PRODUCTION AND RELEASE OF A LOCUS-SPECIFIC RIBONUCLEOPROTEIN PRODUCT IN POLYTENE NUCLEI OF *DROSOPHILA HYDEI*

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ABSTRACT

A specific, 0.1–0.3-μm large ribonucleoprotein complex consisting of a central core with stalklike extensions on top of which 280–320-Å ribonucleoprotein particles are situated is found in an experimentally activated chromosome region, 2-48C, of the polytene chromosomes of *Drosophila hydei*. Alkaline hydrolysis, RNAse digestion, and uranyl-EDTA-lead staining indicated the ribonucleoprotein character of the 280–320-Å particles, whereas the central core seems to be devoid of RNA.

The characteristic complexes are present in the nucleoplasm and at the nuclear membrane, but absent from the cytoplasm. It is suggested that the large RNP complexes are the specific products of the puff at 2-48C. Complexes similar to the ones described have not been observed in any other region of the polytene salivary gland chromosomes of this species.

INTRODUCTION

One of the prominent submicroscopic features of chromosome puffs in Dipteran polytene nuclei is the presence of typical particles within these synthetically active chromosome regions (Beermann and Bahr, 1954; Swift, 1962). The ribonucleoprotein (RNP) nature of these particles, which resemble the perichromatin granules in nonpolytene nuclei (Monneron and Bernhard, 1969), was established by cytochemical analysis (Swift, 1963 and 1965, Swift et al., 1964; Stevens and Swift, 1966). Different puffs within the same polytene chromosome complement can display strikingly different particles with respect to size and shape (Swift, 1965).

One of the largest RNP products thus far described has been observed in the salivary gland chromosomes of *Drosophila virilis* (Swift, 1965) and *Drosophila hydei* (Berendes, 1972), in both instances in a subterminal puff of the second chromosome. This product, previously considered as an aggregation of 150–220-Å particles, can attain a size of 0.3–0.4 μm in diameter. In *Drosophila hydei*, the occurrence of this typical product in the polytene chromosome complement is restricted to chromosome region 2-48C. The characteristic morphology of this product, the fact that its occurrence is restricted to only one chromosome region, and the recent finding of an agent which can selectively induce the activity of this region (Leenders et al., 1973) prompted a study on the formation and fate of this typical RNP particle under various experimental conditions. In order to gain some insight into the possible significance of this product for the metabolism of the cell, particular attention was paid to the questions of whether the particles are released from the chromosome, whether they are stable within the nucleoplasm, and whether they enter the cytoplasm. A further aim of this

study was to determine the most favorable conditions for the synthesis of this product to perform its isolation on a scale which would permit its biochemical analysis.

MATERIALS AND METHODS

Mid-third instar larvae of *Drosophila hydei* (140–150 h after oviposition) raised under standardized conditions (Berendes. 1965) were used throughout this study.

The experimental activation of region 2-48C in the salivary gland chromosomes was performed in three different ways: (*a*) transfer of larvae from 26° to 35°C and maintanance for 1 h at 35°C in a moist chamber (temperature treatment), (*b*) injection of 0.5 ± 0.1 μl of 0.1 M vitamin B_6 (Eastern Chemical Corp., Pequannock, N.J.) in Ringer's solution (see Leenders et al., 1973), (*c*) in vitro incubation of isolated salivary glands in Poels medium (Poels, 1972) supplied with 5.10^{-2} M vitamin B_6. Following 1 h at 35°C the salivary glands were dissected; one gland was stained with aceto-orcein and squashed and the other placed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 0°C for 90 min. From the injected animals, the salivary glands were dissected 4 h after vitamin B_6 injection; one gland being used for analysis of the puffing pattern after staining and squashing in aceto-orcein, the other one fixed as above or fixed for 1 h in 2% OsO_4 in veronal acetate (pH 7.2) at 0°C. Sections of OsO_4-fixed glands were used for alkali hydrolysis (0.5 N NaOH for 1 h). The in vitro-treated glands were all, except for a few used to analyse the puffing pattern, fixed with glutaraldehyde.

