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Chromomeres and Puffing in Experimentally Induced Polytene Chromosomes of *Calliphora erythrocephala* *

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Abstract. In the most advanced types of meroistic ovaries the synthesis of RNA for the growing oocyte in each follicle is taken over by nurse cells, i.e., sister cells of the definite egg cell. In *Calliphora,* the highly polyploid nurse cells (NC) develop a polytene karyotype under conditions of strict brother-sister inbreeding and using a controlled selection technique. A comparison of the polytene NC-chromosomes with those from the pupal bristle forming cells reveals an unexpected discrepancy: while both chromosome complements exhibit a constant banding pattern it is not possible to homologize the two tissue specific patterns by identifying homologous band-sequences. Puffing in NC likewise turns out to be unusual in its extent as well as in that it remains constant during long periods of oogenesis. In a more detailed discussion an interpretation and evaluation of these findings will be attempted.

Introduction

The characteristically banded organization of polytene chromosomes is of great importance for the interpretation of the eucaryotic chromosome structure. The species specific and constant chromomere pattern is usually regarded as being the cytological counterpart of a corresponding linear gene arrangement in the chromosomes. This view is mainly supported by the fact that it is possible to assign individual genes to individual bands and by the phenomenon of a phase- and tissue specific puffing pattern (Beermann, 1962, 1965, 1972).

Recently, several investigators have carried out a cytogenetic fine structure analysis of distinct chromosome regions in *Drosophila* with the aim of testing the theory of the chromomere as being the fundamental chromosomal unit of structure and function (Beermann, 1972; Judd et al., 1972; Lefevre, 1973). These investigations have become particularly challenging since an excess of

* To the memory of Karl Bier

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DNA in the genomes has become known to be a widespread phenomenon. On the level of polytene chromosomes the "DNA-paradox" manifests itself as an excess of DNA in the chromomeres, if the "one-gene one-chromomere" hypothesis is accepted.

According to the above mentioned studies the original "one-gene one-chromomere" idea seems to gain further support and the "excess DNA" is believed to have a regulatory and/or merely "structural" function.

The cytological findings presented in this paper directly relate to the significance of the chromomeric organization, mainly because they cannot be fitted easily into the established concept of a species specific, constant banding pattern. The relevant data have been obtained in the course of analyzing experimentally induced polytene chromosomes in the ovarian nurse cells of *Calliphora erythrocephala*.

NC[1] polytene chromosomes, in a sense, combine the germline-specific functional state of a lampbrush chromosome with the typical soma-specific chromosomal phenotype of a polytenic organization. The present paper describes the techniques applied and the results obtained to improve the cytological quality of the experimentally induced polytene chromosomes (=secondary polytene chromosomes), the banding and puffing pattern of which will be compared with those of somatic polytene chromosomes.

Materials and Methods

The starting material for the brother-sister inbred lines was derived from laboratory stocks and newly established stocks of wild flies. Details of *Calliphora erythrocephala* mass-inbreeding are described by Ribbert (1967). Stages of oogenesis have been classified according to the criteria established by Bier (1963) for *Musca domestica*.

Polytene chromosomes were fixed and stained according to the conventional squash-procedure using aceto-carmine, normally followed by a second staining of the squashed material with lacto-orcein. Details of preparing trichogen cell nuclei can be found in the above mentioned reference.

Photomicrographs were made with a Zeiss photomicroscope, chromosome maps established with a device reflecting the drawing plane into the image plane of the microscopic objectives (Zeiss).

Results

1. The Formation of Secondary Polytene Chromosomes

The meroistic ovary of the diptera is of the polytrophic type. In the case of *Calliphora* there are four synchronous oogonial mitoses, generating a cluster of 16 oocytes, one of which is determined to become the definite egg cell, whereas the remaining 15 oocytes differentiate into so-called nurse cells. The whole unit is subsequently surrounded by a single layered follicle epithelium. Like some other insects with polytrophic meroistic ovaries *Calliphora* shows in its presumptive nurse cells a meiotic synapsis of homologous chromosomes

[1] Abbreviations: TC=trichogene cell(s); NC=nurse cell(s)

resulting in the formation of a typical zygopachytene bouquet. Thereafter the chromosomes of the definite egg cell are quickly condensed into a karyosphere (cf. Bauer, 1938) that neither synthesizes RNA nor passes through a stage of forming typical lampbrush chromosomes (Bier et al., 1967). The prospective nurse cells – on the other hand – become distinguishable by polyploidization and by organizing multiple nucleoli (Ribbert et al., 1969). The mechanisms of determining nurse cells and oocytes are unknown (for review see Telfer, 1975).

During the first steps of polyploidization so-called "primary polytene chromosomes" are formed in the nurse cell nuclei. These chromosomes disintegrate after the reduplication steps from 16n to 32n or 32n to 64n respectively, as a result of an extreme endometaphasic contraction, thereby liberating a corresponding number of individually identifiable diplochromosomes. Upon further growth the nurse cells form highly polyploid nuclei with a reticular structure of chromatin.

As has first been shown by Bier (1957, 1960, 1961), environmental and internal conditions can be found which cause a more or less effective suppression of the endoprophasic contraction in the nurse cells so that individual chromatids are no longer released from the primary polytene chromosomes and big "secondary polytene chromosomes" are built up in the subsequent reduplication steps.

The cytological quality of the secondary polytene chromosomes in *Calliphora* nurse cells is usually not sufficient to be exploited for constructing chromosome maps, on account of incomplete synapsis which causes an indistinct, coarse and variable banding pattern. Generally, the secondary polytene chromosomes are bundles of more or less exactly paired homologous oligotene fibrils (Figs. 2a, b; 3a). Bier has discovered that, besides other conditions, inbreeding may be especially effective in promoting the generation of secondary polytene chromosomes.

2. The Improvement of the Cytological Quality of Secondary Polytene Chromosomes by Selective Inbreeding

Inbreeding was started with twelve single matings, each from different laboratory stocks and newly established "wild"-lines.

Usually, 12–16 single matings (one female with one or sometimes several sib-mates) were reared for each subsequent generation, to get sufficient material for selection. After oviposition squash preparations of the second follicle generation were investigated and each female was classified according to the quality of the polytene chromosomes in her second follicle generation. The offspring of those two females with the best paired giant chromosomes were used to set the matings for the next generation. For reasons of safety, the pupae of the second best subline were kept at 8° C in a refrigerator until the adults of the first quality subline had eclosed.

Figure 1 represents the beginning of a pedigree of such an inbreeding line. Following this procedure 7 lines were established with at least 5 inbred generations and driven up to more than 15 generations in three cases. The chromosomes presented in this paper have been derived from two lines (A and B), which eventually developed the cytologically best organized secondary chromosomes; line A was driven up to the 17th, and line B to the 14th generation.

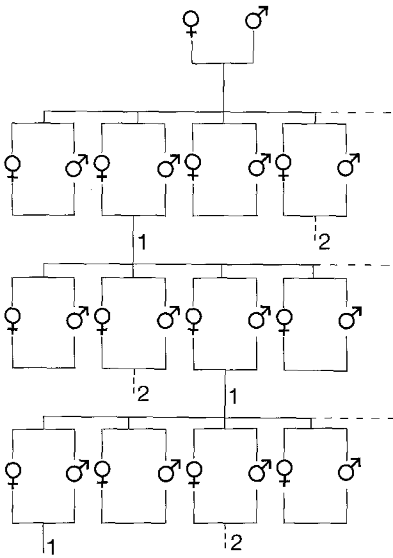

Fig. 1. Diagram of the selective brother-sister inbreeding. The flies for a set of single matings (12 or more) in any successive generation have been selected from the progeny of that female which proved to have developed the cytologically best polytene chromosomes in the second follicle generation (*1*). *2:* Female parents correspondingly showing the second best induced polytene chromosomes

It was not always possible to procure at least 12 single matings per subline and generation, since a decrease of fertility and viability occurred with increasing inbreeding, sometimes reducing the number of offspring drastically. To support the formation of cytologically well organized chromosomes by adequate environmental conditions the flies had to be reared at low temperatures, about 10–16° C. Figure 2 gives an example of the successive improvement of lateral chromatid association with increasing inbreeding for the case of chromosome 2. Line B had developed the best differentiated secondary polytene chromosomes. Females of this line did not at all develop reticular nurse cell nuclei in the 14th generation. About 70% of the nuclei had attained the quality shown in Figures 2e, 4b, 6, 8, and 9. At this stage the best chromosomes no longer displayed any disturbances of their banding pattern. This means that the endometaphasic contraction during the reduplication step from 16n/32n or 32n/64n respectively must have been completely suppressed.

On the other hand, line A never attained maximal chromatid association in its NC chromosomes, in spite of its having been pushed forward to the 17th generation. In addition, we had established several further inbreeding-lines, which did not exceed an intermediary stage of chromosome bundling resembling that shown in Figure 2b, not even after more than 18 generations of inbreeding in one case.

