. This double-stranded DNA contained a mixture of mitochondrial and small circular species, but due to the very small number of the latter molecules in chicken organs, no attempt was made to purify them further.

Preparation of spcDNAs for Electron Microscopy. Contour length measurements from electron micrographs require opened DNA rings, and in order to quantitatively convert a widely heterogeneous population of form I DNAs to form II without degrading the larger molecules, we have used the technique of ethidium bromide/DNase I nicking (11) under the conditions previously described for BSC-1 spcDNA (3). This technique is based upon the selective ethidium bromide inhibition of form II nicking by DNase, so that DNase I- (pancreatic, Worthington DPFF, 2200 units/mg) catalyzed nicking of form I DNA in the presence of ethidium bromide results in the quantitative formation of opened rings containing only *one* single-strand break. In brief, mixtures of form I small circular and mitochondrial DNAs were nicked for 30 min in 200-μl reaction mixtures (30°C) that contained 0.1–1.0 μg of DNA, 0.15 M NaCl, 15 mM $MgCl_2$, 5 mM Tris·HCl at pH 8, ethidium bromide (Calbiochem, A grade) at 100 μg/ml, and 0.04 μg of DNase, and reactions were terminated by the addition of 50 μl of 0.5 M EDTA with chilling on ice; this treatment represented 10 times the incubation time required to nick 50% of form I molecules the size of simian virus 40 DNA. DNase was removed by chloroform extraction, and ethidium bromide by Dowex chromatography.

Electron Microscopy. DNA was mounted for electron microscopy in the absence of cytochrome *c* as follows (12). A 50-μl drop containing 0.1 μg of DNA per ml, 10 mM Tris·HCl at pH 8.5, 1 mM EDTA, 30% (vol/vol) formamide, and 0.01% anthrabis (1,4-bis-[3-(benzyldimethylammonio)propylamine]-9,10-anthraquinone dichloride, supplied by J. O. Thomas) was formed on a piece of Parafilm. After 10 min, a carbon-coated electron microscope grid was briefly touched to the surface of the drop, rinsed in water followed by ethanol (each 15 sec) and rotary shadowed at an angle of 10° with platinum/palladium (80:20). All samples were examined in a Siemens 101 electron microscope.

RESULTS

Isolation of Circular DNA from Chicken Bursas. An outline of the preparative procedure as applied to the isolation of form I circular DNA from 4- to 5-week old chicken bursas is presented in Table 1, which also includes the amounts of total DNA present at each step and the percent recovery of an added form I, ^3H-labeled plasmid DNA marker [*Staphylococcus aureus* plasmid pI258mad34Δ90mer→asi, prepared as previously described (14)]. Three additional preparations (two from embryonic and one from 4- to 5-week-old chicken bursas) containing such a marker resulted in essentially the same pattern of recovery. The efficacy of phenol extraction in removing most of the DNA from the preparation, with good recovery of form I circular DNA in the aqueous phase, was surprising and very helpful. On the basis of a comparison of the molecular weight of plasmid DNA (10×10^6) to that of 4- to 5-week-old bursal small circular DNA (average of 1.6×10^6), and the fact that smaller form I molecules are more resistant to nicking, it may be assumed that the final recovery of form I small circular DNA was at least 6% and probably closer to 30% (in a separate experiment the final percent recovery of an added form I ^3H-labeled simian virus 40 DNA marker of molecular weight 3.4×10^6 was 15%). As noted in *Materials and Methods*, the DNA obtained after step 10 of the preparative procedure contained a mixture of small circular and mitochondrial DNAs.

Table 1. Isolation of circular DNA from bursas of 5-week-old chickens*

Step	Procedure	Total DNA in preparation,† μg	Recovery of plasmid [^3H]DNA, %‡
1	Homogenization		
2	Alkali denaturation	52,000	100
3	Phenol extraction	9,500	88
4	Ethanol precipitation	9,000	83
5	Sephadex chromatography	8,200	78
6	Nitrocellulose chromatography	4,700	75
7	CsCl/ethidium bromide density gradient centrifugation	330	31
8	Hydroxylapatite chromatography	106	29
9	CsCl/ethidium bromide density gradient centrifugation	22	7
10	CsCl density gradient centrifugation	2.4	6

* This preparation began with 10 g of tissue derived from 10 chickens. Form I ^3H-labeled plasmid DNA (1.5 μg; 4×10^4 cpm/μg) was added to the cells before the addition of $NaDodSO_4$. Note that only steps 7 and 9 specifically resolve forms I and II.