All three treatments are effective in the induction of a puff at region 2-48C. Some additional changes in the puffing pattern of the salivary gland chromosomes occur as a consequence of the temperature treatment (Berendes et al., 1965; van Breugel, 1966; Leenders and Berendes, 1972; Leenders et al., 1973) and during in vitro incubation with vitamin B_6 (Leenders et al., 1973). Furthermore, the size of the puff at 2-48C is, in general, significantly smaller after temperature treatment than after in vivo or in vitro application of vitamin B_6. Another significant difference in the behavior of puff 2-48C in relation to the different treatments may be mentioned. Whereas this puff upon its activation by a temperature treatment attains its maximum width within 20 min after transfer of the larvae to 35°C and starts regression after 60 min at 35°C, the puff attains its maximum diameter between 90 and 120 min after injection of vitamin B_6 and in this case puff size remains constant for at least the following 3 h (Leenders et al., 1973). In vitro incubation with vitamin B_6 (5.10^{-2} M) causes growth of the puff diameter up to 2 h.

The glutaraldehyde-fixed glands were extensively washed with cacodylate buffer before postfixation with 1% OsO_4 (1 h). Subsequently, the glands were washed again, dehydrated, and embedded in Epon (Luft, 1961). Random sections were made with an LKB microtome and mounted on Formvar coated grids (Belden Mfg. Co., Chicago, Ill.). The sections were stained with uranyl acetate-lead citrate (Venable and Coggeshall, 1965). Preferential staining of ribonucleoproteins was performed on glutaraldehyde-fixed material with the uranyl-EDTA-lead method according to Bernhard (1969). RNase digestion experiments were carried out on material embedded in glycolmethacrylate (Leduc et al., 1963) by submitting sections, previously treated with Pronase (2 mg/ml) (Calbiochem, San Diego, Calif.) for 2 h at 30°C, to 2 mg/ml RNase (pancreas, Sigma Chemical Co., St. Louis, Mo.) at 30°C overnight. For comparison of the relative number of RNP particles in puff 2-48C induced by different procedures, glutaraldehyde-fixed glands were squashed in 45% acetic acid and the resulting material embedded on the slide. From these slides, chromosome sections including puff 2-48C were selected and sectioned (Sorsa and Sorsa, 1967; Berendes and Meyer, 1968).

RESULTS

In all sections of flat-embedded (squashed) chromosomes displaying a puffed appearance of region 2-48C, large particles 0.1–0.3 μm in diameter are present within this puff. These particles have a globular dense center with stalk-like extensions at the surface on top of which globular particles of 200–300 Å can be present (Fig. 1). In addition to these large particles, smaller particles resembling those at the surface of the larger particles can be found within the puff region. Whether the puff developed as a consequence of either a 1-h temperature treatment or a 4-h treatment in vivo or in vitro with vitamin B_6, the large as well as the small particles are in general distributed randomly over the puffed area.

So far, the large particles just described have never been observed in any other puffed region of the salivary gland chromosomes of *Drosophila hydei*. The occurrence of these particles in region 2-48C is consistently associated with a puffed morphology of this region and with a high level of [^{3}H]uridine incorporation in this puff after pulse labeling (10 min).

Sections through region 2-48C of control glands revealed that neither large nor small particles are present in a nonpuffed state of the region. The number of large particles increases with increasing periods of a puff-inducing treatment. Puffs result-