3. The Genetic Stability of NC Polyteny

Surprisingly the fraction of cytologically well organized secondary polytene chromosomes in a line could not be held stable if the offspring of a controlled brother-sister inbred-line were further kept by mass breeding. Obviously, under

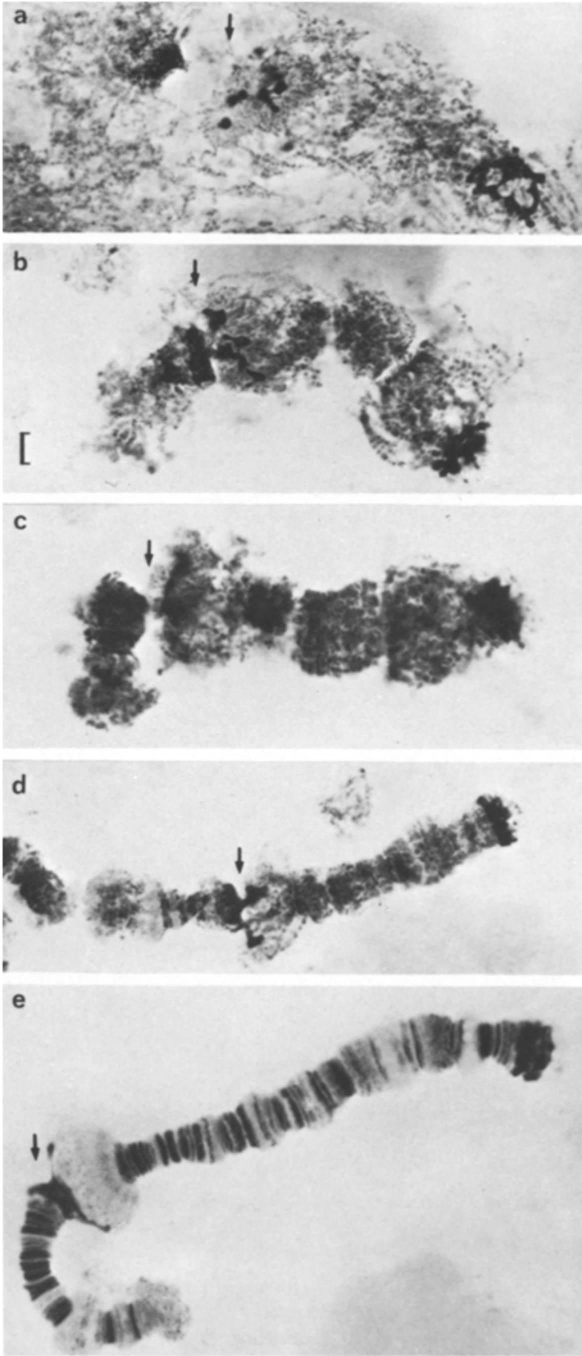

Fig. 2a–e. Improvement of the cytological quality of an induced polytene NC-chromosome, correlated with the increasing degree of inbreeding. Chromosome 2 can be identified by a conspicuous bloc of subterminal heterochromatin even in a state of loose bundling (**a**). Arrows indicate the kinetochore region. Inbreeding had been started using flies of a mass inbred-line with a tendency of chromosome bundling just visible (**a**). **b–e** Optimal synapsis in nurse cell nuclei of the inbred generations 5, 8, 10 and 14

Table 1. Correlation between the relative proportion of cytologically well organized secondary polytene chromosomes and the rate of follicle development.
Imagines eclosed from a 10 h batch of eggs deposited by line A females (14th inbreed generation) have been reared at 16° C with 150 individuals in each cage. After removing flies for a first evaluation (results in a), the number of 150 animals per cage was restored by adding white-eyed mutants to keep the population density constant. (An increasing population density below a certain threshold has a stimulating effect upon developmental velocity of the follicles.) In row 4 the percentage of the ovaries (stage 3a–4) with "best paired" secondary polytene chromosomes (cf. Fig. 2e) is noticed

a Distribution of oogenesis stages 16 days after eclosion							
Developmental stage of follicle	1	2	3a	3b	4	5	6
Number of ovaries	73	36	22	11	7	3	8 = 160
Number of stage 3/4-ovaries with synapsis class I	–	–	5	3	3	–	–
% proportion of class I-ovaries				27			
b Distribution of oogenesis stage 21 days after eclosion							
Developmental stage of follicle	1	2	3a	3b	4	5	6
Number of ovaries	28	14	11	16	1	4	86 = 160
Number of stage 3/4 ovaries with synapsis class I	–	–	8	11	0	–	–
% proportion of class I-ovaries				68			

the latter conditions there is selection against those genetic constitutions which favour synapsis in secondary giant chromosomes.

With progressive inbreeding the synchrony of ovarian development decreases more and more. It is interesting to observe that a better chromosome synapsis correlates with a delayed development of the oocytes (Table 1). This correlation holds also within individual females, i.e., the more slowly developing follicles form the cytologically best organized chromosomes. Moreover, the number of follicles per animal is clearly diminished after inbreeding. All these factors contribute to the counterselection observed in uncontrolled mass-inbreeding.

If flies from two different inbred lines with well paired secondary chromosomes are outcrossed, the nurse cells of the offspring lack any tendency of developing secondary polytene chromosomes (Fig. 3d–f). In addition, the F_1-generations of such crossings exhibit a higher body weight and an increased vitality indicating an optimized genome reaction of the heterosis type.

4. The Banding Pattern of the Secondary Polytene Chromosomes in Nurse Cells: Comparison with the Chromosomes of Trichogen Cells

Fortunately, the secondary polytene chromosomes in the nurse cell nuclei of some of our inbred lines had developed a clarity of their banding pattern,

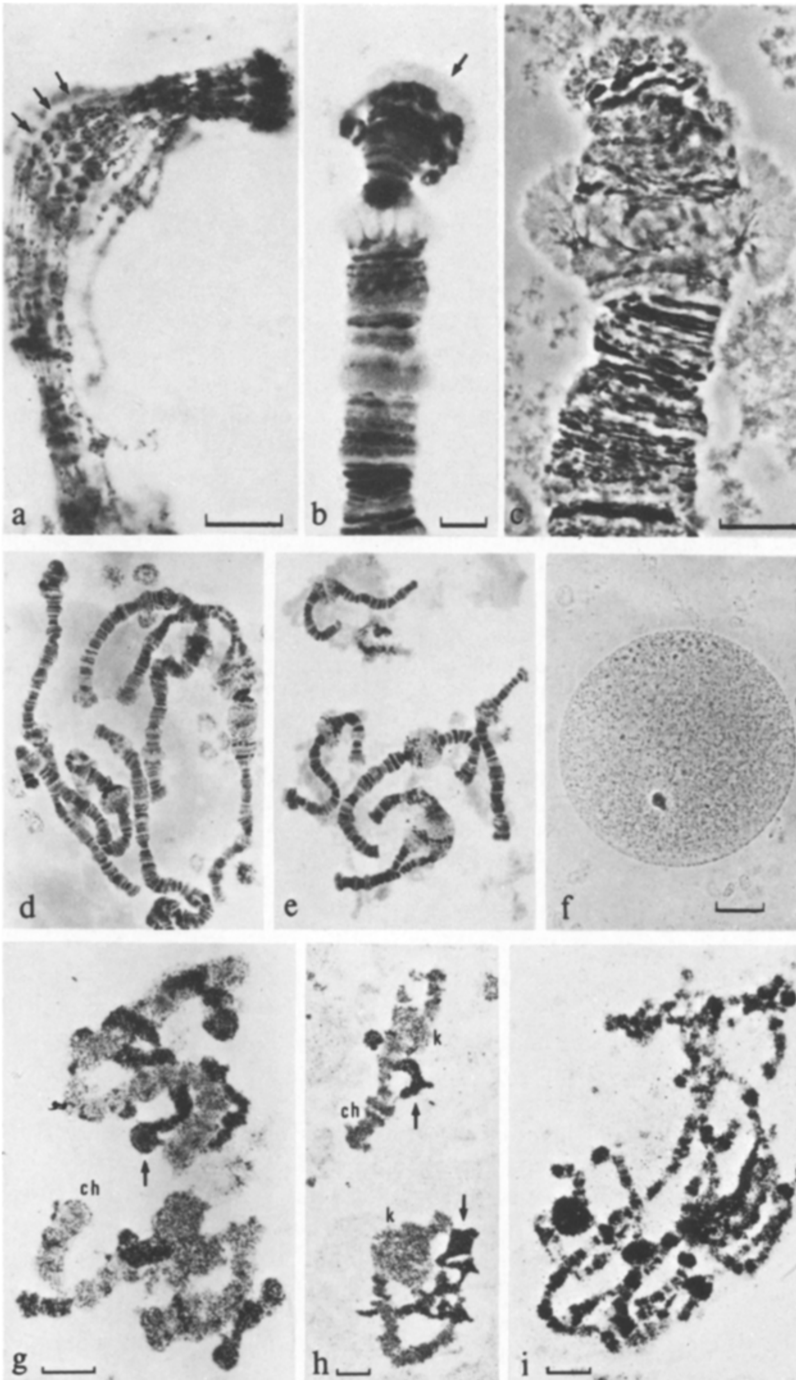

Fig. 3a–c. Cytology of the secondary polytene chromosomes. **a** Chromosome 2 with loosely bundled oligotene fibrils, the homology of which can be recognized at some conspicuous sites (arrows). **b** Puffing in the telomeric region of NC-chromosome 2 (arrow). **c** The only Balbiani ring of the NC-chromosome set in chromosome 5, region 2. **d–f** Results of crossing individuals from two separately grown selective inbreeding lines (A16 and B13) with secondary polytene chromosomes (**d**) and (**e**). All nurse cell nuclei from the progeny exhibit a reticular chromosome structure (**f**). **g–i** Autoradiography after ^{3}H-uridine incubation of NC-chromosomes (**g, h**) and TC-chromosomes (**i**) (incubation times: **g** 10 min, **h** 20 min, **i** 20 min). Homogeneous labelling in NC-chromosomes (**g, h**) as compared with the more discontinuous labelling in TC-chromosomes (**i**). Arrows in **g** and **h** indicate plaques of the multiple nucleolar apparatus. *ch* Chromosome; *k* kinetochorial bulb

the most advanced examples of which clearly exceeded that of the trichogen cell chromosomes analyzed previously (Figs. 4 and 5–8). Chromosome maps for the NC-chromosome complement were therefore even easier to draw than in the case of TC-chromosomes.

Close inspection of the two types of chromosomes revealed striking differences: "weak points", break points, sharp constrictions and sites of "ectopic pairing", which occur at many locations in TC-chromosomes, cannot be detected in the secondary polytene NC-chromosomes (with rare exceptions). The same holds for the asynaptic regions, the frequency and extent of which are distributed non-randomly over the chromosome complement of trichogen cells (Ribbert, 1967): asynapsis in NC-polytene chromosomes is relatively scarce with the exception of chromosome 3 and 5, 1–2 (Figs. 8 and 11).

Figure 4b represents the six chromosomes of the NC-complement in the state of complete synapsis of their chromatids. The smallest one, showing a mere dotlike appearance in mitotic and meiotic metaphases, (e.g.: inset of Fig. 4a) forms a fan with 5 identifiable bands when best developed (Fig. 9) and bears the nucleolus.

Generally speaking, a clear positive correlation between the degree of chromosomal stretching and the precision of the chromomeric arrangement can also be confirmed in the case of NC-chromosomes: the longer the secondary chromosomes, the better their polytene synapsis (Fig. 2).