† Total DNA was measured after completion of the indicated preparative procedure by either the diphenylamine reaction (13) (steps 2–4) or ultraviolet absorbance at 260 nm (steps 5–10).

‡ Percentage of the input plasmid DNA that was recovered in fractions assumed to contain form I circular DNA after completion of the indicated preparative procedure (determined by measuring the radioactivities of aliquots in Aquasol).

Electron Microscopic Analysis of Circular DNAs Isolated from Chicken Bursas. Composite electron micrographs of selected nicked circular DNAs obtained from single preparations of both embryonic and 4- to 5-week-old bursas are shown in Figs. 1 and 2, and the contour length distributions of these small circular DNAs are presented as histograms in Fig. 3. It should be mentioned that although the contrast obtained from the anthrabis procedure of DNA mounting is less than that observed with cytochrome *c* (due to the thinness of the DNA filaments), the anthrabis technique allows for more accuracy in contour length measurements, especially in the case of very small (0.05 μm) circular DNAs. As may be seen by a comparison of the histograms shown in Fig. 3, the major finding of this and other electron microscopic investigations was that the size distribution of small circular DNAs isolated from embryonic and 4- to 5-week-old bursas differed markedly. Embryonic bursal small circular DNA was very heterogeneous, lacked

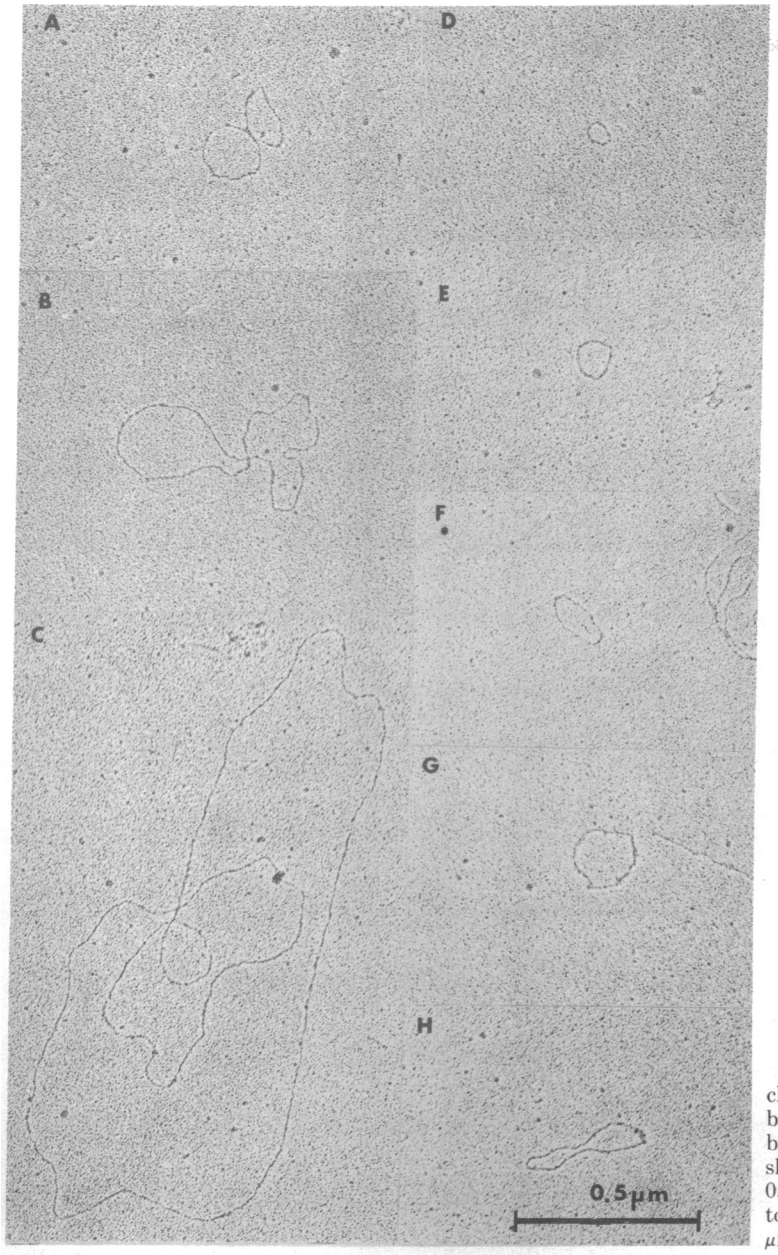

FIG. 1. Small circular DNA isolated from embryonic chicken bursas. Form I molecules were nicked by ethidium bromide/DNase catalysis for 30 min before mounting. The bar indicates 0.5 μm, and the small circular DNA molecules shown have contour lengths, in μm, of (*A*) 0.9, (*B*) 1.86, (*D*) 0.17, (*E*) 0.30, (*F*) 0.39, (*G*) 0.53, (*H*) 0.74. (*C*) Typical mitochondrial DNA molecule with a contour length of 6.0 μm.

distinct size classes, and contained molecules most of which had contour lengths less than 0.4 μm. Four- to 5-week-old bursal small circular DNA, although still heterogenous, contained a major distinct class of molecules 0.8 μm in size; a second distinct class of molecules 1.9 μm in size may also be present, though our data are not yet sufficient to clarify this observation.