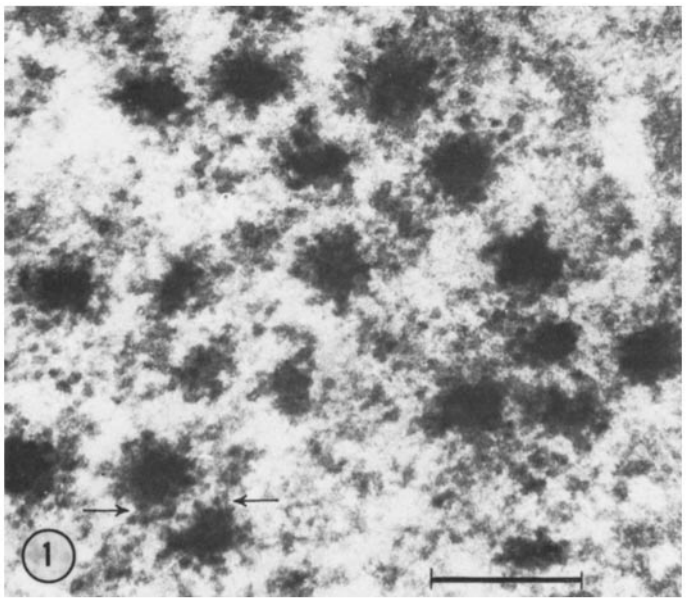

FIGURE 1 Part of puff 2-48C displaying large 0.1–0.3-μm RNP complexes. Section through squashed chromosome with puff at 2-48C induced by a 4-h in vitro vitamin B_6 treatment. Arrows indicate stalklike connections. Scale bar: 300 nm. × 64,400.

ing from a 10-min temperature treatment contain significantly smaller numbers of large particles than those resulting from a 60-min temperature treatment (Derksen, in preparation). In all instances, however, puffs developed as a consequence of in vivo or in vitro treatment with vitamin B_6 contain more large particles than the puffs occurring after a 1-h temperature treatment. The relative number of large particles within the puff, therefore, seems to be correlated with puff size (see Berendes, 1968; Leenders et al., 1973).

This finding was confirmed by observations on puffs at region 2-48C present in sections of whole glands. Whereas it is often difficult to define chromosome regions with regard to their position at the chromosome map in randomly sectioned nuclei, puff 2-48C can be easily detected by the presence of the typical large particles (Figs. 2 A and 3 B), as well as by the presence of the typical telomere structure at the tip of chromosome 2 (Berendes and Meyer, 1968) (Figs. 2 A and 3 B).

Sections through puff 2-48C developed during a 1-h temperature treatment displayed only a few large particles whereas the same region was completely filled with these structures in puffs occurring after a 4-h in vivo or in vitro treatment with vitamin B_6 (Figs. 2 A and B, 3 B).

Within the nucleoplasm of salivary gland cells after a 1-h in vivo temperature treatment, large particles were observed only in a few of the sections screened. The number of particles appearing free of chromosomal structures never exceeded five per section of a nucleus. In sections of nuclei of glands treated in vivo or in vitro with vitamin B_6, the nucleoplasm contained large numbers of the typical large particles distributed randomly over the nucleoplasm (Fig. 4, also see Fig. 3 A). Sometimes clusters of 280–320-Å particles, among which some appeared to be connected by a threadlike structure, were observed in the nucleoplasm. With regard to other 300–400-Å particles present in the nucleoplasm, it is difficult to trace their origin because particles of this size are present in other puffs of the chromosome complement as well as in puff 2-48C (Fig. 3 A).

From the observations described so far, it may be suggested that the large particles are produced within puff 2-48C and are released from this region into the nucleoplasm. Their abundant occurrence in the nucleoplasm of 4-h vitamin B_6-treated glands indicates that the particle is stable within the nucleus.

In contrast to single particles of 300–500 Å in diameter which can be found in positions suggest-

