Experimental Cell Research 69 (1971) 185–192

ARE CHROMOSOME SECONDARY CONSTRICTIONS NUCLEOLAR ORGANIZERS?

A Re-examination Using a Laser Microbeam

M. W. BERNS and W. K. CHENG

Department of Zoology, University of Michigan, Ann Arbor, Mich. 48104, USA

SUMMARY

An argon laser microbeam was used to irradiate regions outside and inside the secondary constrictions of nucleolar organizing chromosomes. Irradiation immediately adjacent to the constriction consistently resulted in the loss of nucleolar organizing capacity. Irradiation 2 μm down the chromosome from the secondary constriction did not affect the capacity to organize a nucleolus. Irradiation directly inside the secondary constriction did not affect the ability to organize a nucleolus in 50 % of the cases. These data are discussed in relation to current ideas that secondary constrictions are nucleolus organizers. Alternative models are presented.

It is generally assumed that nucleolus-associated chromosomal secondary constrictions are nucleolar organizers, and that these regions contain the reiterated ribosomal genes. These facts are attributed to a variety of cytological studies that demonstrate the association of the nucleoli with secondary constrictions [1] and the absence of the nucleoli with a concomitant absence of constrictions [2]. In addition, biochemical analysis has demonstrated correlations between the amount of ribosomal RNA hybridizable to DNA and the number and/or size of the secondary constrictions and nucleoli [3, 4]. Despite this large body of evidence linking secondary constrictions with nucleolus formation, we feel the precise nature of the association remains obscure. In the early literature McClintock [5] demonstrated that in *Zea mays*, even though the nucleoli are associated with the satellited stalk region (secondary constriction), a dark staining region immediately adjacent to the stalk was the real nucleolar organizer. This area could even be divided and translocated by ionizing radiation, and each fragment could produce a nucleolus. She suggested that the stalk region of the satellited chromosome forms merely as a result of chromosome stretching due to accumulation of nucleolar products at the adjacent organizer.

In the more modern literature, despite the evidence linking secondary constriction size and number with the distribution of ribosomal genes, variable function and size of the nucleolar organizer has been demonstrated [3, 6]. For example, size differences in both secondary constrictions and nucleoli have been observed in different cells from the same tissues of the same organism [3]. It has also been demonstrated that in a cell containing several nucleolar organizers, one can

functionally dominate (out-compete) another. Finally, it has been noted by Busch & Smetana [7] that depending upon the dynamics of chromosomal condensation, there may be a large, small, or absent secondary constriction even in the regions where the secondary constriction is normally seen. These data taken together led us to question the clear association often made between nucleolar organizers, secondary constrictions, and ribosomal genes, and to try to determine if secondary constrictions really are nucleolar organizers.

In our earlier studies it was demonstrated that laser microbeam irradiation of the secondary constriction region resulted in a loss of DNA and subsequent loss of nucleolar organizing capacity by that region [8]. However, these initial studies involved irradiation of a chromosomal region considerably larger than the constriction itself, and probably included the chromosome area on either side of the constriction. Further perfection of the technique has allowed more precise irradiation. It is now possible to irradiate within the constriction itself, or regions close to, but outside the constriction. This capability has permitted us to re-examine McClintock's early theory of a nucleolar organizer adjacent to the constriction, and it also has permitted us to approach directly, the question of secondary constriction function.

MATERIAL AND METHODS

The chromosomes of salamander (*Taricha granulosa*) lung epithelium grown in Rose culture chambers were irradiated according to earlier procedures [9]. Cells were sensitized to the laser light by five minutes treatment with acridine orange (0.5 μg/ml). Since the dye intercalates into the DNA helix, the genetic material is selectively sensitized to the laser light [10].

Following dye treatment, the culture chamber was placed on the microscope stage, and an appropriate mitotic cell was chosen for irradiation. Only cells in which the chromosomal secondary constrictions were clearly visible, were chosen. By manipulating the adjustment of the microscope stage it was possible to move the chamber so that a specific region of a particular chromosome could be located under a cross hair on a television monitor complexed with the microscope system. The cross hair indicated the precise focal point of the laser beam.