Another finding derived in part from this electron microscopic study was that 4- to 5-week-old chicken bursas contained several times more form I small circular DNA per cell than embryonic bursas. This calculation was based on the molecular ratios of mitochondrial and small circular DNAs in bursal circular DNA preparations, and on data such as that presented in Table 1. For example, from the data of Table 1 and the electron microscopic observation that 5-week-old bursal circular DNA contains ten times more mitochondrial than small circular molecules, one can estimate the number of form I spcDNAs per cell. Specifically, 2.4 μg of total circular DNA possessing a 10:1 molecular ratio of mitochondrial (molecular weight 12×10^6) to small circular (average molecular weight 1.6×10^6) DNA should contain about 0.03 μg of the latter material, and 0.03 μg of small circular DNA per 52 mg of total cellular DNA [3×10^{12} daltons per diploid chicken cell (15)] corresponds to 1.1 form I molecules per cell before correcting for recovery, and probably about 4 molecules per cell after such a correction. Similar calculations for embryonic bursal small circular DNA preparations (where the ratio of mitochondrial to small circular molecules was about 40:1) resulted in an estimate of 0.2 molecules per cell after making the same correction for recovery. It should be noted that the actual total number of small circular DNA molecules per cell (both forms I and II) cannot be obtained from this data, because the preparative procedure is selective for form I molecules; however, if the stability of closed circular DNAs in chicken bursas is similar to that in BSC-1 (3) and HeLa (1) cells, it may be expected that the amount of form II does not greatly exceed that of form I.

Attempted Isolation of Small Circular DNA from Chicken Liver. Attempts to prepare small circular DNA from livers obtained from both 19-day chicken embryos and 4- to 5-week-old chickens by using the methods presented above for bursas resulted in the finding that such molecules were ex-

