THE BASE COMPOSITION OF NUCLEIC ACIDS IN CHROMOSOMES, PUFFS, NUCLEOLI, AND CYTOPLASM OF *CHIRONOMUS* SALIVARY GLAND CELLS

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ABSTRACT

The base composition of RNA from individually isolated giant chromosomes, puffed chromosome segments, nucleoli, and samples of cytoplasm from *Chironomus* salivary gland cells was determined by microelectrophoresis. Data on the adenine:guanine quotient of the chromosomal DNA were also obtained. The results show that: 1) Chromosomal, nucleolar, and cytoplasmic RNA's differ significantly from each other in base composition. 2) Nucleolar and cytoplasmic RNA's, in spite of the difference, show great similarities with regard to the base composition and are both rich in adenine and uracil. 3) The RNA extracted from chromosome I differs significantly from the RNA's extracted from different segments of chromosome IV, and the latter differ significantly from each other. 4) The values for the RNA:DNA quotients of chromosome segments parallel the development of synthetically active genes, so-called Balbiani rings. 5) The chromosomal RNA does not show a base symmetry in any of the investigated cases, nor is the content of guanine + cytosine the same as that for DNA. The first of these two facts excludes the possibility that the chromosomal RNA is a complete copy of both strands of the chromosomal DNA. In spite of the difference in guanine + cytosine content between the two nucleic acids the RNA may still partly or completely be a single strand copy depending upon how representative the DNA values are for the synthetically active DNA.

The study of the process of information transfer from DNA[1] to protein-synthesizing centers in the cytoplasm has been carried out chiefly in bacteria. It is understandable that technical difficulties should arise when such studies are extended to higher organisms. However, in at least one case, namely the dipteran giant chromosomes, a higher organism may offer a distinct advantage. Here, through polyteny and the phenomenon of puffing (2), products that are known to be due to the activity of single chromosomal loci (3) may accumulate in quantities large enough to be analyzed by special microchemical procedures.

The present paper describes results of microelectrophoretic (5) purine-pyrimidine analyses (microphoresis) of giant chromosomes, nucleoli, and cytoplasm from the salivary gland nuclei of *Chironomus tentans*. One chromosome, the short No. IV element, was further subdivided into three segments, each of which included a very large puff (Balbiani ring). Genetically, a Balbiani ring is

[1] Abbreviations: DNA = deoxyribonucleic acid, RNA = ribonucleic acid, A = adenine, G = guanine, C = cytosine, U = uracil, T = thymine.

equivalent to a single informational gene locus (3) which, in biochemical terms, is characterized by a particularly high synthetic activity as concerns RNA (14). Most of the RNA extracted from the segments of chromosome IV should, therefore, constitute the product of those gene loci represented by the Balbiani rings, an inference which can also be drawn directly from our data. An indication of the general nature of the base composition of the DNA of the giant chromosomes was obtained by measuring its adenine/guanine quotient by means of microphoresis.

METHODS

PREPARATION OF THE MATERIAL: Chromosomes, nucleoli, and cytoplasm were taken from the large cells of the main lobe of the larval salivary gland of *Chironomus tentans* (*cf.* reference 3). Large fourth instar larvae were used throughout. Since the Balbiani rings do not undergo specific changes during metamorphosis it was not deemed necessary to make a distinction between larvae and "prepupae." For similar reasons, we did not determine the sex of the larvae. Within each series of dissections the larvae were collected from a single culture bowl and represented the progeny of a single mating. As judged from the size of the Balbiani rings, the homogeneity within such cultures was excellent. Between different series of dissections where larvae from different cultures had been used some differences in the size of the Balbiani rings were observed. However, since all cultures derive from related inbred stocks, the material as a whole can be considered as genetically homogeneous.