By this procedure, four types of irradiation experiment were conducted; (1) irradiation of the secondary constriction and the distal satellited tip of the chromosome; (2) irradiation of the secondary constriction alone; (3) irradiation of the chromosomal region immediately adjacent to the secondary constriction (on the proximal side of the secondary constriction with respect to the centromere); (4) irradiation up to 2 μm down the chromosome (towards the centromere) from the secondary constriction. Cells were photographed before and after irradiation, and the cells were observed until nucleolus formation was complete.

The microbeam system utilized a high power, pulsed argon laser whose beam is deflected into a Zeiss photomicroscope and focused by the oil immersion ($\times$100, neofluar) objective. Prior to firing the laser the specimen is placed on the microscope as previously described. Appropriate optics permits transmission of the specimen image to a television camera, as well as energy measurements on the laser beam as it passes into the microscope. This system has been described in detail [11]. In the experiments described in this manuscript the approximate energy in the focused spot was 50–60 μJ/per pulse. A total of 2–4 pulses was used in each irradiation. The size of the lesion area was varied from 0.25 to 1.5 μm depending upone the desired lesion size. For irradiation of the constriction alone, or the adjacent site, the lower end of the range was used (0.25–0.50 μm).

RESULTS

The results are summarized in fig. 1. It should be noted that the results for experimental sequences (1) and (2) are consistently repeatable. When a region 1.5–2 μm from the constriction is irradiated, the ability of the nucleolar organizer to function is not affected. This experiment has been repeated three times with the same results, and a typical sequence is depicted in fig. 2 (*a–d*).

When the region adjacent to the constriction (up to about 1 μm from the constriction is irradiated, a nucleolus is not produced (fig. 1, experimental sequence 2). This result has been obtained every time the experiment has been performed (5 times). An actual sequence is illustrated in fig. 3*a–d*. In this particular sequence the chromosome tip remains as a condensed piece of chromatin

(arrow, 3*d*) but eventually disappears. The results involving direct irradiation of the secondary constriction, either alone or with the satellite tip, are not entirely consistent (fig. 1, experimental sequences 3 and 4). In some cases, nucleoli were formed (fig. 4*a–d*), and in other cases nucleoli were not formed (fig. 5*a–d*). In all cases there was a definite alteration to the irradiated region as evidenced by a change in chromosome morphology.

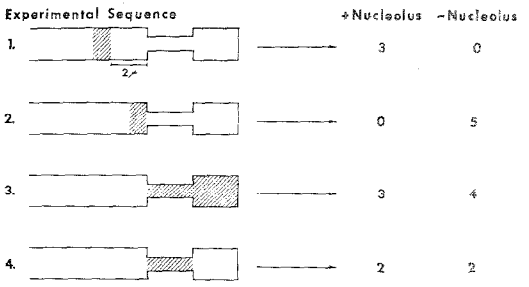

Fig. 1. Summary of microbeam experiments: The portion of the chromosome irradiated is indicated by shading. Sequence 1 involved irradiation of a region up to 2 μm down from the constriction. Sequence 2 was irradiation of the chromosome region immediately adjacent to the constriction. Sequence 3 involved irradiation of both the constriction and the distal satellited tip. Sequence 4 was irradiation of the constriction alone.

DISCUSSION

From the results it would appear that there is a region adjacent to the secondary constriction that in some way is involved in nucleolar formation. The fact that five repeats of this experiment had the same results is strong evidence for this. Furthermore, it appears that this region is localized near the constriction, since irradiation 1.5–2 μm from the constriction does not affect nucleolar formation (experimental sequence 1). Further support for the existence of a control region adjacent to the secondary constriction comes from a series of experiments in which we irradiated the chromosome region immediately adjacent to the nucleolus in prophase [12]. These experiments will be reported in detail elsewhere (Ohnuki & Berns), however, they are diagrammatically summarized in fig. 6. Note that in sequence 3, irradiation of the "juxtanucleolar" region of one nucleolus results in the production of two daughter cells with one nucleolus each. Apparently the irradiation of the region adjacent to the nucleolus can affect a reduction in nucleolar organization at the subsequent interphase. This region would be analogous to the region adjacent to the chromosomal secondary constriction, as well as the region McClintock described in *Zea mays*. The fact that direct irradiation of the nucleolus did not alter the ability to form nucleoli (sequence 4) would further suggest a primary role of the adjacent site, and indeed, no role to the intranucleolar chromatin (i.e., the secondary constriction DNA). However, the possibility that the nucleolar material shields the DNA from the laser, cannot be entirely ruled out.