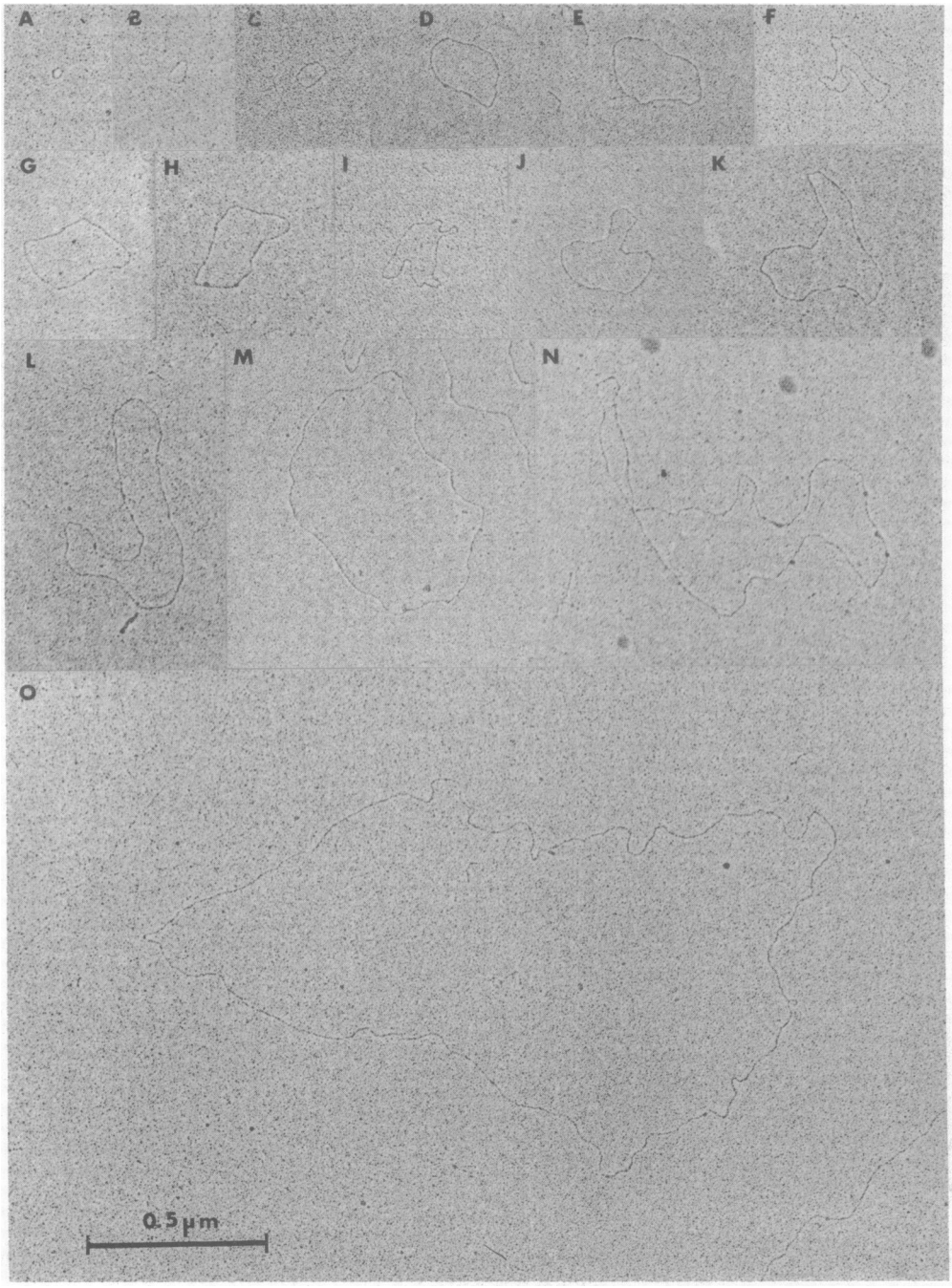

FIG. 2. spcDNA isolated from bursas of 4- to 5-week-old chickens. Form I molecules were nicked by ethidium bromide/DNase catalysis for 30 min before mounting. The bar indicates 0.5 μm and the small circular DNA molecules shown have contour lengths, in μm, of (*A*) 0.09, (*B*) 0.18, (*C*) 0.24, (*D*) 0.61, (*E*) 0.73, (*F*) 0.77, (*G*) 0.78, (*H*) 0.78, (*I*) 0.82, (*J*) 0.87, (*K*) 1.30, (*L*) 1.85, (*M*) 1.98, (*N*) 3.16. (*O*) Typical mitochondrial DNA molecule with a contour length of 5.85 μm.

tremely rare. However, mitochondrial DNA was isolated in amounts similar to those obtained from bursal preparations.

DISCUSSION

It is difficult to explain why there should be such a difference between the small circular DNA molecules found in the bursas of 19-day embryonic and 4- to 5-week-old chickens, because, by the 19th day of embryonic life, development of the bursa is virtually completed (16) and lymphocytes within it resemble those found in this organ at 4–5 weeks, at least in terms of the immunoglobulin isotypes expressed on the cell surface. Some quantitative change in the function of the bursa (rather than a qualitative one) may be responsible for the observed changes in the small circular DNAs isolable from this tissue at these two points in the life cycle of the chicken.