The salivary glands were dissected in body fluid and immediately fixed in buffered isotonic formalin (5 per cent in M/60 phosphate buffer of pH 7.1 containing 0.2 per cent NaCl) for 30 minutes. This was followed by a brief wash in distilled water which served as the medium for further dissection as well. Microdissection of single cells was carried out by hand under a binocular (Leitz Greenough). The nuclear membrane was torn open, with tungsten needles, and the chromosomes were freed from the nucleus by gentle agitation. They could then be drawn up individually into a mouth pipette and transferred to coverslips where they were air dried. Homologous chromosomes from different nuclei of the same pair of glands were put together on the same coverslip but arranged individually in order to permit the further dissection into chromosome segments (see below). There is no difficulty in identifying the four different chromosomes of *C. tentans* individually so that errors in classification are excluded (see Fig. 1). In the case of chromosome IV all inversion heterozygotes were discarded, which is an obvious prerequisite for obtaining clear separations of the chromosomal segments from each other. For the preparation of nucleoli the nucleolar chromosomes were transferred to coverslips and air dried. The nucleolar mass was then freed from the attached chromosome as much as possible. No distinction was made between nucleoli isolated from chromosomes II or III. Samples of cytoplasm were prepared by placing enucleated cells on coverslips for air drying.

The preparations were posttreated as follows before being sent to Gothenburg for microchemical analysis: One series (lot No. 1, sent to Gothenburg on May 19, 1961) was treated with 5 per cent TCA (trichloracetic acid) at 4°C for 5 minutes; the next series (lot No. 2, sent to Gothenburg on October 21, 1961), with 10 per cent PCA (perchloric acid) at 4°C for 5 minutes. The treatment was followed, in all cases, by water, acetone, and finally air drying.

Lot No. 1 comprised 30 coverslips with 104 No. IV chromosomes from 9 animals; 5 coverslips, each with 5 or 6 nucleoli from 2 animals; and 3 coverslips, each with the cytoplasm of one cell from a different animal (2 of these were fixed with acetic alcohol 1:3).

Lot No. 2 comprised 20 coverslips with 260 No. IV chromosomes from 20 animals; 12 coverslips with 93 No. I chromosomes from 12 animals; 1 coverslip with 12 nucleoli from 2 or 3 animals; 1 coverslip with the cytoplasm of one cell.

ANALYTICAL PROCEDURES: The fixed and dried salivary gland constituents were hydrated with 0.01 N acetic acid and covered with liquid paraffin in an oil chamber arrangement. They were rearranged and, in the case of the chromosomes, cut according to requirements, using the de Fonbrune micromanipulator equipped with two glass needles.

The No. I chromosomes were used whole and were collected in groups of 17 to 28 for each analysis. The No. IV chromosomes were divided into three segments and designated IV *u* (upper), IV *m* (middle) and IV *l* (lower). The middle and upper segments were separated between their Balbiani rings; and the lower segments were taken off as close to the Balbiani ring of the middle segments as possible (see Fig. 2). Thirty to eighty segments were used for each analysis. RNA was extracted with ribonuclease and analyzed by microphoresis as described earlier (5), but digested at 37°C instead of room temperature.

In cases with a low RNA/protein quotient, such as certain chromosomal segments, the increased amount of material necessary for a microphoretic separation may give rise to an increased contamination of RNA by proteins. If no precautions are taken the effect is to increase the values for uridylic acid. Evidence for such contamination was obtained in some analyses of lot No. 1. In view of this, lot No. 2 was postfixed with 96 per cent ethanol for 18 hours, a treatment which largely eliminates contaminations.