Our results involving irradiation of the secondary constrictions directly (experimental sequences 3, 4) are less easy to interpret. The fact that in some instances nucleoli can be produced following irradiation of the constriction is, perhaps, significant. This means that, (1) either the irradiation did not completely destroy the DNA in the constriction region, therefore permitting organization of a nucleolus by the remaining ribosomal cistrons, and (2) if the DNA was completely destroyed, the production of a nucleolus would mean that the adjacent region is capable of producing a nucleolus, and therefore, would contain the ribosomal genes.

The fact that nucleoli were not produced in several cases when the constriction alone was irradiated, would suggest that the constric-

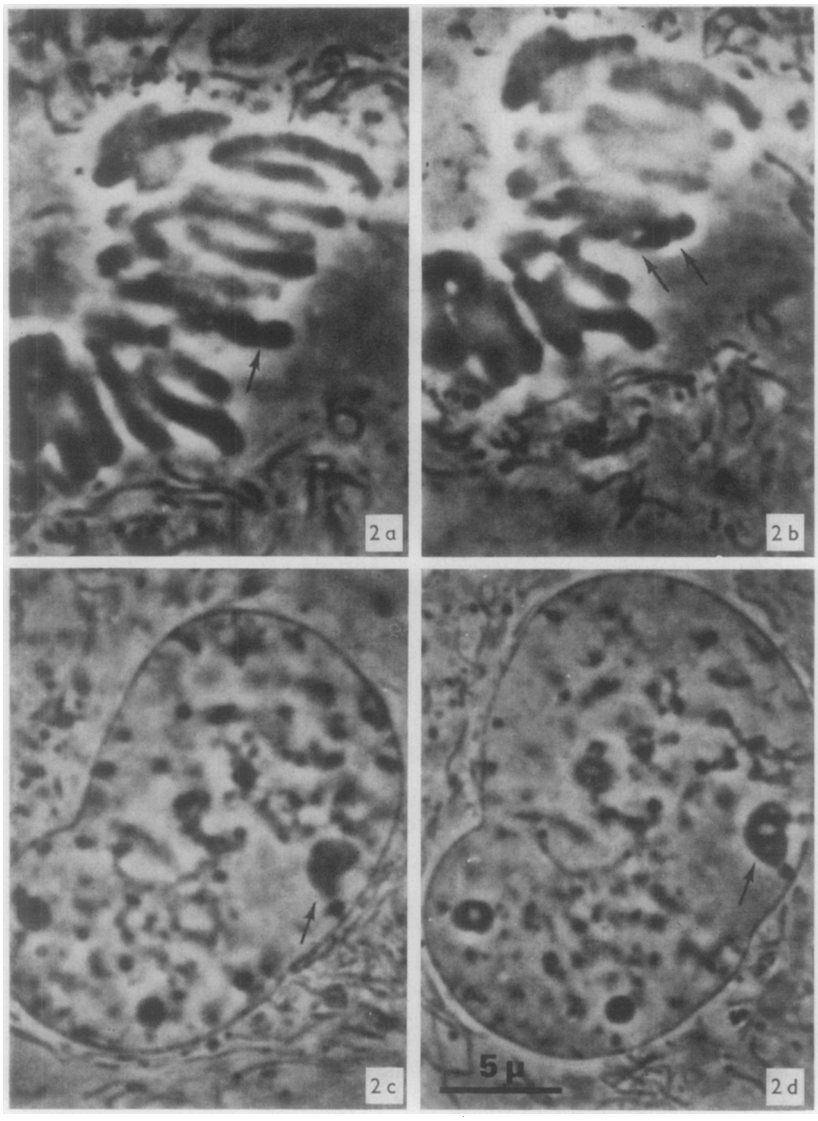

Fig. 2. Irradiation two microns from the constriction (sequence 1, fig. 1): (*a*) Chromosomes pre-irradiation (arrow indicates the secondary constriction); (*b*) Post-irradiation, left arrow indicates lesion, right arrow indicates unaffected secondary constriction; (*c*) cell 2 h post-irradiation, arrow indicates nucleolus formed in association with the secondary constriction; (*d*) same cell 24 h post-irradiation.

