MOLECULAR HYBRIDIZATION OF MOUSE SATELLITE DNA-COMPLEMENTARY RNA IN ULTRATHIN SECTIONS PREPARED FOR ELECTRON MICROSCOPY

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SUMMARY

The feasibility of in situ hybridization in tissue sections prepared for electron microscopy has been examined using mouse satellite DNA-complementary RNA and mouse L cells. The results obtained are encouraging, although certain technical aspects require further clarification. In interphase cells, hybrid-forming sites occur in chromatin patches positioned along the nuclear envelope. It is also confirmed that satellite DNA occurs in nucleolus-associated chromatin. The results suggest that satellite sequences are present in intranucleolar and perinucleolar chromatin. A similar distribution is indicated for ribosomal cistrons.

INTRODUCTION

The technique of in situ hybridization of nucleic acids to cytological preparations developed by John, Birnstiel & Jones (1969) and Gall & Pardue (1969) has proved to be a powerful tool for localizing well-defined stretches of DNA within the genome. Localization of particular DNA sequences in interphase nuclei and subcellular entities would, however, be greatly facilitated if the procedure could be extended to electron-microscope preparations. The feasibility of molecular hybridization in ultrathin sections has so far been demonstrated in only one instance (Jacob, Todd, Birnstiel & Bird, 1971) and remains to be examined in other situations. The present report summarizes data obtained in attempts to locate mouse satellite DNA at the ultrastructural level using satellite-complementary RNA and mouse cells.

From in situ hybridization experiments at the light-microscope level (Jones, 1970; Pardue & Gall, 1970; Jones & Robertson, 1970), it has been established that satellite DNA is predominantly located in pericentromeric heterochromatin of all chromosomes except the Y. Biochemical experiments (Schildkraut & Maio, 1968) and cytological hybridization studies (Jones, 1970; Rae & Franke, 1972) have also located satellite DNA in nucleoli. Knowledge of these facts makes the mouse cell a favourable target for attempting in situ hybridization in ultrathin sections with a view to evaluating the practicability of the approach and the specificity of the reaction.
MATERIALS AND METHODS

Preparation of target cells for electron microscopy

Mouse L cells growing on Falcon plastic dishes were washed gently with Dulbecco buffer to remove as much of the growth medium as possible. The cells were then detached with a rubber policeman, transferred to a centrifuge tube and spun gently for about 5 min at 1000 g. The supernatant was then poured off and the cells were resuspended in 2.5 % glutaraldehyde (Taab Laboratories, Reading, England) in 0.1 M phosphate buffer, pH 7.2. After about 20 min the fixed cells were pelleted by centrifugation, the supernatant discarded and the cells resuspended in the buffer. After a 10-min wash the cells were spun to a firm pellet and embedded in glycol methacrylate (Polysciences Inc., Rydal, Pa., U.S.A.). Polymerization was effected by ultraviolet light at a low temperature. Sections showing gold interference colour (approx. 120 nm thick) were collected on nickel or gold grids (Mason and Morton, Harrow, England) covered with Formvar and carbon.