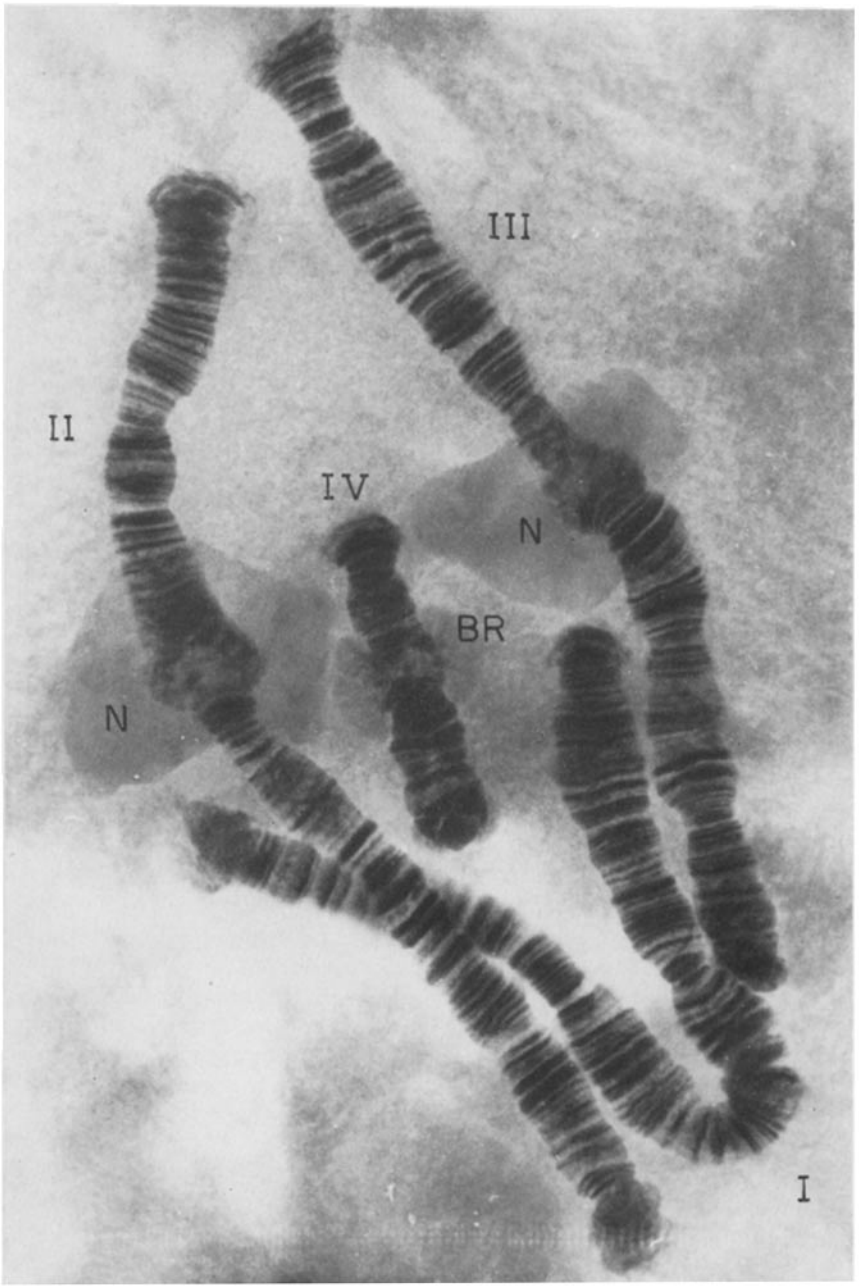

FIGURE 1

The salivary gland chromosomes (I, II, III, IV) of *Chironomus tentans*. *N* = nucleoli, *BR* = Balbiani rings. × 750.