tion does contain the ribosomal genes. However, it is also possible that in these cases the irradiation area inadvertently included the adjacent "sensitive" region. This is possible because when attempting to irradiate the constriction it is necessary to irradiate as close to the condensed chromatin as possible. It might also be suggested that the irradiation of the adjacent site inadvertently altered the constriction region, thus explaining the results of experimental sequence 2 (fig. 1). This seems highly unlikely for two reasons: (1) irradiation of up to 1 μm down the chromosome from the secondary

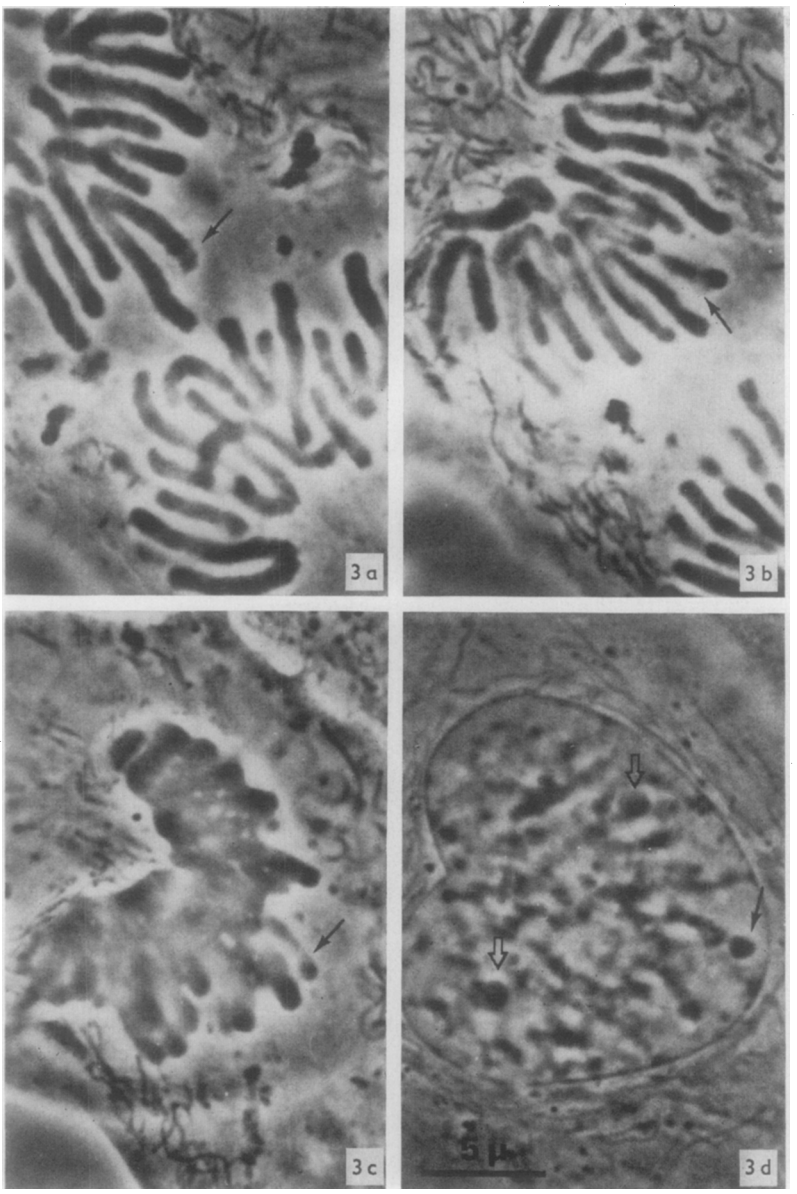

Fig. 3. Irradiation immediately adjacent to the secondary constriction (sequence 2, fig. 1): (*a*) Chromosomes pre-irradiation, arrow indicates secondary constriction; (*b*) post-irradiation, the lesion area and the secondary constriction are indistinguishable because they are both of the same phase density; (*c*) 20 min post-irradiation, note the chromosome tip next to the arrow; (*d*) 1 h post irradiation, large arrows indicate nucleoli forming, small arrow indicates the chromosome tip adjacent to the secondary constriction illustrated in (*a*) above; it completely disappeared by 24 h.

constriction, using a 0.25–0.50 μm visible lesion area could still affect the ability to organize a nucleolus; (2) 50% of the cases of direct constriction irradiation still resulted in nucleolus formation. This latter result would imply that if the nucleolar genes are located in the constriction they could sustain considerable damage and still organize a