The possibility remains that the 0.8-μm circular DNA molecules found in the bursas of 4- to 5-week-old chickens are not of chicken origin. The bursas of our 4- to 5-week-old chickens could contain viruses or bacteria (possibly with associated plasmids or bacteriophages) that could be the origin of these small circular DNAs. Though most bacterial and animal viruses have genomes substantially larger than 2400 base pairs (the content of a 0.8-μm circular DNA), the presumptive hepatitis B virus particle contains a circular DNA of contour length 0.78 μm (17). Alternatively, the 0.8-μm circular DNA found in bursas of 4- to 5-week-old chickens could represent self-replicating plasmid or organelle DNA, though molecules of this size were not easily detectable in chicken liver or in the bursas of 19-day chicken embryos. Small circular DNAs could also represent functional amplified genes, products of induction of integrated viral genomes, products of DNA degradation, or other side products of DNA metabolism (presumably nonfunctional).

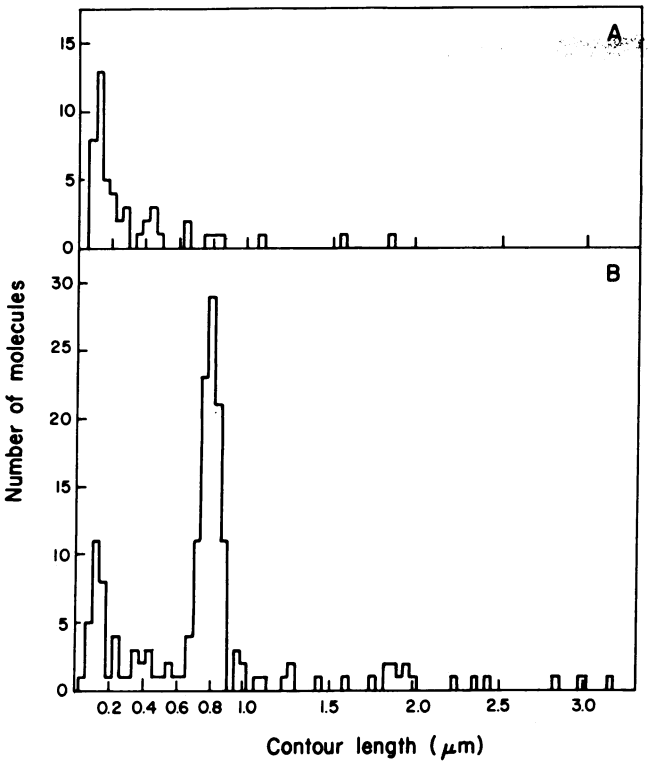

FIG. 3. Contour length distributions of small circular DNAs isolated from bursas of embryonic and 4- to 5-week-old chickens. Electron micrographic plates were projected onto a ground glass screen, and contour lengths of the projected molecules were measured by using a Numonics graphics calculator; magnification was determined from a diffraction grating. Papilloma DNA molecules mounted, photographed, and measured under the same conditions had an average contour length of 2.45 μm (5). (*A*) Histogram of small circular DNA isolated from embryonic bursas. A total of 51 molecules were measured, and the average contour length was 0.39 μm. (*B*) Histogram of small circular DNA isolated from 4- to 5-week-old bursas. A total of 172 molecules were measured, and the average contour length was 0.81 μm.

Of greatest interest to us are the possible implications of our results to the molecular mechanisms involved in the development of lymphocytes in the bursa. A very attractive hypothesis is that these 0.8-μm circular molecules are the product of intramolecular recombination events, which produce a circular DNA and result in the joining of segments of DNA that are not contiguous in the germ line. Somatic rearrangement of the DNA of B lymphocytes probably does occur in the course of their development, bringing into close proximity the genetic information for the constant and variable portions of an immunoglobulin polypeptide chain (6–8). Several roles can be envisioned for intramolecular recombination involving excision of circular DNA, and gene joining, in the development of B lymphocytes. The excised genetic material could represent an immunoglobulin constant region gene or genes along with spacer DNA, and such excisions could serve to join a single immunoglobulin variable region gene to several different constant region genes in succession, as may occur in the development of IgG- and IgA-producing cells from those producing IgM. Also, intramolecular recombination could serve to bring one of many variable region genes into close proximity with a constant region gene, and circular DNAs could be excised as a result of similar intramolecular recombination between variable region genes [both possible mechanisms for the generation of antibody diversity (6, 18–20)]. In the latter case, the variable region gene remaining in the chromosome could differ extensively from both of the germ line genes from which it was produced, especially if one postulates the formation of heteroduplex DNA [which may occur commonly in recombination (21) and which could be repaired in many different ways to yield genes of different primary structure]. A few germ line variable region genes, differing slightly in base sequence (especially in the hypervariable regions), could produce an enormous number of recombinant variable region genes with base sequence variations concentrated in hypervariable segments. Finally, small circular DNAs in the B lymphocyte could represent variable region genes that have been excised to be reintegrated at a new position in the chromosomal DNA (6), or mutator elements that induce changes in the base sequence of chromosomal variable region genes.