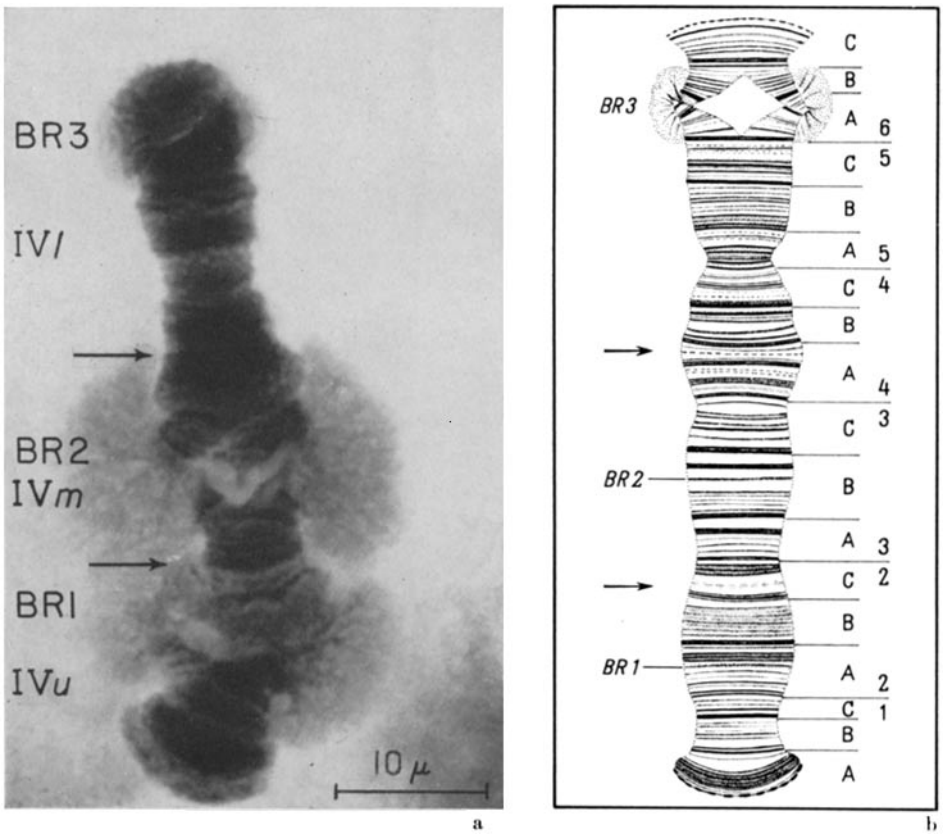

FIGURE 2

Chromosome IV from the salivary glands of *Chironomus tentans*. (*a*) The three chromosomal segments used in the analysis of RNA (IV *u*, IV *m*, IV *l*) and their approximate delimitation (arrows). *BR 1*, *BR 2*, and *BR 3* = the three Balbiani rings. (*b*) Cytological map showing the banding and length relations when *BR 1* and *BR 2* are not developed (bands of origin indicated for both, after Beermann, 1952). × 2000.

Some separations of RNA from whole No. IV chromosomes and one from IV *l* showed evidence of contamination, the former to a moderate degree. These are not included among the results.

RESULTS

The base compositions of RNA extracted from isolated chromosomes, chromosome segments, nucleoli, and cytoplasm are given in Table I. Photographs of separations, representative for each kind of material, are shown in Fig. 3. It can be seen that the RNA from all sources was characterized by a high adenine content; in the case of nucleolar and cytoplasmic RNA's, by a relatively high uracil content as well. The RNA's from the latter two sources were very similar, but still significantly different with regard to their G/U quotients, the higher quotient being exhibited by cytoplasmic RNA.

All chromosomal RNA's were found to differ from the nucleolar and cytoplasmic RNA's (Table II). There was, however, a wide variation in compositional values between the RNA's from the different chromosomal sources. The greatest variations are to be found in the A/U ratio (Table I). There are significant statistical differences in this ratio between IV *u* and IV *m* as well as between each of these and chromosome I. It is true that IV *l* differs from the other segments also; however, the few values obtained in this case do not usually permit the conclusion to be drawn that significant differences do exist. An exception is the case of IV *l versus* IV *m*, where the difference shows a relatively high t-value.

In order to make comparisons possible between chromosomal RNA and DNA, a few preliminary analyses of DNA composition were carried out and will be reported. Chromosome segments, which had been extracted for RNA, were incubated with 1 N HCl in the oil chamber using liquid paraffin chromosome IV, section *IA*) will necessarily be higher.