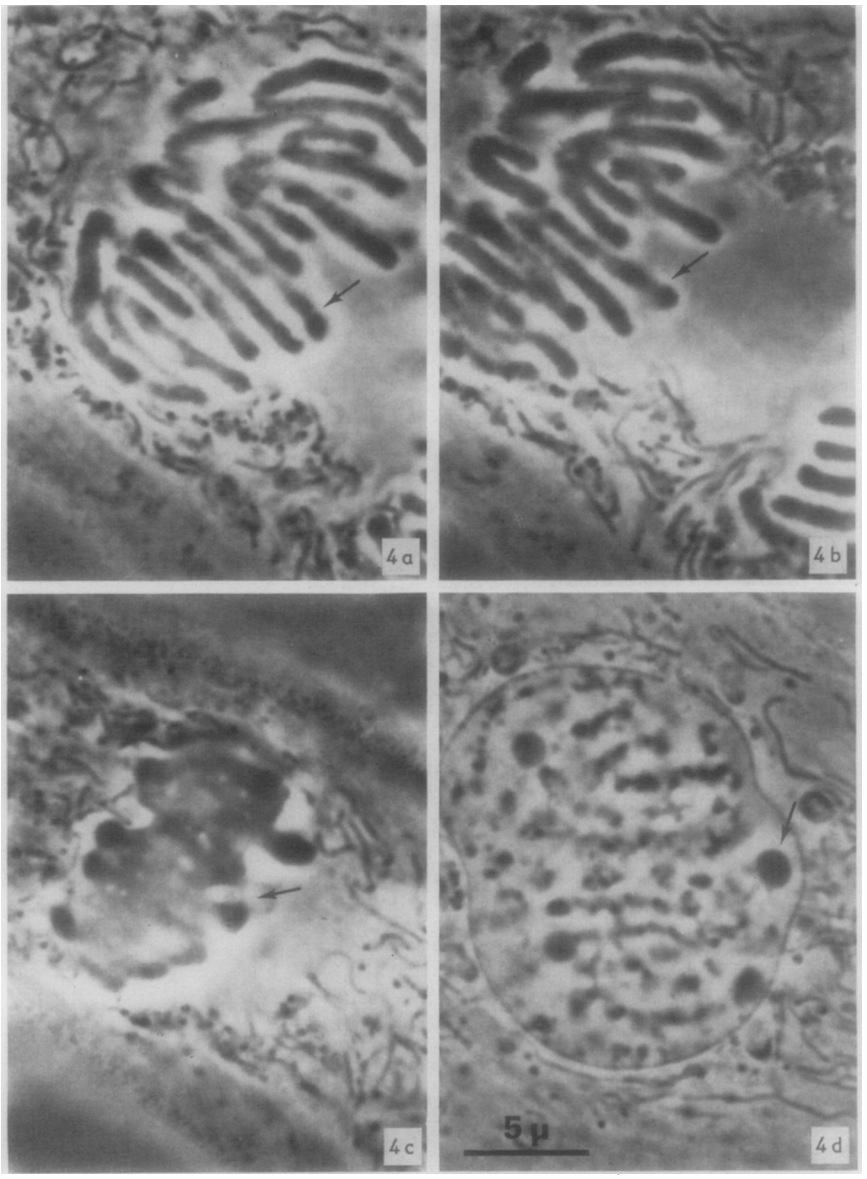

Fig. 4. Nucleolus produced following irradiation of secondary constriction directly (sequence 4, fig. 1): (*a*) Arrow illustrates secondary constriction prior to irradiation; (*b*) post-irradiation, (*c*) 20 min post-irradiation, (*d*) 1 h post-irradiation, arrow indicates nucleolus produced in association with the irradiated region of the chromosome.

nucleolus. Inadvertent damage to the constriction resulting from irradiation of the adjacent site should not cause such a consistent loss of nucleolar organizing capacity unless some critical function resides in the adjacent region.

From these results, what can we say about the role of the secondary constriction in nucleolus formation? We feel the data indicate that there is a region adjacent to the secondary constriction that plays a major role in nucleolar organization. If this region