Only chromosomes of optimal cytological structure were used to prepare chromosome maps (cf. Plate 1). Map constitution was initiated with the help of camera-lucida-drawings of an individual chromosome complement from a follicle of stage 3b. In the second step the maps were corrected and completed on the basis of microphotographs and by direct microscopical inspection. The reproducibility of the banding pattern turned out to be extremely good especially because there was no interference by such phenomena as fragmentation, ectopic-pairing, and changes of the puffing pattern.

On comparing the polytene chromosome maps from NC with those from the pupal trichogen and pulvilli forming cells which had been analyzed previously (Ribbert, 1967, 1972), it became immediately clear that while a constant banding pattern could easily be established for each of the two types of polytene chromosomes, it was not possible to homologize the two types of chromosomes with each other by the method of identifying homologous banding-sequences (Ribbert, 1975). Therefore, parameters like chromosome lengths, the number of chromomeres per chromosome and the ratio of arm lengths had to be used to arrange a comparable order of the two different chromosome complements. Table 2 presents these data derived from the 5 long chromosomes of trichogen and nurse cells, completed by the length ratios of metaphase chromosomes. Unfortunately, these parameters did not always differ sufficiently from one another so as to correlate TC- and NC-chromosomes unambiguously. There is but one exception: in both cell types a striking subterminal block of heterochromatin is present in the long arm of one of the chromosomes which therefore seems to mark homologous chromosomes. This chromosome according to its total length and the number of its chromomeres should rank as number 2 (Table 2, column 3, 10). Therefore, its previous designation in the TCN-complement has been changed from "3" to "2". Because of its undoubted homology

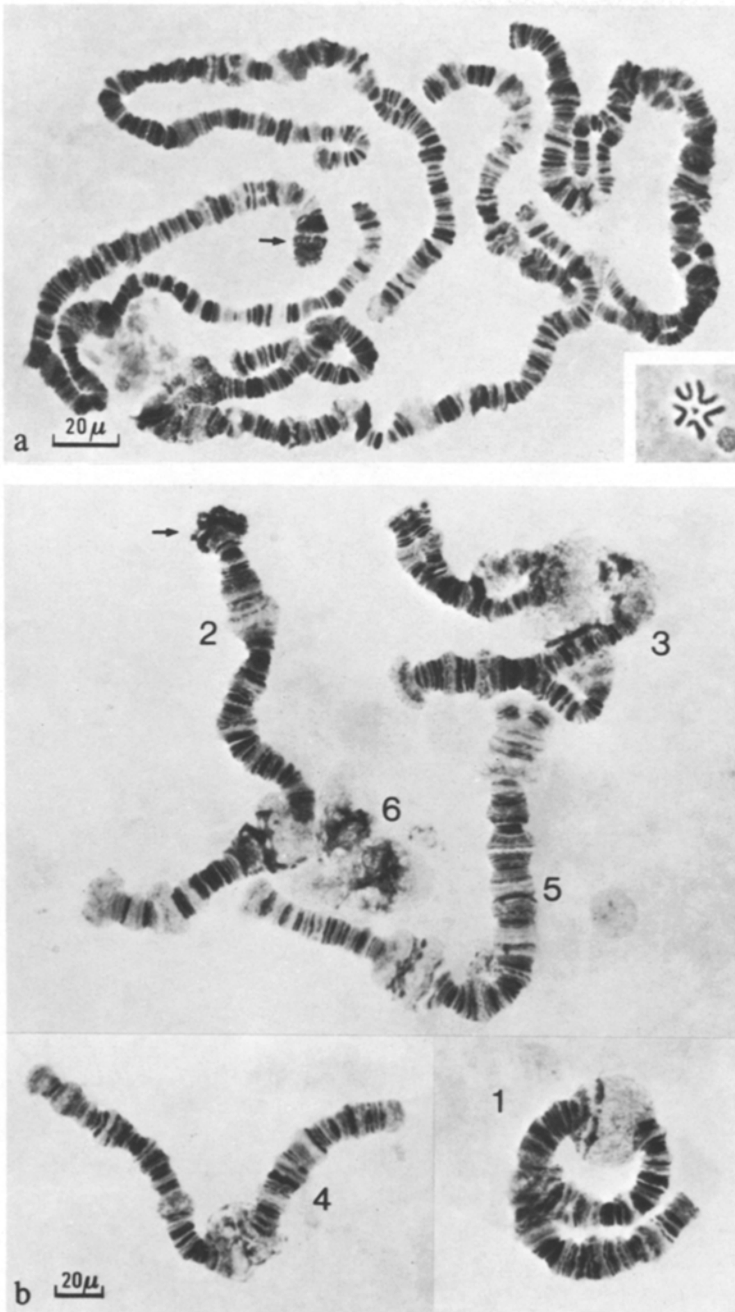

Fig. 4 a and b. The chromosome complements of *Calliphora*. **a** Chromosomes from a pupal bristle forming cell (without chromosome 4); inset: pseudohaploid (somatically paired) metaphase derived from a larval brain neuroblast. **b** Chromosome complement of stage 4 nurse cells with secondary polytene chromosomes

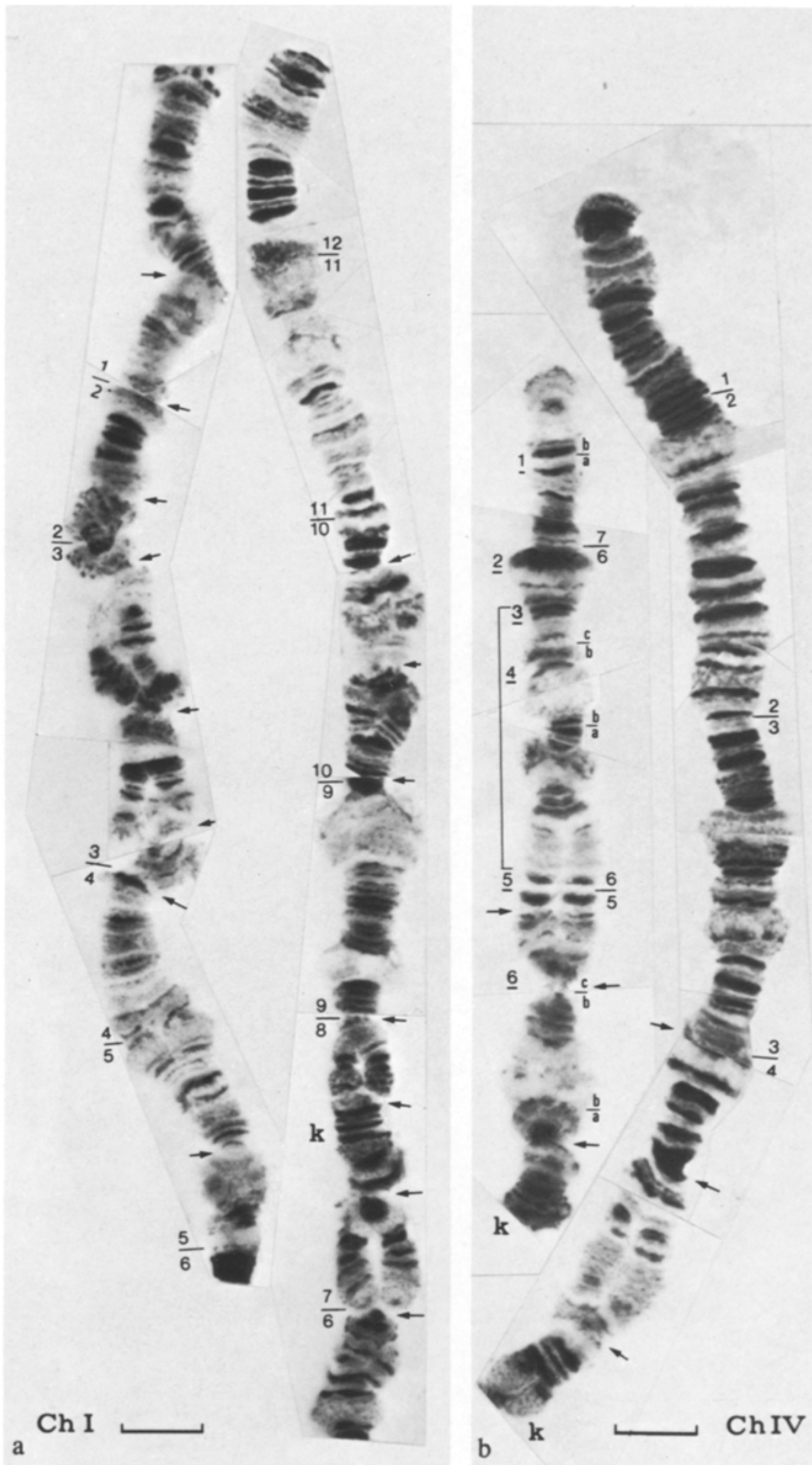

Fig. 5a and b. Polytene chromosomes number 1 (**a**) and 4 (**b**) of pupal bristle forming cells from flies of inbred line B. The two arms of each TC-chromosome have been placed side by side in reversed order. Break points (weak points) are marked by arrows. *k* Kinetochorial bulb (see text for further explanation)