A corresponding method for determining RNA amounts was not applied. It is, however, possible to make a very rough estimation of the amounts involved from the intensity of the ultraviolet ab-

TABLE I

Base Composition of RNA Isolated from Various Cellular Components of Chironomus Salivary Gland Cells
Mean values of molar proportions in per cent of the sum ±SEM

	Adenine	Guanine	Cytosine	Uracil	$\frac{\text{Adenine}}{\text{Uracil}}$	$\frac{\text{Guanine}+}{\text{cytosine}}$	n
Chromosome I	29.4 ± 0.5	19.8 ± 1.0	27.7 ± 0.8	23.1 ± 0.6	1.27	47.5	4
Chromosome IV							
Upper parts (IV *u*)	35.7 ± 0.6	20.6 ± 1.7	23.2 ± 1.2	20.8 ± 0.8	1.72	43.8	5
Middle parts (IV *m*)	38.0 ± 0.6	20.5 ± 0.6	24.5 ± 0.6	17.1 ± 0.6	2.22	45.0	6
Lower parts (IV *l*)	31.2 ± 2.2	22.0 ± 2.0	26.4 ± 1.9	20.2 ± 1.4	1.54	48.4	3
Nucleoli*	30.6 ± 0.8	20.1 ± 0.5	22.1 ± 0.6	27.1 ± 0.6	1.13	42.2	13
Cytoplasm	29.4 ± 0.4	22.9 ± 0.3	22.1 ± 0.4	25.7 ± 0.3	1.14	45.0	7

* One nucleolar analysis excluded, showing the following composition: adenine 41.5, guanine 19.7, cytosine 20.4, and uracil 18.5.

saturated with 1 N HCl. Incubation was allowed to take place at 37°C for 18 hr and the extracts were analyzed afterwards by microphoresis for adenine and guanine. The determinations of the A/G ratio show high values, corresponding to about 35 per cent adenine and 15 per cent guanine in a complementary DNA structure (see Table III).

In a few cases, a known amount of cytidine (2 to 3 μμM) was dissolved in a volume of hydrochloric acid used for incubating the chromosome segments. This offered a way of determining the absolute amounts of DNA. Values obtained according to this method are given in Table III. It can be seen that chromosome I contains 2.5 times as much DNA as chromosome IV, which is in good agreement with the relative dimensions of these chromosomes (*cf.* Fig. 1). The values for the segments IV *u* and IV *m*, particularly the latter, seem to be too high in proportion to the apparent chromosome length they constitute. This, however, is to be expected since the formation of a Balbiani ring considerably shortens the chromosome section involved (2). The true length relations as revealed in chromosomes where Balbiani rings are not present (Fig. 2) come much closer to the DNA values. Furthermore, in short chromosome segments, the weight of local variations in DNA concentration (*e.g.* heavy bands at the lower end of

TABLE II

Significance of Determined Differences in Adenine: Uracil Ratio among Various Cellular Components of Salivary Gland Cells

	t-Value*	Degrees of freedom	P-value (upper limit)
IV upper *vs.* IV lower	1.16	6	insignif.
IV upper *vs.* IV middle	4.12	9	0.01
IV middle *vs.* IV lower	4.04	7	0.01
I whole *vs.* IV upper	5.87	7	0.01
I whole *vs.* IV middle	8.49	8	0.001
I whole *vs.* IV lower	2.09	5	0.1
Nucleoli *vs.* IV upper	7.84	16	0.001
Nucleoli *vs.* IV middle	9.83	17	0.001
Nucleoli *vs.* IV lower	3.00	14	0.01
Nucleoli *vs.* I whole	2.22	15	0.05
Cytoplasm *vs.* IV upper	8.80	10	0.001
Cytoplasm *vs.* IV middle	10.3	11	0.001
Cytoplasm *vs.* IV lower	3.01	8	0.02
Cytoplasm *vs.* I whole	2.84	9	0.02
Cytoplasm *vs.* nucleoli (Guanine:Uracil ratio)	5.25	18	0.001

* Statistical significance.