Preparation of satellite-complementary RNA

Satellite DNA was isolated from caesium chloride gradients of mouse liver DNA prepared according to the method of Marmur (1961). The crude satellite fraction was then purified through 2 further CsCl gradients and finally analysed in the Beckman Model E analytical ultracentrifuge, where it was observed to form a single symmetrical band at 1.691 g cm⁻³. Tritium-labelled, purified satellite DNA obtained by such a procedure has been checked for site specificity against metaphase chromosomes in situ hybridization experiments at the light-microscope level. Ten microgrammes of pure satellite DNA were transcribed into complementary RNA using *Escherichia coli* RNA polymerase prepared according to the method of Chamberlain & Berg (1962). Approximately equimolar (0.4 mM) amounts of each of the tritium-labelled nucleoside triphosphates, ATP (22.7 Ci/mM), UTP (14.0 Ci/mM), CTP (20.8 Ci/mM) and GTP (9.9 Ci/mM) in solution (Radiochemical Centre, Amersham, England) were lyophilized together with the DNA in water in a 10-ml conical centrifuge tube. To the lyophilizate was added 0.3 ml of 0.1 M Tris-HCl buffer (pH 7.9 at 30 °C) containing 1.6 mM spermidine, 1 μl mercaptoethanol (1 ml = 1.168 g, Sigma), 1.5 mM MnCl₂, 10 mM MgCl₂ and 5 units of RNA polymerase. The reaction was carried out for 90 min at 30 °C. After incubation, 5 mM MgCl₂ and 40 μg/ml RNase-free DNase (Worthington) were added and digestion was carried out for 10 min at 37 °C; 500 μg non-radioactive *E. coli* RNA were added as carrier and the mixture made 0.2 % in sodium lauryl sulphate and 0.1 M in NaCl. After 2 min at 37 °C, the mixture was shaken with an equal volume of water-saturated phenol for 10 min at room temperature. After 10 min centrifugation at 12100 g in a SS-34 rotor of a Sorvall RC2B centrifuge, the upper aqueous layer was removed, set aside, and the phenol once more extracted with an equal volume of water. The 2 aqueous fractions were combined and applied to a 1.7x15 cm column of Sephadex G 50 equilibrated with 0.1 SSC (SSC = 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.2). The column was developed with 0.1 SSC and 0.5-ml fractions were collected. The radioactive excluded fractions containing carrier and c-RNA were lyophilized, and then adjusted to 4 x SSC for use. The final specific activity obtained was estimated to be approximately 1.2 x 10⁸ dpm/μg. The possibility that polymerized nucleotides were the product of polynucleotide phosphorylase contamination in RNA polymerase was excluded since enzyme controls exhibited lack of incorporation into TCA-insoluble material on the omission of DNA. The product of primed reactions was RNase-sensitive and chromosomally site-specific.

Preparation of Xenopus ribosomal RNA

The ³H-labelled 28S ribosomal RNA used in one set of experiments reported here was synthesized in vivo using cultures of a *Xenopus* kidney cell line as previously described (Jacob *et al.* 1971). The specific activity of the sample kindly supplied by Dr Birnstiel was 1.5 x 10⁸ dpm/μg.
In situ hybridization in EM preparations

In situ hybridization and autoradiographic localization of labelled molecules

The procedure for hybridization was essentially as described before (Jacob et al. 1971) in experiments with ribosomal RNA. Ultrathin sections were taken through 0.2 % solution of bovine pancreatic RNase (Koch-Light Laboratories) for periods ranging from 30 to 60 min at 37 °C, 0.01 % protease (from Streptomyces griseus, repurified type VI, Sigma) for 30–45 min at 37 °C, and 0.1 M NaOH for 1 or 2 h at room temperature, before they were floated on ²H-c-RNA (3.3 x 10⁴ or 9.3 x 10⁴ dpm/μl) for about 5 h at 70 °C. After this, the sections were treated with 0.05 % RNase for 15–30 min at 37 °C, stained with uranyl acetate in ethanol and covered with a layer of carbon. The preparations were finally coated with a monolayer of pregelled Ilford L-4 nuclear emulsion by the method of Caro & Van Tubergen (1962), which has several advantages over other coating methods (Jacob, 1971).