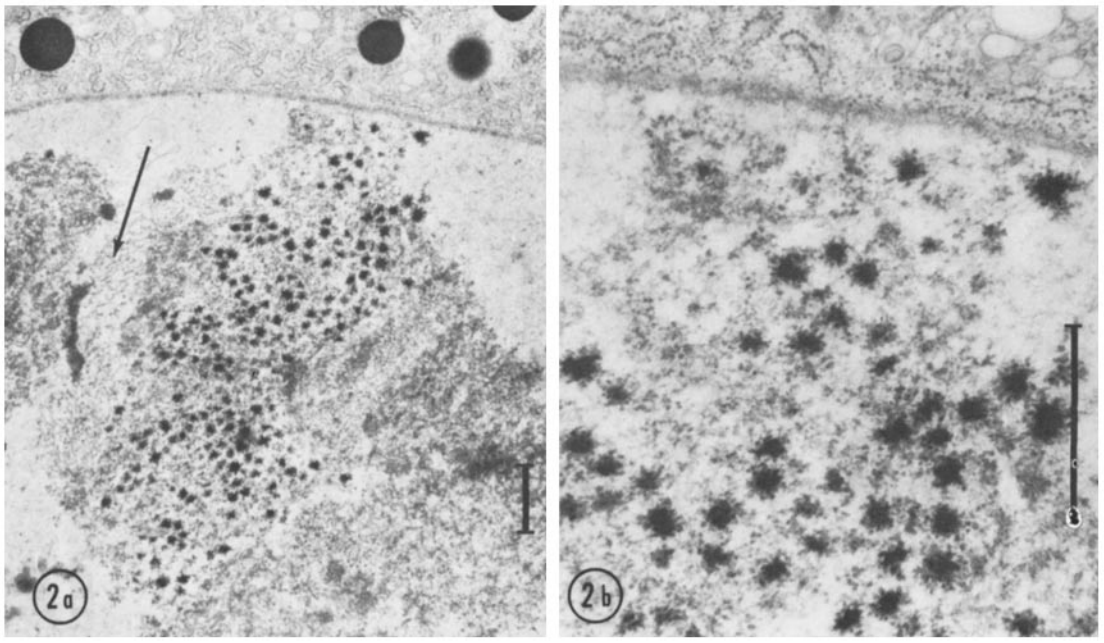

FIGURE 2 Section through nucleus displaying puff 2-48C induced by 4-h in vivo vitamin B_6 treatment. Arrow indicates the telomere position. Figs. 2 *a* and *b* are magnifications. Scale bar: 1 μm. *a*, × 8,700. *b*, × 25,550.

ing passage through the nuclear membrane (Fig. 5 A–C) and which can be seen in the cytoplasm enveloped in an outpocketing of the outer membrane, the large 0.1–0.3-μm particles have never been observed in the cytoplasm. On the other hand, these large particles have been found associated with the nuclear membrane (Fig. 6) in configurations that indicate the passage of the smaller subunits (Fig. 6 B). Whereas this type of association has been observed only in a few cases, clusters of small granules (200–300 Å) are frequently found associated with the membrane. These clusters often display connections between the individual small particles. As may be evident from Fig. 7, the small particles could pass the nuclear membrane through the nuclear pores. It should be mentioned that neither clusters nor strings of interconnected small granules were found outside the nucleus.

The RNP nature of the large particles present in puff 2-48C and their morphological equivalent in the nucleoplasm has been inferred from the uranyl-EDTA-lead staining method, alkaline hydrolysis, and enzyme degradation experiments. The results of the uranyl-EDTA-lead staining procedure (Fig. 8 A) indicate that the large particles consist of a central matrix devoid of RNA, at the surface of which smaller RNA-containing particles are arranged in a stringlike fashion indicating a physical connection between at least some of the smaller particles. Pronase followed by ribonuclease digestion resulted in the loss of the small particles at the surface of the dense central matrix, a result which was essentially the same as that of alkaline hydrolysis (Fig. 8 B).

DISCUSSION

The present observations on the submicroscopic organization of region 2-48C after the induction of a puff by various treatments indicate a quantitative relationship between puff size and the mumber of large (0.1–0.3 μm) RNP complexes.

It was shown earlier by autoradiography that puff 2-48C incorporates [^{3}H]uridine during short in vivo or in vitro pulses given at any moment after the onset of puff induction (Berendes, 1968; Berendes and co-workers, unpublished). On the other hand, autoradiographical analysis of the incorporation of tritiated amino acids gave no indication for a specific labeling of the protein components which are known to accumulate within the puff region during development of its