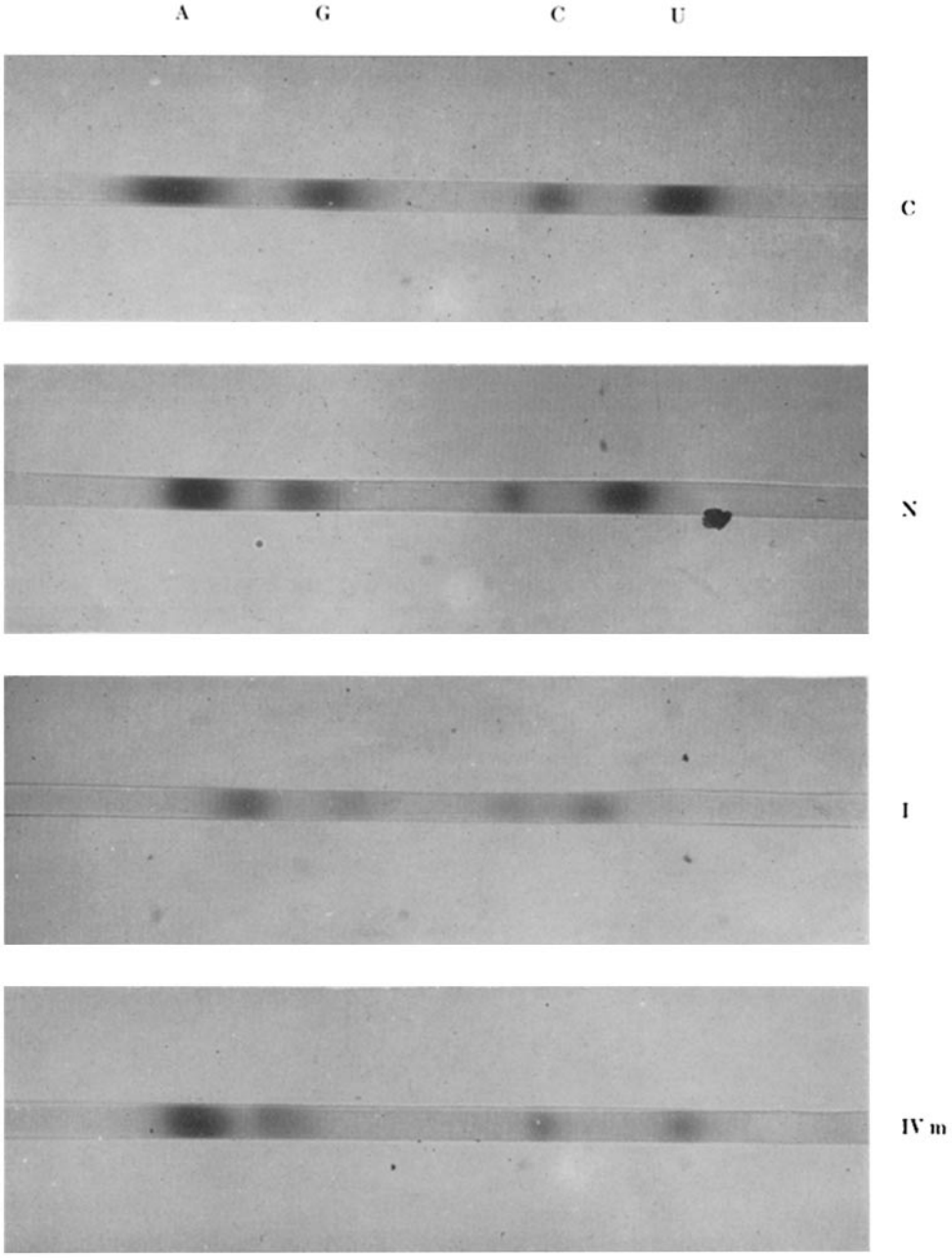

FIGURE 3

Microphoretic separations at 257 mμ of RNA hydrolysates from cytoplasm (*C*), nucleoli (*N*), the first chromosome (I) and the middle segment of the fourth chromosome (IV *m*) from *Chironomus* salivary gland cells. *A*, *G*, *C*, and *U* stand for adenine, guanine, cytidylic acid, and uridylic acid, respectively. × 150.

sorption of the separated bands. These estimations can be used for obtaining round figures for the RNA/DNA quotients in the different chromosomal segments. These data are given in Table III. There is an obvious increase in this ratio as one passes from segments with low synthetic activity

to those of higher activity (as judged by the size of the Balbiani rings).