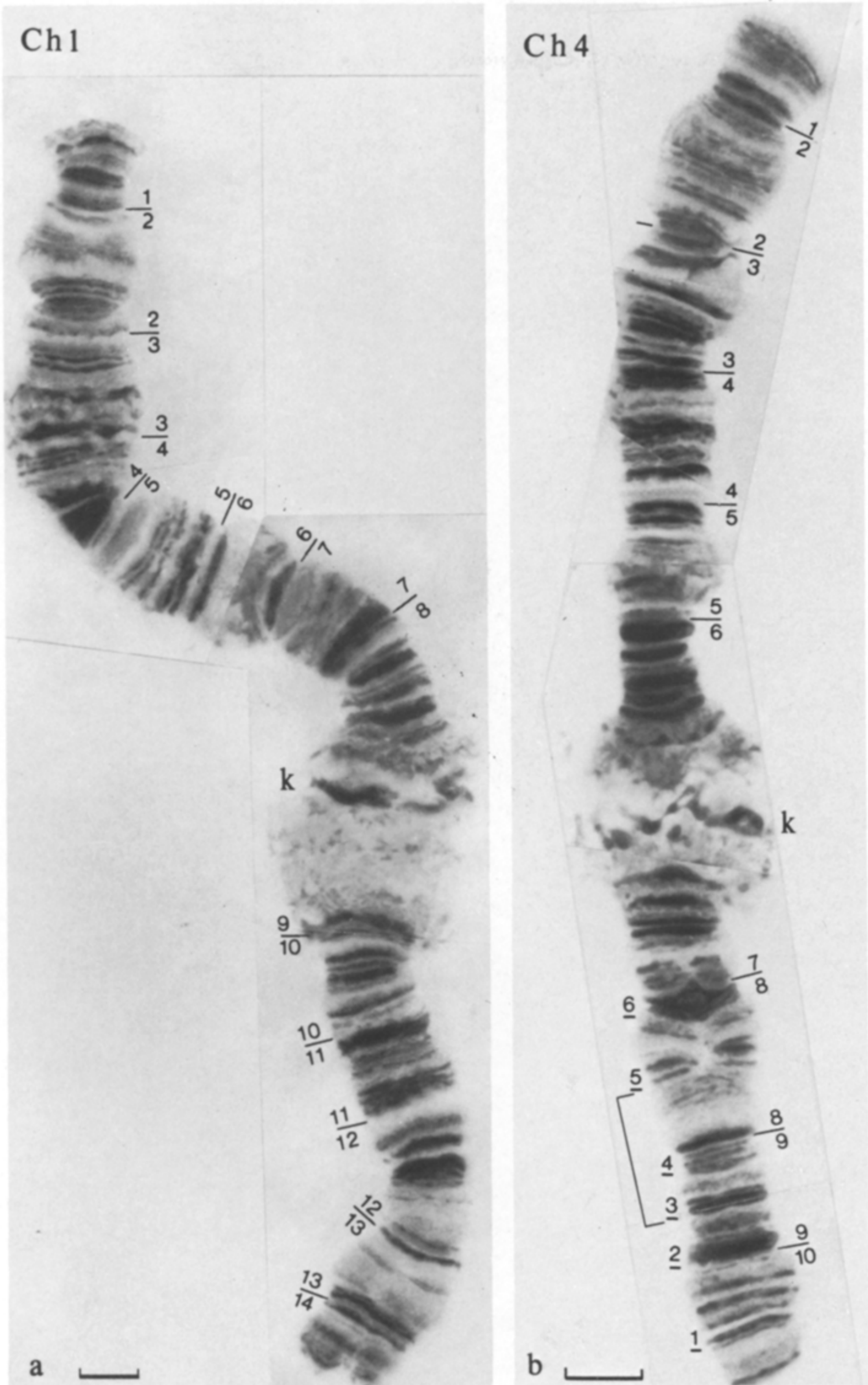

Fig. 6a and b. Experimentally induced polytene chromosomes number 1 (**a**) and 4 (**b**) of nurse cells prepared from flies of line B after more than 14 brother-sister inbred generations. The numbering of sections in TC-chromosomes (Fig. 5) and NC-chromosomes respectively have been made independently from one another, therefore they do *not* indicate homologous positions

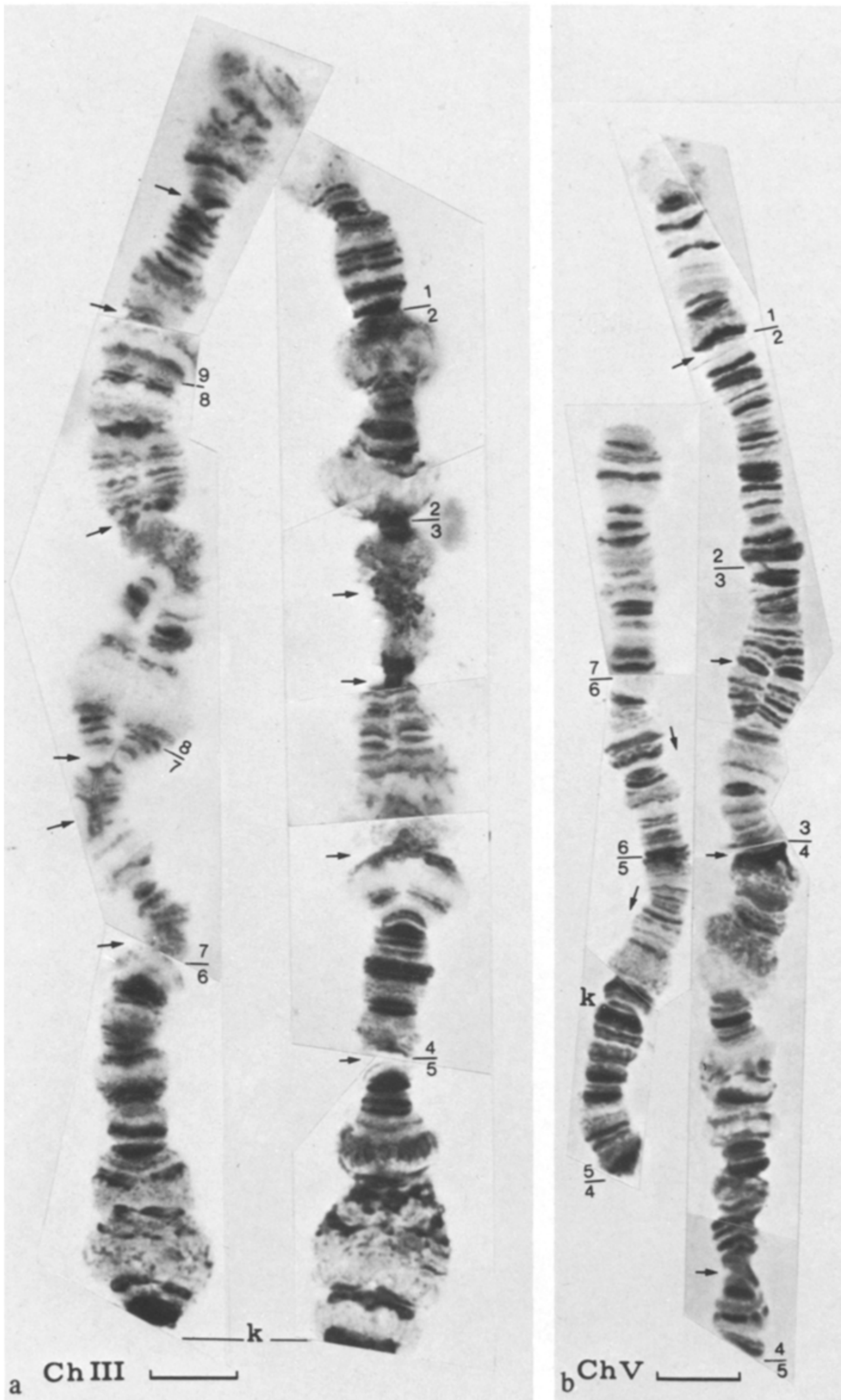

Fig. 7a and b. Polytene chromosomes number 3 (**a**) and 5 (**b**) of pupal bristle forming cells from flies of inbred line B. Break points (weak points) are marked by arrows, perpendicular to the chromosome axis

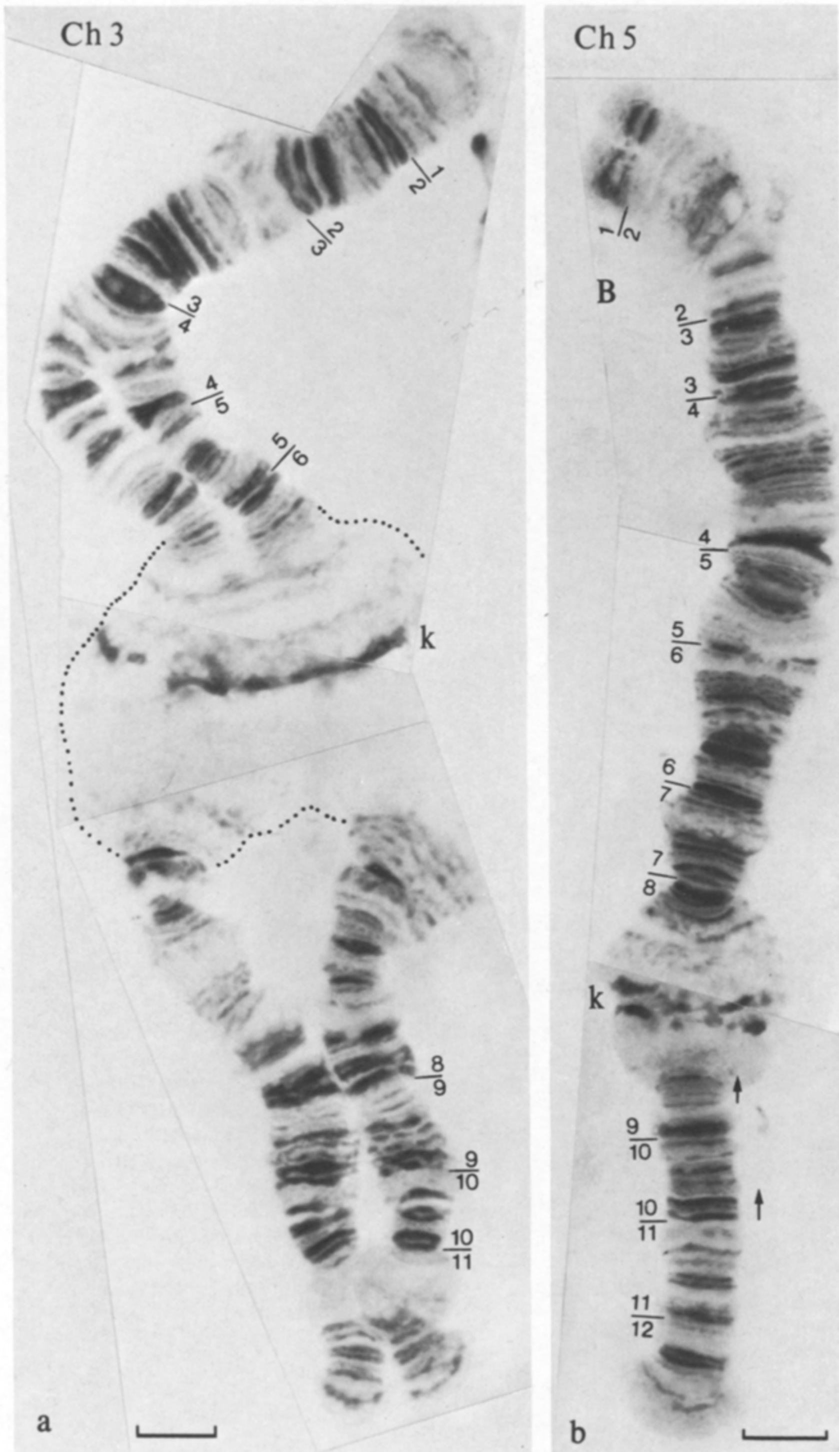

Fig. 8 a and b. Experimentally induced polytene chromosomes number 3 (**a**) and 5 (**b**) of nurse cells prepared from flies of line B after more than 14 brother-sister inbred generations