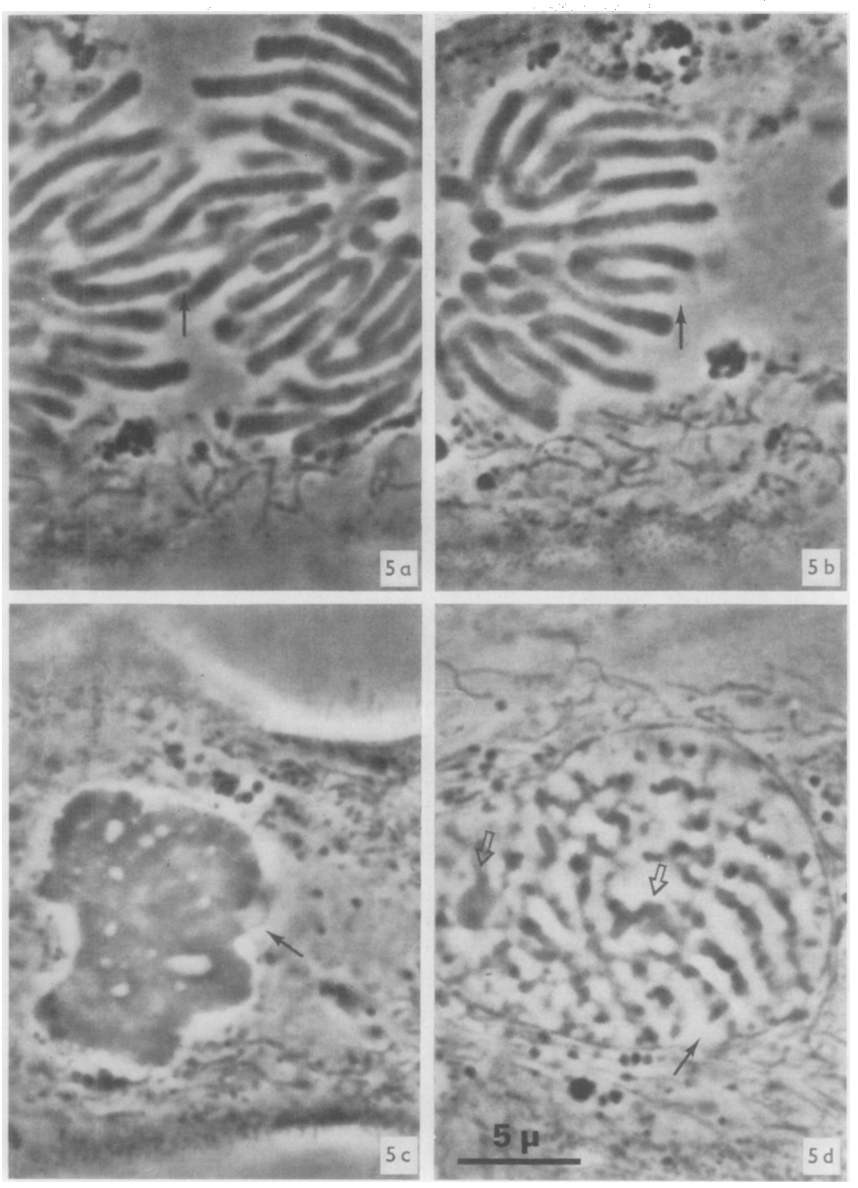

Fig. 5. Lack of nucleolus production following irradiation of secondary constriction and adjacent satellited tip (sequence 3, fig. 1): (*a*) Arrow indicates secondary constriction prior to irradiation; (*b*) post-irradiation, arrow indicates irradiated region of the chromosome; (*c*) 20 min post-irradiation; (*d*) 1 h post-irradiation, large arrows indicate nucleoli forming, small arrow indicates site where a third nucleolus should have been produced in association with the secondary constriction chromosome that was irradiated.

contains the ribosomal cistrons, then the formation of a secondary constriction would be merely a passive phenomenon resulting from synthesis and accumulation of products at the adjacent site. This idea would not be inconsistent with those studies relating secondary constriction absence with nucleolar and ribosomal cistron absence. If the constriction results after the fact (i.e., it is formed as a result of nucleolus synthesis and is visible

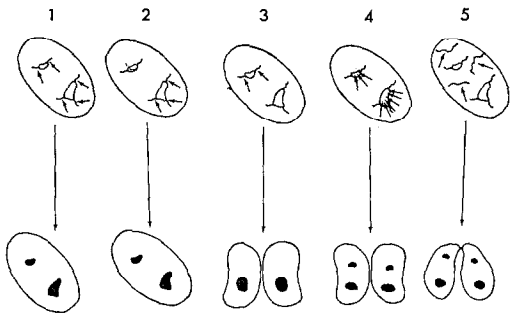

Fig. 6. Summary of experiments involving irradiation of nucleolar associated chromosomes in prophase: Arrows indicate sites of irradiation. In sequences 1 and 2, cell division was inhibited and the cells returned to the interphase condition; in sequence 3, the cells continued through division and produced daughter cells with a reduced nucleolar number; sequences 4 and 5 were controls.