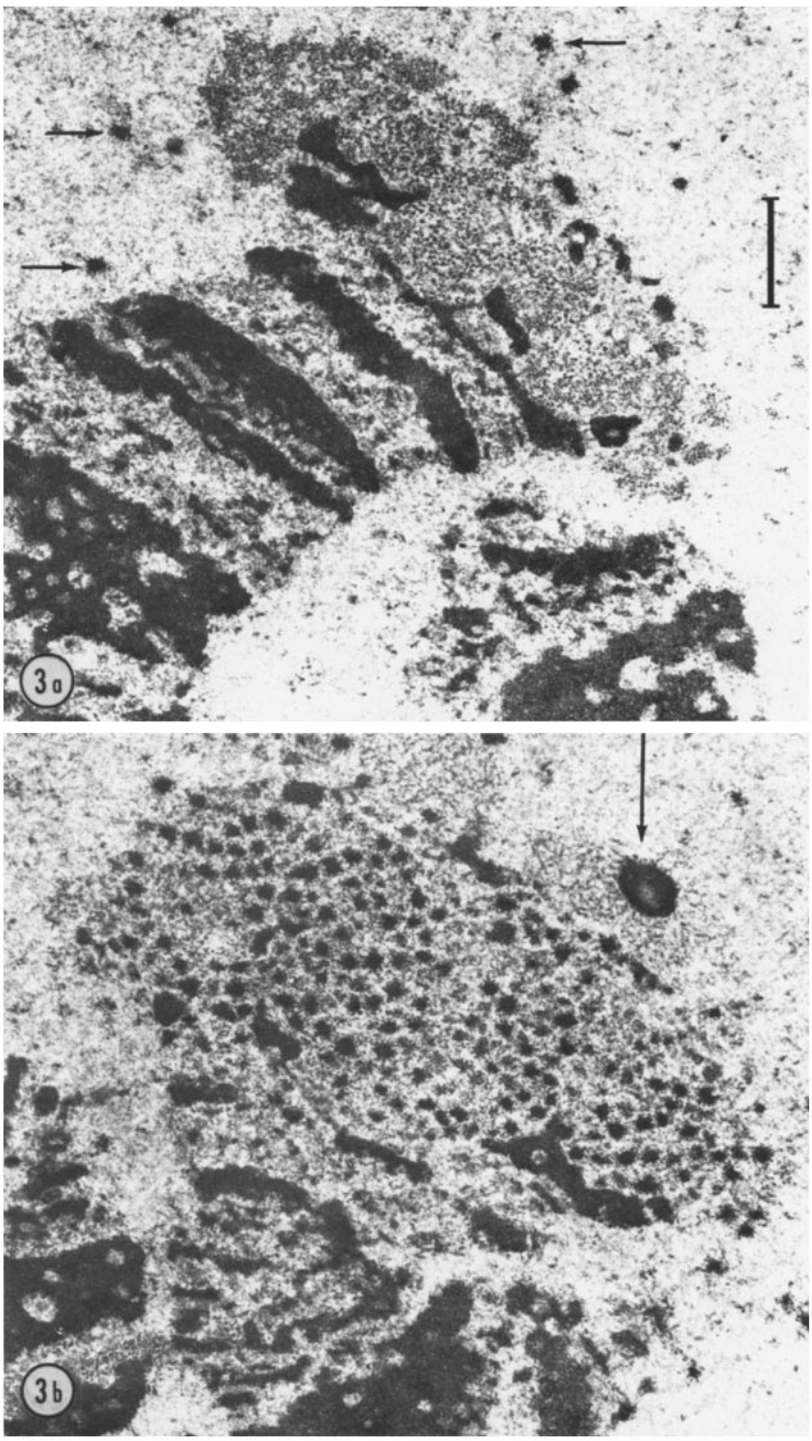

FIGURE 3 Sections through a puff at the tip of the X chromosome (*a*) showing accumulation of 300–500-Å RNP particles and, through puff 2-48C (*b*) displaying a random distribution of large RNP complexes. Both micrographs are from the same nucleus. Puff 2-48C was induced by 4-h vitamin B_6 treatment in vitro. The dense region at the upper right of photograph *b* is the telomere region (arrow). Large RNP complexes (arrows) resembling those in puff 2-48C can be seen in the nucleoplasm around the tip of the X chromosome (*a*). Scale bar: 1 μm. × 14,400.