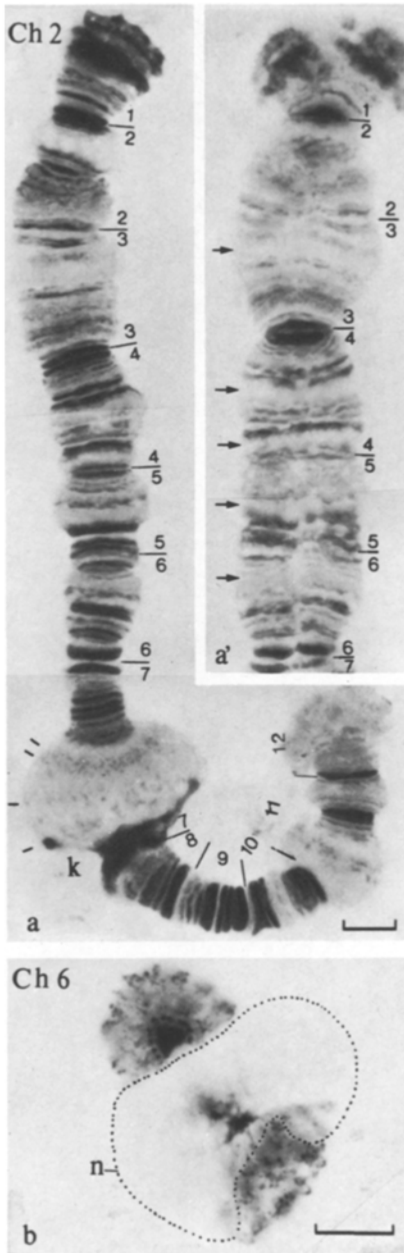

Fig. 9 a and b. NC-chromosomes 2 and 6. **a** and **a'** Chromosome 2, two extreme variants derived from nurse cells of the same follicle stage 3 b/4 grown at different temperatures. **a** Rearing temperature was 14° C. **a'** Flies have been transferred from 14° C to 21° C two days before preparation. Quantitatively enhanced puffing can be observed especially at sites marked with arrows. **b** Chromosome 6: the border of the chromosome-attached central nucleolus has been marked by a dotted line

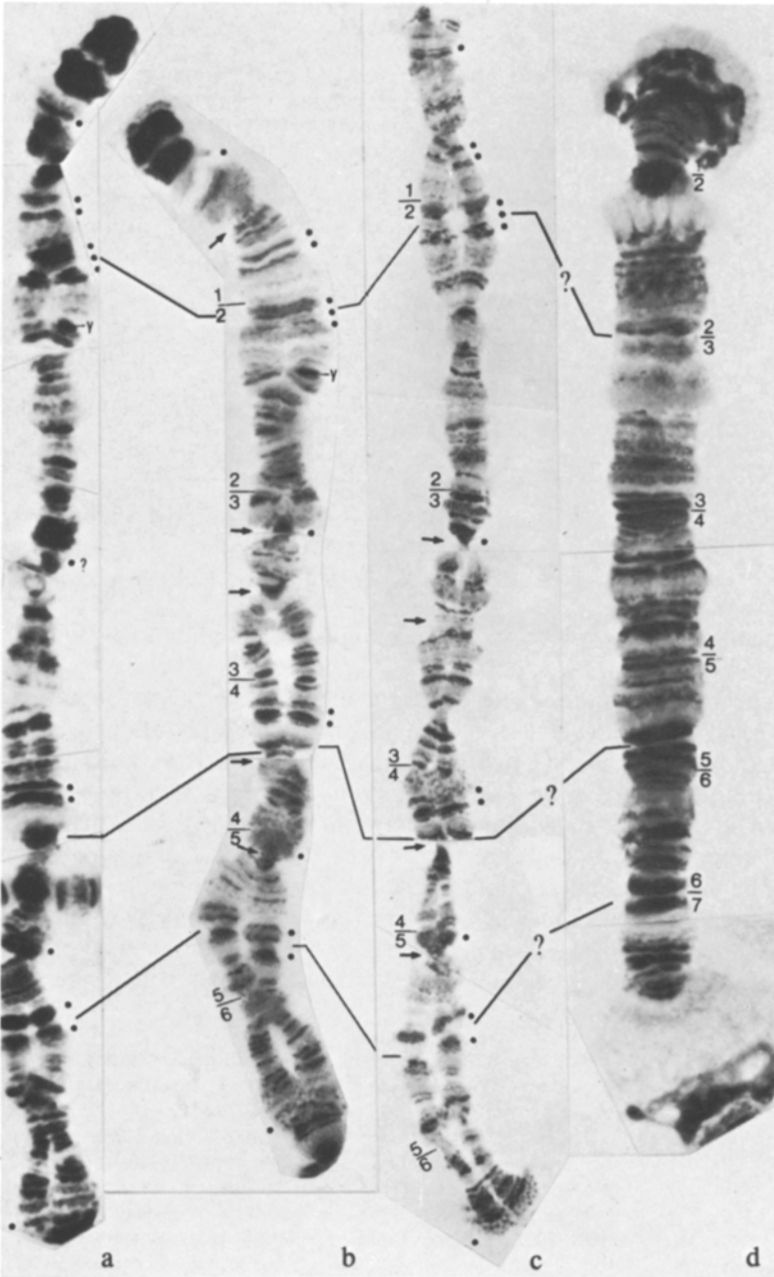

Fig. 10a–d. Phenotype of the left arm of chromosome 2 derived from different tissues. **a** Footpad giant cell of *Calliphora* pupae. **b** Pupal bristle forming cell of *Calliphora*. **c** Pupal bristle forming cell of *Lucilia sericata*. **d** Secondary polytene chromosome of a *Calliphora* nurse cell. – Lines and dots facilitate identification of some of the homologous chromosome segments. In comparison to *Calliphora*, *Lucilia* chromosome 2 is lacking the characteristic subterminal bloc of heterochromatin and parts of the centromere-associated heterochromatin, exhibiting otherwise a euchromatic banding pattern identical with that of *Calliphora*. *y* (in **a** and **b**) marks the Y-chromosome-specific band in *Calliphora* (cf. Ribbert, 1967). Even the positions with a high tendency of breakage ("weak-points", arrows in **b** and **c**) are identical in TC-chromosomes of *Lucilia* and *Calliphora*; in contrast polytene NC-chromosomes of *Calliphora* do not show weak-points

Table 2. Absolute measurements and relative proportions of some chromosomal parameters

Chromosome number		Absolute length (μm)		% of the total genome length			Number of (chromosomal) bands						Left arm/right arm				
							NC			TC			Length (μm)			Bands	
NC[a] map	TC map	NC	TC	NC	TC	mitosis	left arm	right arm	sum	left arm	right arm	sum	NC	TC	mitosis	NC	TC
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	1	269	368	24	24	28	100	78	178	200	125	325	1.8	1.7	1.7	1.3	1.6
2	3	251	305	21	21	19	114	53	167	195	80	275	2.0	2.2	2.0	2.2	2.4
3	2	217	336	20	21	23	78	64	142	110	140	250	1.2	1.1	1.2	1.2	0.8
4	4	181	268	17	18	16	105	59	164	150	105	255	1.4	1.6	1.4	1.8	1.4
5	5	190	260	17	16	14	100	51	151	170	85	255	2.3	2.0	2.0	2.0	2.0
Total:		1108	1537						802			1360					

[a] Ribbert, 1967

this chromosome was taken as a starting point in the attempt to assign the other four chromosomes.

The approach was to optimize the correspondence of the chromosomal parameters listed in Table 2. To facilitate a comparison, photographs of the presumably homologous TCN- and NCN-chromosomes were placed on pages facing one another (Figs. 5–8). The corresponding photomicrographs of chromosome 2 are those seen in Figure 9 (including chromosome 6) and Figure 10. Because of their good reproducibility the armlength relations (Table 2, columns 14–16) were used as the main criterion. Following the rule for establishing TC-chromosome maps, the longer chromosome arm always was designated as the left one. Each chromosome shows a "kinetochorial" swelling containing one prominent heterochromatic band, which is assumed to mark the position of the true kinetochore.

In the case of chromosomes 2–5 this procedure can be applied without problems, but for chromosome 1 the situation is somewhat ambiguous, because there are two heterochromatic bands at quite different positions which could be kinetochorial landmarks. If one elects the prominent band at position 8/9, an armlength ratio of 1.4 can be calculated, which is quite different from that of the longest TC-chromosome in trichogen cells and mitotic metaphases (cf. Table 2, column 15, 16). The ratio determined by the band at position 9/10 is $1.7 \pm 0{,}07$ and fits in fairly well with the values derived from TC- and metaphase chromosomes; in addition to this correspondence one must take into account that almost all the heterochromatic material of the TC-chromosome 1 that is associated with the kinetochore is located within the left arm, and since the proximal heterochromatic chromosome regions of TC-chromosomes probably correspond to the kinetochorial swellings of the NC-chromosomes, it seems reasonable to homologize band 9/6 with the position of the kinetochore.