in metaphase because the nucleolar material has disappeared in preceding prophase) then one would not expect to see a secondary constriction if the organizer (ribosomal cistrons) has been deleted. Likewise, the presence of a reduced number of ribosomal cistrons in the adjacent region might result in the production of a smaller secondary constriction because the subsequent nucleolus is smaller and therefore stretches the chromosomes less.

However, the possibility that the secondary constriction does contain the ribosomal cistrons cannot be entirely ruled out. It is possible that the sensitive region adjacent to the constriction in some way controls the ribosomal cistrons within the constriction. By turning off the control region, one could in effect, prevent the ribosomal cistrons from functioning. This model, also, would not be inconsistent with our data.

This work was supported by a grant from the National Science Foundation, GB 24457.

REFERENCES

1. Hsu, T C, Brinkley, B R & Arrighi, F E, Chromosoma (Berlin) 23 (1967) 137.
2. Kahn, J, Quart j microscop sci 103 (1962) 407.
3. Miller, L & Brown, D D, Chromosoma (Berlin) 28 (1969) 430.
4. Wallace, H & Birnsteil, M C, Biochim biophys acta 114 (1966) 293.
5. McClintock, B, Z Zellforsch mikroskop Anat 21 (1934) 294.
6. Rafferty, K A & Sherwin, R W, Cytogenetics 8 (1969) 427.
7. Busch, H & Smetana, K, The nucleolus, p. 118. Academic Press, New York (1970).
8. Berns, M W, Ohnuki, Y, Rounds, D E & Olson, R S, Exptl cell res 60 (1970) 133.
9. Berns, M W, Rounds, D E & Olson, R S, Exptl cell res 56 (1969) 292.
10. Berns, M W, Olson, R S & Rounds, D E, Nature (London) 221 (1969) 74.
11. Berns, M W, Exptl cell res 65 (1971) 470.
12. Berns, M W & Ohnuki, Y, J cell biol 47 (1970) 18*a*.

Received May 18, 1971