DISCUSSION

There may be justification for giving some comments regarding the extent to which the obtained data can be assumed to be representative for the *in vivo* conditions. It was shown for Carnoy-fixed starfish oocytes that the fixation and subsequent isolation procedure leaves RNA in a suitable state for quantitative RNA determination and base analysis (7). In the present work there are stages which cannot be checked easily with macrochemical control experiments. However, speaks against artefacts inherent in the preparative procedure.

It immediately becomes apparent, from an inspection of the results on chromosomal RNA, that, in no case, is there a symmetry in base composition of the same kind as that which has been found for DNA. While leaving several alternatives open to explain this result, *e.g.* heterogeneous origin or secondary modifications of RNA, the data make one thing clear, *viz.* that the chromosomal RNA cannot to any higher percentage represent a copy of both strands of complementary DNA molecules. Thus, it is obvious that there is no close resemblance between chromosomal RNA in *Chironomus*

TABLE III

Adenine:Guanine Ratio and Content of DNA in Chironomus Giant Chromosomes

Values in parenthesis give figures expressed as fractions of 50. Data on DNA content refer to single determinations

	Adenine:guanine ratio ±SEM	n	DNA content in μμg	Estimated RNA:DNA ratio
Chromosome I*	3.71 ± 0.29 (39.4:10.6)	4	457; 582	1:20
Chromosome IV				
Upper parts (IV *u*)	2.58 ± 0.12 (36.0:14.0)	4	78	1:5
Middle parts (IV *m*)	2.15 ± 0.20 (34.2:15.8)	5	69	1:3
Lower parts (IV *l*)	2.48 ± 0.13 (35.6:14.4)	3	105	1:10
Whole chromosomes	2.54 ± 0.09 (35.9:14.1)	3	244	—

* Extended analyses, while confirming the results on chromosome IV, have given somewhat lower adenine values (37.8 per cent) for chromosome I.

the substitution of formalin for Carnoy's fluid as a fixative seems reasonably safe in view of the findings of Hartleib *et al.* (10) that the nucleic acid phosphorus is conserved during formalin fixation. Comparisons between the RNA contents of isolated Purkinje nerve cells which were treated either with Carnoy's fluid or Lillie's buffered formalin for 3 hours show inconsiderably lower values for the cells from the formalin-fixed tissue, and base analyses of starfish ovaries demonstrate relatively small differences between the values obtained for formalin-fixed and freeze-dried tissue (Edström and Gall, unpublished data). The completeness of the RNA extractions was controlled in a few cases by carrying out renewed, prolonged digestions and also by staining part of the material after RNA had been extracted. Absence of basophilia was regularly observed for Balbiani rings, nucleolar substance, and cytoplasm. The reproducibility of the values obtained also and the DNA-like "informational RNA" in *E. coli* (11) or yeast cells (21, 24).

It might be permitted also to pose the question whether the data are compatible with the hypothetical view that the chromosomal RNA only consists of unmodified copies of the single DNA chain, with the full understanding that a positive answer will not permit any conclusions to be drawn as to the origin of chromosomal RNA. An answer to the question may possiby be obtained through a comparison between the G-C contents in the chromosomal RNA and DNA. This is because the G-C content has to be the same in a DNA duplex and each one of its constituting strands. As is evident from Table III, the relative G-C content of DNA should be of the order of 30 per cent, a value that differs significantly from the corresponding value for chromosomal RNA (see Table I). The composition of the chromosomal RNA is consequently not the one to be expected for a

product that is copied from the single DNA chain unless one assumes that a fraction of DNA, particularly rich in guanine and cytosine, is synthetically active.