OBSERVATIONS

Following in situ hybridization of mouse satellite DNA-complementary RNA on to ultrathin sections of mouse cells, autoradiographic preparations can be obtained with silver grains restricted almost entirely to nuclear regions. Some technical problems have, however, been encountered. One of these is variability in labelling intensity between closely placed sections. Factors such as the unavoidable variation in thickness of sections cut with an ultramicrotome (Williams, 1969) and the variable location of the target material in relation to the cut surface (Bernhard & Tournier, 1962) may account for this. Another problem was the presence of background grains, which other workers have also reported in hybridization studies (Amaldi & Buongiorno-Nardelli, 1971; Gall, Cohen & Polan, 1971) in conventional cytological preparations. When hybridization was carried out with labelled Xenopus ribosomal RNA synthesized in vivo, background was usually low or negligible. In experiments with mouse RNA transcribed in vitro, the background tended to be higher and variable. This is probably caused by the presence in c-RNA of RNase-resistant duplexes which may stick to sections and especially to the supporting Formvar-carbon membrane of grids. Data presented in this report are from grids or areas of grids with low background; the background ranged from 1 grain per 72 to 270 μm² in the cytoplasm of the cells analysed. It may be possible to avoid this problem altogether by the use of c-RNA transcribed from isolated strands of satellite DNA or by the use of H or L strands of DNA labelled in vivo prior to extraction (Jones, 1970).

The preparations shown (Figs. 1–4) are representative of interphase cells obtained from several experiments. In Fig. 1, fairly large patches or blocks of chromatin are associated with the nuclear envelope, while more diffuse chromatin is distributed throughout the nucleus. In the nucleus in Fig. 2, most of the chromatin, including the nucleolus-associated chromatin, is in compact form and appears more electron-dense than in the previous instance. A different organization of chromatin is evident in another interphase nucleus (Fig. 3), where chromatin patches of intermediate size and density are dispersed amongst large amounts of diffuse chromatin. The silver grains present in Fig. 1 can be ascribed to radioactivity present in the chromatin patches associated with the nuclear envelope. Hybrid-forming sites are apparently more dispersed in the nucleus shown in Fig. 3. Most of the grains in Fig. 2 are localized
over the chromatin associated with nucleoli, suggesting that this chromatin is rich in satellite DNA sequences. The distribution of satellite DNA in the nucleolus-associated chromatin is better illustrated in Fig. 4. Many grains in this instance are localized over the chromatin surrounding the nucleoli. Stretches of chromatin can also be seen extending into the interior of the nucleoli and some of these are also clearly labelled. The autoradiographic resolution obtainable in electron-microscope preparations permits such an interpretation and it would be difficult to attribute the grains in question to radioactive sources in perinucleolar chromatin.

It has been demonstrated earlier (Brown, Weber & Sinclair, 1967; Pardue, Gerbi, Eckhardt & Gall, 1970) that ribosomal RNA from one eukaryote can hybridize with ribosomal DNA from another eukaryote. An in situ hybridization experiment was therefore performed on electron-microscope sections of mouse cells using 3H-labelled, Xenopus ribosomal RNA. The outcome of this was interesting, in that the distribution of silver grains on nucleoli (Figs. 5, 6) is similar to that obtained after hybridizing satellite-complementary RNA to sections of mouse cells.

DISCUSSION

The electron-microscope hybridization experiments with satellite-complementary RNA show that in some of the interphase cells, hybrid-forming sites are located in chromatin patches apposed to the nuclear envelope, as was pointed out in the preliminary report (Jacob, 1972). A recent light-microscope study of mouse liver and testis cells (Rae & Franke, 1972) also demonstrated that hybridization occurs with heterochromatin blocks associated with nuclear envelope. In view of the well established location of satellite DNA in centromeric regions of metaphase chromosomes (Pardue & Gall, 1970; Jones, 1970), the simplest explanation of the present observation would be that, at some particular stage of interphase, the centromeric heterochromatin comes to be positioned predominantly along the nuclear envelope. This event may be related to the phenomenon of chromosome condensation to the envelope during early prophase of mitosis as reported in several electron-microscope investigations (Porter & Machado, 1960; Robbins & Gonatas, 1964; Comings & Okada, 1970). Alternatively, such a location of satellite DNA-containing chromatin may be indicative of the period of replication of the DNA. It is known (cf. Flamm, 1972) that most of the satellite DNA in mouse replicates in the third quarter of S-phase. But what is highly controversial is whether replication or initiation of replication of nuclear DNA in eukaryotes occurs in association with the nuclear membrane or away from it (Comings & Kakefuda, 1968; Mizuno, Stoops & Peiffer, 1971; Fakan, Turner, Pagano & Hancock, 1972; O'Brien, Sanyal & Stanton, 1972; Ockey, 1972; Huberman, Tsai & Deich, 1973; Wise & Prescott, 1973).