In summary, the data presented here are consistent with the supposition that intramolecular recombination resulting in the excision of small circular DNA may be a mechanism involved in the ontogeny of B lymphocytes. Of course, the presence of small circular DNA in developing chicken bursas is also consistent with many other possible origins. In any event, we hope that examination of the structure of these molecules, and the nature of their homology to the DNAs of embryonic and differentiated cells, will yield major insights into the structure and function of eukaryotic genes.

We thank Messrs. Horace Lozina and Heinz Annus for expert technical assistance and Dr. John Thomas for both the use of his electron microscope facilities and much helpful advice. This research was supported by Grant VC-256 from the American Cancer Society and Grant CA-16239 from the U.S. Public Health Service. R.D. was supported by Training Grant 5T32 CA-09161-02, also from the U.S. Public Health Service.

1. Smith, C. A. & Vinograd, J. (1972) *J. Mol. Biol.* **69**, 163–178.
2. Rush, M. G., Eason, R. & Vinograd, J. (1971) *Biochim. Biophys. Acta* **228**, 585–594.
3. DeLap, R. J., Rush, M. G., Zouzias, D. & Khan, S. (1978) *Plasmid*, **1**, 508–521.
4. Stanfield, S. & Helinski, D. R. (1976) *Cell* **9**, 333–345.
5. DeLap, R. J., Friedman-Kien, A. & Rush, M. G. (1976) *Virology* **74**, 268–272.
6. Hozumi, N. & Tonegawa, S. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3628–3632.
7. Tonegawa, S., Hozumi, N., Matthyssens, G. & Schuller, R. (1976) *Cold Spring Harbor Symp. Quant. Biol.* **41**, 877–889.
8. Brack, C. & Tonegawa, S. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5652–5656.
9. Rush, M. G. & Warner, R. C. (1970) *J. Biol. Chem.* **245**, 2704–2708.
10. Rush, M. G., Kleinschmidt, A. K., Hellmann, W. & Warner, R. C. (1967) *Proc. Natl. Acad. Sci. USA* **58**, 1676–1683.
11. Greenfield, L., Simpson, L. & Kaplan, D. (1975) *Biochim. Biophys. Acta* **407**, 365–375.
12. Thomas, J. O., Sternberg, N. & Weisberg, R. (1978) *J. Mol. Biol.* **123**, 149–162.
13. Burton, K. (1956) *Biochem. J.* **62**, 315–323.
14. Rush, M. G., Gordon, C. N., Novick, R. P. & Warner, R. C. (1969) *Proc. Natl. Acad. Sci. USA* **63**, 1304–1310.
15. Sober, H., ed. (1966) *Handbook of Biochemistry* (CRC Press, Cleveland, OH), pp. 14–58.
16. Eisen, H. N. (1974) *Immunology* (Harper & Row, Hagerstown, MD), pp. 473–503.
17. Robinson, W. S., Clayton, D. A. & Greenman, R. L. (1974) *J. Virol.* **14**, 384–391.
18. Smithies, O. (1967) *Cold Spring Harbor Symp. Quant. Biol.* **32**, 161–168.
19. Edelman, G. M. & Gally, J. A. (1967) *Proc. Natl. Acad. Sci. USA* **57**, 353–358.
20. Whitehouse, H. L. K. (1967) *Nature (London)* **215**, 371–374.
21. Radding, C. M. (1973) *Annu. Rev. Genet.* **7**, 87–111.