In this way the numbering of chromosomes as used throughout this paper was derived (Table 2, column 1). Table 2, column 2, shows the revised numbering of the TC-chromosomes according to the above mentioned criteria. There is one uncertainty as to whether or not the left and right arms of chromosome 3 have been assigned correctly. If the ratios of band numbers (cf. Table 2, col-

umn 17 and 18) are taken as a criterion, an arrangement reverse to that in the chromosomal map would follow.

The extent of divergence of the banding pattern between TC- and NC-polytene chromosomes, as well as some similarities which can be detected in certain regions, will be illustrated by a few examples. Since chromosome 2 (new designation) is the only one which can be homologized in both cell types without resorting to a correspondence in the band pattern, the cytological situation here should be discussed first. In Figure 10b and c, the long arms of chromosome 2 from trichogen cells of *Calliphora erythrocephala* and *Lucilia sericata* are placed side by side. Though there are considerable differences as to the amount and distribution of heterochromatin (e.g., *Lucilia* lacks the subterminal and some of the kinetochorial heterochromatin) it is not difficult to homologize almost every single band out of a total of about 200 that can be identified in the left arm of chromosome 2. Thus, it is possible in this case to recognize the homologous chromosomes from two *different* dipteran *genera* solely by virtue of their banding pattern even within relatively small segments of their chromosomal complements. Neither is there any problem to homologize chromosomes from different *somatic* tissues of the same species (Fig. 10a, b). In striking contrast, any attempt at homologizing chromosomes of NC and TC within the same species fails completely. Clearly, a schematic band to band comparison cannot be expected to be successful due to the mere fact that the chromomere number of TC-chromosomes exceeds that of NC-chromosomes by a factor of 1.7 (cf. Table 2, rows 10 and 13).

A more promising approach seemed to be searching for prominent cytological landmarks (e.g., a sequence of distinct bands) within about the same chromosomal region, i.e., within the same relative distance from the kinetochore or the chromosome ends respectively. The situation may be followed with the help of Figure 9a and by directly comparing Figure 10b and d. Major homologous bands have been marked by dots in Figure 10a–c. At a first glance one might believe to detect some similarities even in the puffing pattern, e.g., at the distal end of chromosome 2, where a hyperboloid like structure characterizes section 2a in TC-chromosomes and a similar structure can be detected in NC-chromosomes (region 2/3) likewise. In region 6/7 of the NC-chromosomes a prominent pair of thick bands has developed at about the same distance from the kinetochore as a similar pair of bands in TC-chromosomes (section 4b). However, if one proceeds from these markers into the distal or into the proximal direction, it is not possible to detect *sequence* homologies of the banding patterns over a longer range. The same experience can be made if any other major band is used as a starting point. Thus, even local cytological peculiarities like "thick bands" cannot be homologized unambiguously. The attempt of homologization becomes all the more difficult, since other helpful cytological parameters such as intrachromosomal constrictions, specific break-points and sites of "ectopic pairing", all of which characterise the TC-chromosomes, are absent from NC-chromosomes.

A more convincing similarity between banding patterns of TC- and NC-chromosomes can be seen in the right arm of chromosome 4; the presumably homologous groups of bands have been marked with identical numerals (under-

lined) each in Figures 5b and 6b. Taken this homologization to be correct, it is noteworthy that the breakpoint at the border 5b/c of TC-chromosome 4, which is that with the highest relative breakage frequency (86%) of all the listed 33 break points within the TC-chromosome complement (cf. Ribbert, 1967), corresponds to an extremely thick band at position 7/8 of the NC secondary polytene chromosome 4. Despite some gross over-all-similarity of the banding pattern within this region it is still impossible to establish a consistent band-to-band alignment. Thus the sequence 3/4 in the NC-chromosome 4, for example, marked with a bracket in Figure 6b, can at best be resolved into 14 bands and will match a sequence of 27 bands in TC-chromosome 4, from which 22 bands can be seen in the photomicrograph of the actual chromosome 4 in Figure 5b (bracket), reaching from IV, 6a–3 to IV, 6c–4 (cf. TC-chromosome map, Ribbert, 1967). Some similarity is also indicated in the case of chromosome 5 when one compares the NC-regions 10 and 11, marked by arrows parallel to the chromosome axis (Fig. 7b) with the group of bands in region 6/7 (Fig. 8b) of the TC-chromosome.

On the other hand, the dissimilarities of the banding pattern of chromosomes 1 and 3 proved to be so extensive that not even a single chromosomal segment could be homologized with confidence. This is all the more remarkable because the banding patterns, especially those of the chromosomes 1, are anything but monotonous, neither in NC nor in TC. In both cell types the chromosomes exhibit a conspicuous amount of intercalary heterochromatin in form of deeply stained thick bands (or groups of thick bands) in NC nuclei and likewise strongly stained but thinner bands, often with a tendency towards ectopic pairing and/or high breakage, in TC nuclei. But even in those cases, where the distribution of prominent cytological markers shows some positional correspondence, it does not help to identify sequence homologies in the neighborhood of these landmarks, as already mentioned above.

Generally speaking, it is the NC-chromosome complement which exhibits considerably more prominent single or thick bands or clusters of them; on the other hand its total number of bands is only about 60% of that of the TC-chromosome set. To illustrate this, note for example the accumulation of major bands in the proximal half of the right arm of NC-chromosome 2 (Figs. 9a, 11f). As mentioned above, the homologization of this chromosome is possible without reasonable doubts. The region marked by the kinetochore (k) and the position 10/11 (i.e., region 8–10) in NC-chromosomes makes up half the length of the right arm. Besides the single puffed area in region 10 it consists totally of clusters of extremely thick bands. In the actual chromosome of Figure 11f, 16 bands can be resolved within this region; since in very rare cases region 8 (Fig. 9a) displays an additional set of 6 densely stained bands, instead of the normally occurring β-heterochromatin-like appearance, shown in Figure 11f (bracket), the number of bands in NC-chromosome region 8–10 totals up to maximally 22 bands. If, in a very formalistic way, half the contour-length of the right arm of TC chromosome 2 is taken for comparison (Fig. 11e), we find 35 bands (at least) matching the 16 (or 22) bands of the NC-chromosome. Considering that this nearly unpuffed NC-chromosome segment obviously makes up much more than half the total amount of the chromatin within

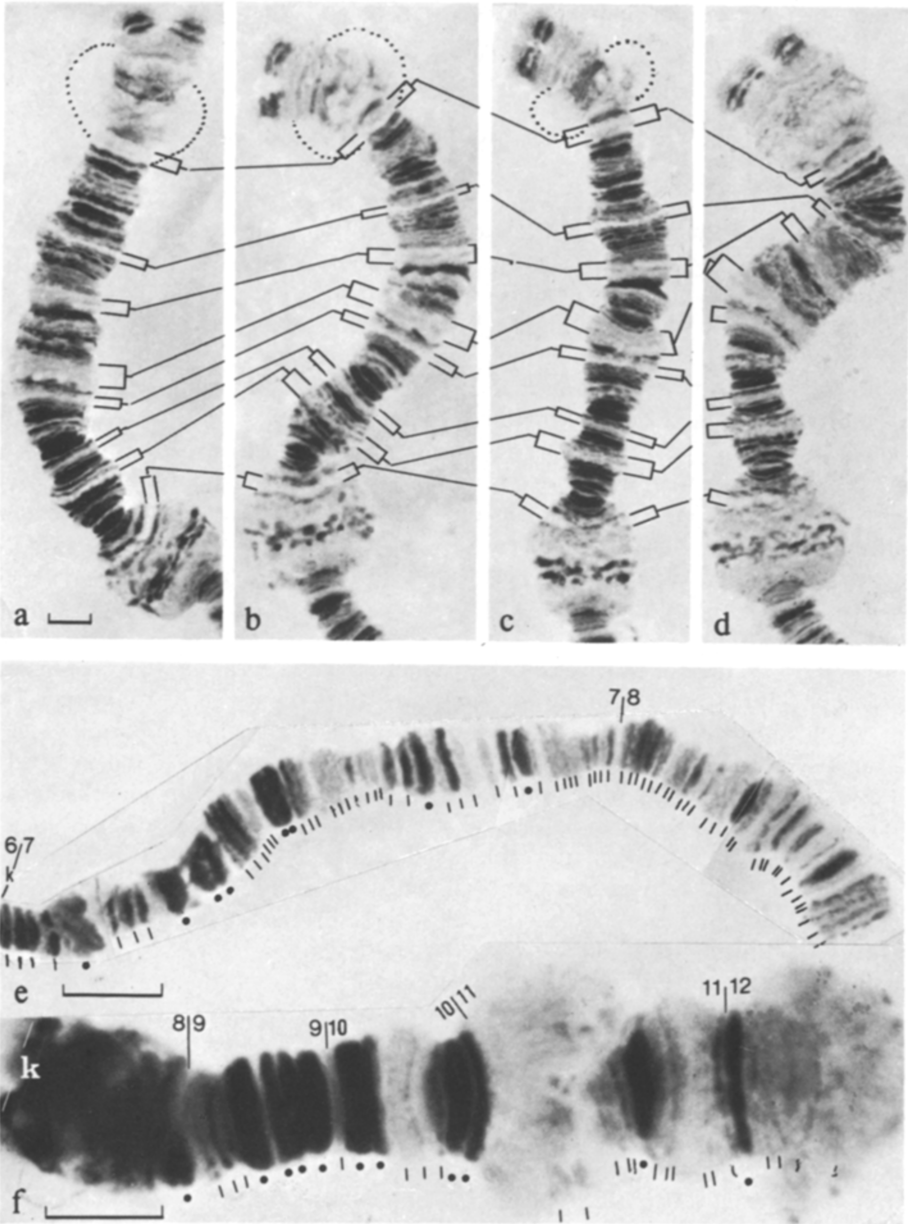

Fig. 11a–d. Left end of NC-chromosome 5 from nurse cells of developmental stages 2–3a (**a** and **b**) stage 3b (**c**) and stage 4b (**d**), showing that there is no appreciable qualitative change of the puffing pattern during this period of oogenesis where more than 90% of the RNA is synthesized. **e, f** Right arm of chromosome 2 derived from a trichogen cell (**e**) and from stage 3/4 nurse cell of an inbred line (**f**). Dashes indicate bands of "normal" dimensions, dots those of unusual thickness (> 1 μm)

the right arm of chromosome 2, the discrepancy between the two types of chromosomes becomes even more significant.