Our observations with the electron microscope on the localization of label over nucleoli are consistent with the fact known for some time (Schildkraut & Maio, 1968; Mattoccia & Comings, 1971), that DNA sedimenting with nucleoli is considerably enriched in satellite sequences compared with DNA from whole nuclei. That peri-nucleolar chromatin is rich in satellite DNA was recently shown by the light-micro-
In situ hybridization in EM preparations

In situ hybridization experiments of Rae & Franke (1972). With the increased optical and autoradiographic resolution provided by the present electron-microscope preparations, hybrid-forming sites are also demonstrable in the chromatin strips which penetrate interphase nucleoli.

In mouse cells hybridized with *Xenopus* ribosomal RNA, the indications are that the ribosomal cistrons are contained in chromatin within and around nucleoli. Such a distribution of ribosomal cistrons was also indicated in *Xenopus* cells (Jacob *et al.* 1971). It is now generally accepted that ribosomal DNA is localized in nucleolus organizer segments of chromosomes, but one of the still-unanswered questions is the precise location and extent of the organizer in interphase cell nucleoli (Lafontaine & Lord, 1973). From the above-mentioned observations relating to this question, it can be expected that analysis of hybrid-forming sites at the ultrastructural level will prove useful for further clarification of this and other similar problems in molecular cytology.

As pointed out earlier in this report, our hybridization experiments suggest that ribosomal DNA as well as satellite DNA in mouse cells have a similar location in relation to nucleoli. The nucleolus organizers of mouse chromosomes are situated near the centromeric heterochromatin (Levan, Hsu & Stich, 1962) which contains the light satellite DNA (Jones, 1970; Pardue & Gall, 1970), but electron-microscope hybridization experiments indicate that ribosomal cistrons and satellite sequences are probably more intimately linked than hitherto assumed. Any speculation on the possible significance of a close spatial association between ribosomal DNA and other highly repetitive DNA sequences must await further study and confirmation in many more organisms.

The technique of *in situ* hybridization in ultrathin sections is in its infancy and the first results obtained are encouraging. More work will, however, be needed before the potential usefulness of this procedure can be evaluated fully. A parallel approach that also requires to be explored further is that of examining hybrid-forming sites in electron-microscope autoradiographic preparations of cells after carrying out *in situ* hybridization in them by conventional procedure. The first step in this direction has already been taken by Rae & Franke (1972). Development of these techniques may prove useful in our attempts to gain further insight into the molecular organization of the genome.

This work was aided by grants from the Medical Research Council and the World Health Organization.

REFERENCES


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(Received 5 June 1973)
Figs. 1-4. Autoradiographs of ultrathin sections of mouse L cells following in situ hybridization using \(^3\)H-RNA complementary to mouse satellite DNA. The bars indicate 1 \(\mu\)m. In Fig. 1 the silver grains (arrows) are located exclusively on chromatin patches associated with the nuclear envelope. In Fig. 2 most of the grains (arrows) overlie the nucleolus-associated chromatin. Label is obviously more dispersed in the interphase nucleus shown in Fig. 3: many grains overlie small chromatin patches in the central region of the nucleus in addition to those over the nucleolus-associated chromatin. The distribution of grains over nucleolus-associated chromatin is shown especially well in Fig. 4: both intranucleolar chromatin (inc) and perinucleolar chromatin (pnc) are labelled. \(n\), nucleolus.
Figs. 5, 6. Mouse L cells hybridized with $^3$H-28S ribosomal RNA of *Xenopus*. Practically all the silver grains (arrows) in Fig. 5 are over nucleolar chromatin. Intranucleolar labelling is indicated in Fig. 6. *n*, nucleolus. Bars represent 1 μm.