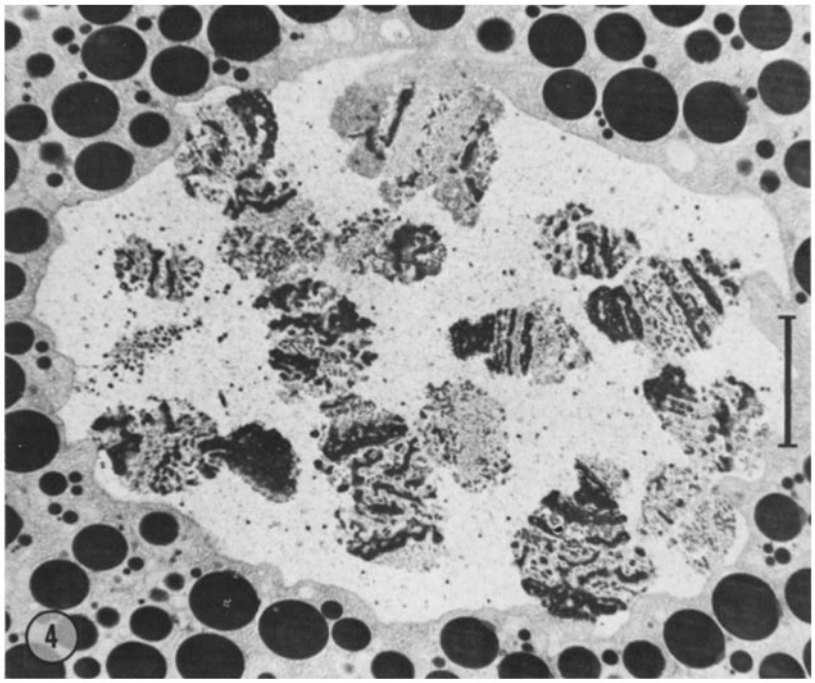

FIGURE 4 Low power micrograph of salivary gland nucleus after 4-h vitamin B_6 treatment in vitro, showing numerous large RNP complexes in the nucleoplasm. Scale bar: 5 μm. × 3,450.

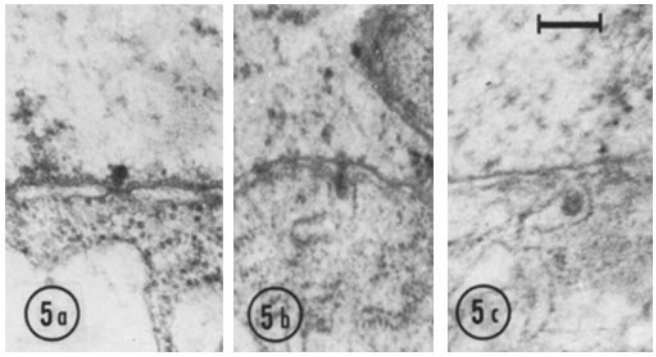

FIGURE 5 The occurrence of single 300–500-Å RNP particles at the inside (*a*), within (*b*), and at the outside (*c*) of the nuclear membrane. Scale bar: 200 nm. × 42,000.

puffed state (Berendes, 1968; Holt, 1970 and 1971).

On account of these findings it could be suggested that the large RNP complexes present in puff 2-48C are composed of RNA transcribed within the puff region and protein(s) which were already available in the cell before development of the puff was initiated. Thus far, however, it can neither be excluded that the [^{3}H]uridine incorporation actually reflects a local accumulation of labeled RNA synthesized elsewhere on the chromosomal DNA, nor that the association of the large RNP complexes with region 2-48C results from an accumulation of the complexes within the puff.

On the other hand, because of the frequent occurrence of small single 200–300-Å particles within the puff region and the presence of simi-

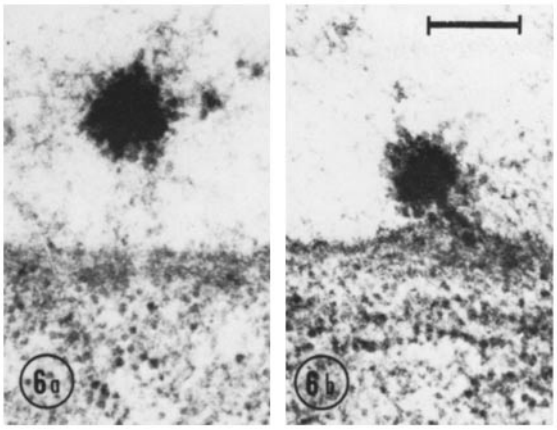

FIGURE 6 The occurrence of large 0.1–0.3-μm RNP complexes inside (*a*) and associated with (*b*) the nuclear membrane. Scale bar: 200 nm. × 56,000.