It is not possible to discern with certainty any rules governing the base composition of chromosomal RNA in view of the few examples studied. From the available data only one trend is evident. The A/U ratio parallels both the RNA/DNA ratio and the synthetic activity of the chromosome segments, as judged from the development of Balbiani rings. However, this parallelism seems to be fortuitous, since analyses to be reported of fourth chromosomes in which BR 3 shows a higher degree of development than BR 1 give values for the A/U quotient that are unusually low for BR 3.

An estimation of the size of the fraction of RNA extracted from segments IV *u* and IV *m* that originates in the Balbiani rings may be made. On the assumption that the quotient estimated from chromosome I for RNA/DNA, *i.e.* $\frac{1}{20}$, is representative for all regions of low synthetic activity and with the knowledge of the values for the quotients of the segments in question, *i.e.*, $\frac{1}{5}$ and $\frac{1}{3}$, respectively, it can be estimated that these fractions are 75 and 85 per cent, respectively.

The results of several investigations on different kinds of material, including giant chromosomes, indicate that the chromatin or chromosomes is the source of nucleolar RNA (14, 8, 22, 20, 18, 16). In the present study, there is no indication of a common origin for any of the investigated samples of chromosomal RNA and nucleolar RNA. In view of the varying base composition of chromosomal RNA, these results are not contradictory, of course, to those of earlier investigators.

The results of autoradiographic investigations indicate the nucleolus to be a source of the major part of cytoplasmic RNA (1, 23). It is true that such investigations are subject to errors, such as the unknown influences of the turnover rates of various precursors (9), self absorption (16), as well as cellular radiation damage with the use of tritium-labeled compounds (17). Nonetheless, these results are supported by the microirradiation experiments of Perry (15) and microphoretic base analyses of isolated nucleoli and cytoplasm from spider (6) and starfish (7) oocytes. Further support is furnished by the present data on *Chironomus*. These results seem even more informative than earlier base analyses in the respect that both the nucleolar and cytoplasmic RNA's are strikingly different from both the ribosomal RNA that is usually encountered in animal tissues and the soluble RNA (12).

Although the nucleolar and cytoplasmic RNA's are similar there is a highly significant difference between them in the G/U quotient. As was pointed out earlier (7), such findings do not exclude even the extreme alternative that practically all cytoplasmic RNA present at a given moment is derived from the nucleolus. If nucleolar RNA is transported to the cytoplasm and if RNA is retained there, as seems to be the case in growing or proliferating tissues, including *Drosophila* salivary gland cells (13), any change in the composition of the nucleolar RNA during the life of the cell will give rise to differences since the cytoplasmic RNA would represent an integral of all nucleolar RNA produced. It has been found, for starfish oocytes, that there are significant changes in base composition of nucleolar RNA during the growth of the oocyte (Edström, unpublished).

In *E. coli*, the fraction of the RNA synthesized on the chromosomes that acts as a "messenger" RNA is present in very low amounts (4). In *Chironomus* there is no sign of a contribution of chromosomal RNA to the cytoplasmic RNA, as judged by the base data, an expected finding in view of the results on *E. coli*. Cytoplasmic RNA, the bulk of which is ribosomal and is found usually to be rich in guanine and cytosine, shows an unusual base composition, in *Chironomus*, for an animal tissue. Similar results, *i.e.* a relatively high A-U content, have been obtained earlier (19) in analyses on *Drosophila* eggs. Furthermore, since, in experiments to be published, RNA of A-U type was extracted from whole *Chironomus* larvae, there is nothing which indicates that this composition is characteristic for polytenic tissues only. It is interesting to note that DNA from *Chironomus* giant chromosomes deviates in composition from the DNA of other known animal sources in a similar fashion, *i.e.* it has an unusually high A-T content, and it is tempting to believe that the parallel behaviour of the two nucleic acids in this respect is not fortuitous.

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