5. The Pattern of Activity in Secondary Polytene Chromosomes of Nurse Cells

As it is not possible to make a direct comparison between the puffing patterns of TC- and NC-chromosomes. Because of the poor or even lacking correspondence of the banding patterns, one is confined to state the more general aspects of the chromosome puffing in both cell types.

One of the most outstanding cytological peculiarities of NC-chromosomes is the conspicuous swellings adjacent to the kinetochore in each of the 5 long chromosomes (Figs. 6, 8, 9). Probably they correspond to the "kinetochorial baskets", of the TC-chromosomes (Ribbert, 1967). However, there are important differences between the two structures as to their dimension and their ability of synthesizing RNA. Whereas the kinetochorial baskets of the trichogen cells never show any sign of enhanced RNA synthesis in autoradiographs, short time incubations (3–20 min) with ^{3}H-uridine resulted in a strong and RNase-sensitive incorporation into the kinetochorial bulbs of the NC-chromosomes (Fig. 3h).

The bulb diameters can exceed that of an adjacent unpuffed chromosome region by factors from 3.2 for chromosome 5 to more than 5 for chromosome 3. Nevertheless, these structures are not puffs of the Balbiani Ring-type, since the torus-like shape, characteristic for Balbianirings (cf. Fig. 3c), is not developed at these sites in any of the oogenesis stages at which secondary polytene chromosomes can be analyzed. It looks rather, as if a whole sequence of active loci, located closely together, but nevertheless interrupted by some small inactive zones, builds up the huge kinetochorial bulbs. In good squash preparations mostly very faint and granular bands, still with a surprisingly high lateral order of their subchromomeres (e.g., marked by dashes in Fig. 9a) and clearly distinguishable from the densely stained kinetochorial heterochromatin bands, can be seen spread over the entire width of the kinetochorial bulbs. Such a "clustering" of individual moderately activated loci is not confined to the kinetochorial bulbs, but can also be found in other chromosome regions. Compare, e.g., chromosome 4, region 8 (Fig. 6b), or region 2/3, chromosome 2 and especially region 11/12 of chromosome 2 (Fig. 9a). The latter region comprises about half the distal length of the right arm of chromosome 2 and looks "totally puffed", only interrupted by two thick bands in the middle of region 11. But in this case, too, a series of faint granular bands (up to 21) are more or less evenly distributed over the puffed area.

The overall extent of puffing in NC-chromosomes seems to be considerably higher than that in TC-chromosomes. But there is hardly a way to substantiate this qualitative impression quantitatively, because none of the commonly used procedures was successful to determine the number of individually activated chromosome sites within the puffed areas, mentioned above.

Autoradiographies after short time incubation with ^{3}H-uridine have revealed

a considerably homogeneous labelling over NC-polytene chromosomes (Ribbert et al., 1969). This strongly contrasts to the wellknown discontinuous pattern of incorporated RNA-precursors in polytene chromosomes, such as the bristle forming cells of *Calliphora* (cf. Fig. 3g–i). To assess this phenomenon one must remind, however, that these autoradiographs have been prepared from chromosomes the cytological quality of which normally did not exceed that shown in Figure 2d. Thus it might well be that small longitudinal displacements of the oligotene chromosomal subfibrils, still existing in secondary polytene chromosomes of this cytological quality, are partially responsible for the observed homogeneous distribution of silver grains. Nevertheless, it should be emphasized, as already done by Ribbert et al. (1969), that an autoradiographic label after short time incubation with ^{3}H-uridine is not confined to puffs and Balbiani rings, but is also found in "normal" band-interband areas.

The autoradiographic technique allows at least a semiquantitative estimation of the different levels of RNA synthesis in NC-nuclei and TC-nuclei: if identical in vivo mixtures of precursors and salt solutions as well as identical incubation times are used, the polytene chromosomes from TC-cells need an 8–10-fold exposition time for developing the same total amount of silver grains as polytene chromosomes derived from a nurse cell nucleus of equal size.

The *multiple nucleoli,* as they occur in wild-type NC-nuclei, are maintained if cytological well organized secondary polytene chromosomes have been induced. In comparison, the polytene nuclei of trichogen cells only develop a *single* nucleolus at the site of the chromosomal nucleolus organizer in chromosome 6. Cytological and autoradiographic analysis of this peculiar multiple nucleolar apparatus in polytene nuclei have shown an autonomous RNA synthesis, i.e., independent of chromosomes. With the help of sensitive histochemical reactions it was even possible to demonstrate extrachromosomal DNA within the central core of each of the multiple nucleoli (Ribbert et al., 1969). The autoradiographs of squashed secondary polytene chromosomes after ^{3}H-uridine incorporation (Fig. 3g, h) exhibit the randomly distributed multiple nucleoli in the form of densely labelled plaques.

In principle, an analysis of a sequential change of the puffing pattern should be possible, beginning with oogenesis stage 2 until the degeneration of the nurse cells in early stage 5 becomes visible. Within this period the bulk of the RNA (i.e., more than 95% of the amount finally stored in the mature egg) is synthesized by the nurse chamber. In addition to considerable quantities of nucleoside-triphosphates (0.4 µg) a mature egg (stage 6) contains more than 2 µg of macromolecular ribonucleic acid (> 20,000 dalton), which is quite the same amount that can be isolated quantitatively from the fully developed larval brain with its 200,000 diploid cells. More than 2% of this macromolecular RNA is of the mRNA-type, judged by the criterion that these molecules contain long sequences of poly-A (Kirchhoff, in preparation). This enormous over-all synthesis of RNA required for the growing oocytes should be reflected qualitatively and quantitatively in the puffing of the secondary polytene chromosomes, since the nurse cells are the only source of RNA for the growing *Calliphora* oocyte. The analysis of chromosomes derived from nurse cells of various developmental stages revealed that the puffing pattern did not change during the whole

period (Fig. 11a–d) during which secondary polytene chromosomes can be analyzed and in which more than 90% of the macromolecular RNAs are synthesized. The only clear exception are the Balbiani-ring located in region 2 of chromosome 5 (Figs. 3c, 11a–d) which shows maximal unfolding towards the middle and end of oogenesis (stage 4/5) and a transitory puffing within the subterminal heterochromatin bloc of chromosome 2 in stage 3/4 follicles (Fig. 3b).

There is some influence of the temperature to which the flies are exposed after secondary polytene chromosomes have already been developed. Figure 9 shows two specimens of chromosome 2, both derived from stage 3b follicles of flies grown at 14° C (a), and (a'); at 21° C, after transfer from 14° C to 21° C for 2 days. Puffing was enhanced quantitatively (cf. especially sites marked by arrows) but not changed qualitatively.

Discussion

An understanding of the unique banding phenotype of polytene chromosomes essentially requires an insight into the molecular mechanisms contributing to the discontinuous condensation of chromosome fibres in interphase nuclei. It is reasonable to assume that these condensating mechanisms operating in the longitudinal direction and over a limited segment of a chromosomal fibre are likewise responsible for the lateral pairing of sister chromatids and homologous chromosomes as well in cases where the "somatic synapsis" becomes effective, thus eventually leading to the well known individually identifiable bands of polytene chromosomes in certain polyploid nuclei. The prerequisites for the production of this discontinuous longitudinal chromosomal subdivision seems to be a common feature of all eukaryotes, since the relative rare occurrence of polytene chromosomes has been found spread all over the animal and plant kingdom and within quite unrelated taxonomic categories (Protozoa: Ammermann, 1964; Collembola: Cassagneau, 1968; Plants: Tschermack-Wöss, 1963; Nagl, 1962; Diptera: Balbiani, 1881).

The experimental factors which are effective to induce secondary polytene chromosomes in *Calliphora*, show that the quality of the somatic synapsis and thereupon the expression of the synaptic forces strongly depends on the genetic background as well as on environmental influence. Since crosses of two inbred lines with cytologically well organized secondary polytene chromosomes produce a F_1-progeny with totally reticular nurse cell nuclei it is obvious that an enhanced homozygosity of some polyfactorial system is a prerequisite that a polytene phenotype of nurse cell chromosomes is expressed rather than the normal reticular structure. It might well be that this genetical influence is of a more indirect nature. Thus, continuous brother-sister-inbreeding of our *Calliphora* lines was accompanied by a successive decrease of vitality and fertility combined with a retardation of the total ovarian development and an increasing instability of the normally absolute synchronous growth of nurse cell units of an ovary. It turned out that just those ovaries with slowly and asynchronously growing follicles had a greater tendency of developing secondary polytene chromosomes.

In any case, the cytologically identifiable effect of inbreeding and low rearing

temperatures is the suppression of the endoprophase-contraction of the primary polytene chromosomes. This normogenetic cycle of condensation and decondensation is confined to definite early steps of reduplication of the primary polytene chromosomes in wild-type stocks of *Calliphora.*

So far, the secondary polytene chromosome system of *Calliphora* detected by Bier (1957, 1960) is the only case in which experimental procedures were successful in producing giant chromosomes of good cytological quality in polyploid cell nuclei the chromatin arrangement of which normally is reticular. To assess this cytological phenomenon one must bear in mind, however, that all Diptera (except the Tipulidae) are capable of forming polytene chromosomes in polyploid nuclei and that the reticular chromosome arrangement found in the ovarian nurse cells of most Diptera rather is an exception. Moreover, there are some species (8% out of a sample of 190) with a naturally occurring tendency of forming polytene chromosomes in grown-up nurse cells, most of them clustered within the two families of Agromycidae and Dolichopidae, respectively (Stalker, 1954). Some of these species, e.g., those of the genus *Anopheles* (cf. Coluzzi, 1975) develop quite a good chromomere pattern. In *Drosophila* the semilethal mutation *fes* causes the development of polytene chromosomes (King, 1957) in ovarian nurse cells, unfortunately of poor cytological quality. The possibility of obtaining well banded polytene NC-chromosomes in *Calliphora* provided the attractive chance of comparing the NC banding pattern with that of the somatic polytene chromosomes, e.g., from the trichogen cells.