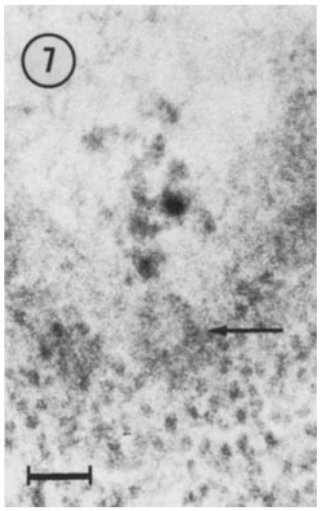

FIGURE 7 Group of 200–300-Å RNP particles situated in the neighbourhood of a nuclear pore (oblique section) (arrow). A connection between some of the particles can be seen. Scale bar: 100 mμ. × 82,000.

larly sized particles associated with the large 0.1–0.3-μm complexes, it may be suggested that the complexes arise by association of the small particles which may include the primary products of transcription. This assumption is supported by autoradiographical evidence for a undirectional migration of [^{3}H]uridine-labeled material within the puff region (Berendes, 1968 and 1972). Moreover, in early stages of puff development at 2-48C the large complexes are restricted in their distribution to a particular region of the puff which could be essentially the endpoint of the intrapuff migratory process to which the [^{3}H]uridine-labeled material is subjected. If this were the case, the site of transcription would be different from the site at which the complexes accumulate.

Recently, the activity of region 2-48C has been related to certain steps in the respiratory metabolism (Leenders et al., 1973). This relationship could be brought about by the synthesis of a specific RNA in region 2-48C followed by its release from the puff in the form of RNP and subsequent passage of the RNP, or part of it, through the nuclear membrane. A release of the large complexes from the puff may be assumed from the abundant occurrence of the complexes within the nucleoplasm, detached from chromosomal material, and their presence close to or at the nuclear membrane. It may be pointed out that the number of complexes occurring free in the nucleoplasm is far greater after vitamin B_6 treatments than after the application of a temperature treatment. As has already been mentioned, both the size of the puff and the number of large complexes it contains are greater after vitamin B_6 treatments than after a temperature treatment. The question of whether the abundant occurrence of complexes in nuclei of vitamin B_6-treated glands is a result of a high rate of production of these structures, an inefficient transport to the cytoplasm, or a combination of these possibilities remains to be answered. In any case, it can be concluded that the vitamin B_6 treatments, so far, provide the best point of departure for the isolation of the complexes by biochemical methods.

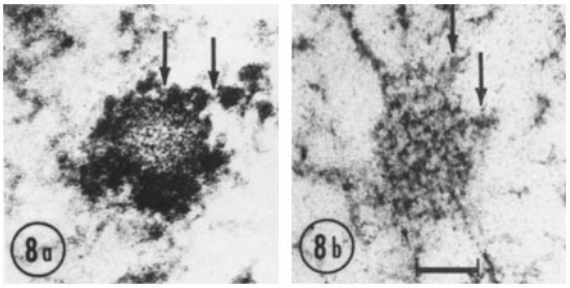

FIGURE 8 Large 0.1–0.3-μm RNP complexes following uranyl-EDTA-lead staining of a section of glutaraldehyde-fixed material (*a*) and after alkaline hydrolysis performed on a section of OsO_4-fixed material (*b*). A connection between the stained 200–300-Å particles at the surface of the complexes can be seen (arrows). In *b* only the central core and remainders of the stalks (arrows) are distinguishable. Scale bar: 100 nm. × 80,000.

In contrast to our observations that single RNP particles of 400–500 Å do occur in the cytoplasm included in outpocketings of the outer nuclear membrane, the large complexes have never been found outside the nuclear membrane. Only in a few instances has the complete complex been seen closely associated with the nuclear membrane. Individual particles of 200–300 Å, sometimes connected with each other in a string-like configuration, have been observed more frequently at the inner nuclear membrane. These structures may represent dissociated complexes. In these cases the non-RNA-containing central part of the complex has disappeared. It thus may be suggested that the macromolecular constituent(s) of the central part of the complex remain within the nucleus. A similar behavior has recently been suggested for protein components involved in the intranuclear transport of "informofers" (Lukanidin et al., 1972 *a* and *b*).

It may be concluded that the selective induction of the abundant production of the large RNP complexes by vitamin B_6 provides the opportunity for an elegant approach to the isolation and characterization of a product associated with the activity of a defined chromosome region of a eukaryotic cell.

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