The anticipation that the polytene phenotype of the nurse cell chromosomes, which in principle is a functional equivalent to the lampbrush configuration of gametocyte chromosomes synthesizing RNA, would essentially deviate somehow or other from "true somatic" polytene chromosomes, has found a somewhat unexpected confirmation in that the banding pattern of both chromosome types differs to such a large degree that for the major part of the genome an unambiguous homologization by detecting identical sequences of bands was not possible. This conclusion does not readily meet the expectations of the hypothesis of a tissue-invariant constancy of the banding pattern, if the latter is claimed to be of general validity. But it is exactly the pretension of general validity which makes up the core of this conception, because it includes a specific interpretation of the banded chromosome phenotype, i.e., reduced to a short formula, the "one-band one-gene hypothesis" which goes back to Muller et al. (1935). At the moment several explanations can still be offered, from which within the scope of this article only some major aspects shall be discussed.

On the lowest molecular level of a protein-DNA-interaction the nucleosome concept of homonomous packing elements not oriented on sequential subdivisions of the DNA has been well established during the last years (Elgin and Weintraub, 1975; Kornberg, 1977). With regard to our present knowledge of a continuous DNA-histone fiber being the basic structural component of eukaryotic chromosome, the aperiodic banding pattern of polytene chromosomes therefore positively demands that a corresponding molecular subdivision of the DNA becomes effective on the next or even higher level of the chromatin structure.

Because of the rigidity of the aperiodic banding pattern and the high specific-

ity of the somatic synapsis down to the level of single bands which especially become obvious by studies of inversions, translocations and duplications, it is hard to believe that the chromomeres represent only packing elements of the chromosomal fibers *without* any relation to a sequence specific, aperiodic subdivision of the DNA itself. Furthermore, all mechanisms including some kind of developmental processes with continuously changing quantitative parameters would be less attractive as a model to explain the highly reproducible aperiodic chromomere pattern, because the requirements of the precision for regulating these processes would be unreasonably high. This is an objection to models which try to explain the specific characteristics of individual bands to be the result, e.g., of locus-specific differential magnification processes, or to take another example, a mere "freezing" of the continuously changing interphase condensation pattern, which latter for example can be demonstrated by experimentally induced premature condensation of the interphase chromosomes in cells which have been fused with mitotic metaphase cells (Röhme, 1974). Still it is not plausible that such regulating processes could produce two different and equally rigid patterns within the same species. In short, it looks much more reasonable to interpret the chromomere pattern as the cytological pendant of a molecular subdivision of the DNA itself.

A straightforward and suggestive reification of this hypothesis is the one-chromomere one-gene theorem which directly correlates the band-interband structure of polytene chromosomes with the positions and boundaries of individual genes. Thus, it seems reasonable to discuss first whether or not it is possible to consider any known phenomenon of the chromosomal physiology in eukaryotes which might explain a substantial change of the banding pattern without affecting the above mentioned conception. Principally we know of two major processes which may affect the visible structure and/or the number of bands, these are: (i) a local disproportional reduplication of certain chromosomal sites, (ii) the puffing phenomenon.

A locally limited underreduplication in polytene chromosomes is a well known phenomenon which was detected on a cytological level (Heitz, 1934) and later confirmed by biochemical investigations (Hennig et al., 1971; Dickson et al., 1971; Gall et al., 1971). Until now the only case of a cytologically identifiable localized overreduplication – besides the amplification of ribosomal DNA – is that of the DNA-puffs in Sciarid species (Breuer et al., 1955; Gabrusiewicz-Garcia, 1964; Crouse and Keyl, 1978). The comparative cytology of TC- and NC-polytene chromosomes strongly supports the suspicion that local underreduplication might occur in somatic polytene chromosomes, e.g., from trichogen cells, salivary gland cells, cells of Malpighian tubules etc., but not in the secondary polytene chromosomes of the abortive oocytes: it can be observed that the conspicuous locus-specific breakages, the constrictions and the sites of ectopic-pairing in TC-polytene chromosomes, all of which can be considered to be indicative of underreduplication, are totally absent in secondary NC-polytene chromosomes. Moreover, in some cases (e.g. NC 4, 7/8; TC IV, 5b/c; cf. Figs. 5, 6) the mutual substitution of break-points in the TC-genome within intercalary heterochromatin-bands, which in addition show a high tendency of ectopic

pairing, and extremely thick heterochromatic crossbands in the NC-genome can be made plausible to some extent.

Every cytologist knows about the difficulties in demonstrating the reproducibility of the banding pattern within certain chromosome regions. Apart from the effect of site-specific breakages this is mostly due to chromosomal puffing. In cases where the initial processes of puffing can be analyzed cytologically the very first modifications of the chromosomal phenotype always prove to be confined to single bands. But to the same extent as the puff after this initiation step gradually unfolds, an increasing number of adjacent cross bands may become passively included into the zone of disintegration (cf. Beermann, 1962). Thus the observed differences of the banding pattern in somatic and germline polytene chromosomes might in principle be explained as being caused by disparate puffing if a comparable extent of differences in transcriptional activities between the NC- and TC-genomes is assumed to exist.

There are at least two facts supporting the view that differences in the spectrum of transcription between various somatic tissues might be much smaller than those between any kind of somatic cells from the germ line: (i) The existence of a germline confined chromosome phenotype during meiotic RNA-synthesis, i.e., the lampbrush configuration of diplotene chromosomes, (ii) the early transcription of numerous genes whose transcripts will be utilized not before either the next cell generation (e.g. as in the case of male fertility factors of *Drosophila,* Hess, 1971) or early embryogenesis of the progeny (cf. Davidson, 1977).

Both phenomena may be related. The invariant puffing pattern of nurse cell chromosomes can freely be interpreted to be the polytenic counterpart to the well known unaltered loop-pattern of lampbrush chromosomes. As to the huge amounts of template active RNAs with a considerable over-all-complexity which are stored in oocytes (comp. Davidson, 1977).

The question remains, of course, whether the far reaching differences of the banding pattern in NC- and TC-chromosomes can be fully explained by differential puffing and locally limited out-of-step reduplications. Especially because of the cases of those chromosome regions where "weak points" and extensive puffing are absent in either of the chromosome types one should consider alternative interpretations of the observed banding pattern differences.

The greater proportion of thick bands in nurse cell polytene chromosomes could be explained, at least partly, as a consequence of clustering of germline confined inactivated genes, the corresponding chromosome segments of which, organized in bands and interbands of normal dimensions in somatic cells, appear as deeply stained, condensed chromatin in the polytene chromosomes of NC-nuclei. The apparent lower band count in NC-chromosomes is thus perhaps not only due to an enhanced number of active sites but in part also the result of clustering of inactive ones. From this viewpoint the simplest explanation would reduce the very nature of the banding pattern to a cell specific relict of chromosomal interphase condensation. This leads back to models, which in principle had been proposed in the early days of autoradiographic studies on polytene chromosomes (Fujita, 1965) or, on even more speculative grounds,

already in classical times of polytene chromosome research (Koltzoff, 1934). Crick (1970) has proposed a model confining the positions of structural genes exclusively to interbands. The idea of ascribing all interbands together with puffs and Balbiani-rings to the general class of chromosomal activity structures would also be in accord with the homogeneous distribution of the autoradiographic label in secondary polytene chromosomes of *Calliphora* after short time incubations of ^{3}H-uridine (Ribbert et al., 1969) and with the low-level-transcription in all interbands, which has recently been suggested from autoradiographic studies in *Drosophila* (Zhimulev et al., 1975). Results obtained by immunofluorescence techniques which showed RNA polymerase B to be present not only in puffed chromosome regions but in all interbands point in the same direction (Jamrich et al., 1977).

General Conclusions

Considering the precision and rigidity of polytenic banding patterns of a given species in a specific tissue and at a specific developmental stage we are still faced with various possible models of explanation in all of which the discontinuous condensation pattern is attributed to an underlying subdivision of the DNA itself. Within the range over which this general postulate is valid three more specified conceptions may be established which do not necessarily exclude each other:

(1) The banding pattern might be a mere (de)condensation pattern, specific e.g. for the S- or G2-phase respectively, interbands being only low condensated linkers between the folding domains which are distributed along the chromosome without any relation to genes or higher functional units.

(2) It might reflect condensation compartments with their relative positions congruent to some functional units of the chromosome, e.g., transcriptional or reduplication units; interbands being linkers or the primary control sites of the attached functional units.

(3) The *interband*-pattern, inclusively puffs and Balbiani-rings, is the cytological counterpart of a correspondingly distributed pattern of active chromosome sites and the bands are indicating no other structural prefixed order as that of the inactive and condensated portion of the genome.

Conception (2) seems to be the most favoured one in present discussions and represents to some extent the succession view of the classic one-gene one-band hypothesis. As to the black box "functional unit" it promises to hide still a lot of scientific delicacies; on the other hand, it runs the risk to be burdened with all kinds of chromosome properties and functions, the repository of which chromomeres have been held out already in the past.

Conception (3) includes alterations of banding pattern per se and conception (1) is principally capable to comprise such phenomena; but within the scope of hypothesis (2) this is only possible if a very speculative extension is allowed in that the eukaryotic chromosome is said to be able to change number and quality of operational units by differential folding mechanisms, a view in princi-

ple proposed by Lima-de-Faria (1975). With not so much theoretical overburdening, interferences by mechanisms according to conception (1) and/or (3) or by tissue specific localized out-of-step reduplications might be responsible for the development of different banding patterns.

It is beyond the scope of this paper to discuss all important aspects of the banding phenotype of polytene chromosomes; but to make a final assessment as to one main point dealt with in this work it becomes clear from cytological and molecular biological evidences, gained within the last decade, that our picture of the chromosome structure is evolving rapidly. It is evident, that the classical one-band one-gene theorem in its simplest version cannot further be considered as valid and needs at least substantial modification, and we are at present not close to that point where a clearly out-lined new picture, equally regarding all important experimental observations and data, can be drawn. Nevertheless, the actual vivid discussion which arose about the subject can be taken as evidence that a more generally accepted model might be established in the near future.

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Plate 1. Maps of the five autosomes (1–5) in *Calliphora erythrocephala* nurse cells drawn from optimally developed chromosomes after selective inbreeding (see p. 276)