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10. In addition to the Kurile Islands earthquake (1963), I also analyzed its largest aftershock plus the Alaskan (1964), the Rat Island (1965), and the Tokachi-oki (1968) earthquakes. A detailed report on the results for these earthquakes will be presented elsewhere.

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Immunofluorescent Detection of Nuclear Double-Stranded RNA in situ in Vero and Mosquito Cells

Abstract. *Double-stranded RNA (dsRNA) was detected in situ by indirect immunofluorescence with antibodies to dsRNA. It was seen in nuclei of Vero and* Aedes albopictus *cells, but not in BHK cells, KB cells, chick embryo fibroblasts, or HeLa cells. Reactive dsRNA was present in the nucleoplasm, but not in nucleoli or cytoplasm. Extracted RNA from the whole cell contained from 0.08 percent (BHK) to 0.46 percent (HeLa) dsRNA, as estimated by serological methods. This dsRNA, found in molecules having the size distribution of heterogeneous nuclear RNA, did not renature rapidly after denaturation. The quantity of dsRNA in total extracted RNA did not correlate with the presence or absence of nuclear staining in situ.*

A number of reports have described double-stranded RNA (dsRNA) in extracted nuclear RNA, particularly in the large, heterogeneous nuclear RNA (hnRNA) fraction (*1*-*5*). Interpretation of the possible functional significance of dsRNA from in vitro studies of extracted RNA had been limited, since it is unknown whether any double-stranded regions exist in native intracellular structures. A significant portion of the dsRNA probably forms only during deproteinization, and consists either of intermolecular complexes (*6*) or of intrachain complementary sequences that have annealed during phenol extraction (*6*-*8*). However, a small fraction of dsRNA has been identified in nuclear ribonucleoprotein particles prepared without deproteinization (*7*).

Antibodies that specifically recognize dsRNA (anti-dsRNA) can be used to examine whether dsRNA exists in native intracellular structures. Similar techniques have served to identify reovirus dsRNA (*9*) and Sindbis and dengue virus replicative form RNA (*10, 11*) in infected cells and in extracted RNA; the viral dsRNA could be visualized in situ by immunofluorescence without extraction and deproteinization (*9, 10*). In these experiments, control or uninfected BHK or KB cells showed little or no fluorescence when tested with the combination of anti-dsRNA prepared in rabbits (rabbit anti-dsRNA) and fluorescein-labeled antibodies to the rabbit globulin prepared in goats (goat anti-rabbit globulin) (*10*). With these same reagents, we have observed that other cell lines display a striking nuclear fluorescence, specifically localized in the nucleoplasm. We report here (i) the in situ demonstration of dsRNA in the nucleoplasm of uninfected Vero cells and the *Aedes albopictus* mosquito cell line of Singh (*13*) and (ii) the size distribution and nuclear origin of extracted RNA that contains serologically reactive dsRNA. The antibodies for these experiments recognize dsRNA structure specifically (*14, 15*). Both the gamma globulin fraction of serum and antibodies that had been immunospecifically purified with heteroduplex molecules of polyinosinic acid and polycytidylic acid [poly(I)·poly(C)] (*10, 15*) gave similar results.

When acetone-fixed Vero cells were tested with purified rabbit anti-dsRNA followed by fluorescein-labeled goat anti-rabbit globulin, there was diffuse staining of the nucleoplasm in virtually all cells; in contrast, the nucleoli and the cytoplasm were unstained (Fig. 1a). Vero cells, tested at varying times over a period of several years, reacted similarly. When normal rabbit immunoglobulin was used instead of purified antibody, no staining was seen. Unlike Vero cell nuclei, BHK cell nuclei were not stained by the antibody reagents (Fig. 1b). *Aedes albopictus* cells were stained with the gamma globulin fraction from anti-dsRNA (Fig. 1c), but not with gamma globulin from normal serum. The specific fluorescence was confined to the nucleoplasm. The reactive antigen in these cells gave a speckled pattern, rather than the more homogeneous pattern seen in Vero cells.

The immunofluorescence reaction was specific; it was blocked by poly(I)·poly(C) (Fig. 1d), but not by total yeast RNA or calf DNA. The appearance of faintly stained nucleoli against the dark nuclear background seen with poly(I)·poly(C)-absorbed serum was not specific immune fluorescence; it was also observed in some cells stained with normal rabbit gamma globulin.

As with BHK cells, the nuclei of chick embyro fibroblasts and KB cells consistently failed to stain with anti-dsRNA and fluorescent anti-globulin. HeLa cells were also negative in the one test done. Occasionally, a few stained granules were seen in the cytoplasm of these cell lines.

Since different cell lines vary in their immunofluorescent reactivity, the total phenol-extracted RNA from several cell lines was tested in quantitative complement-fixation assays with purified anti-dsRNA. Although positive reactions were seen, the total amount of RNA required was from 200 to 1200 times the amount of purely double-stranded poly(I)·poly(C) that was reactive. From these results, we estimated that from 0.08 percent (BHK) to 0.46 percent (HeLa) of the total RNA behaved as if it were double-stranded (Table 1). The dsRNA represented a higher percentage

Table 1. Serological estimation of dsRNA in total RNA extracted from varying cell lines. RNA was extracted (*12*) from either whole cells (2 to 5 × 10^8) or isolated nuclei. Varying concentrations of RNA were tested with purified antibodies to dsRNA in quantitative complement fixation assays (*22*). The amount of dsRNA was estimated by comparison with the amount of poly(I) · poly(C) required for a standard reaction curve (*11, 12*). The values for total RNA from BHK, Vero, and *A. albopictus* cells are averages of four separate experiments.

Cell line	Percentage of extracted RNA reactive with antibodies to dsRNA	
	Total	Nuclear
BHK	0.08 ± 0.027	
Vero	0.29 ± 0.1	0.51
A. albopictus	0.2 ± 0.05	0.7
HeLa	0.46	
KB	0.1	

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of the RNA extracted from nuclei than of whole cell RNA (Table 1).

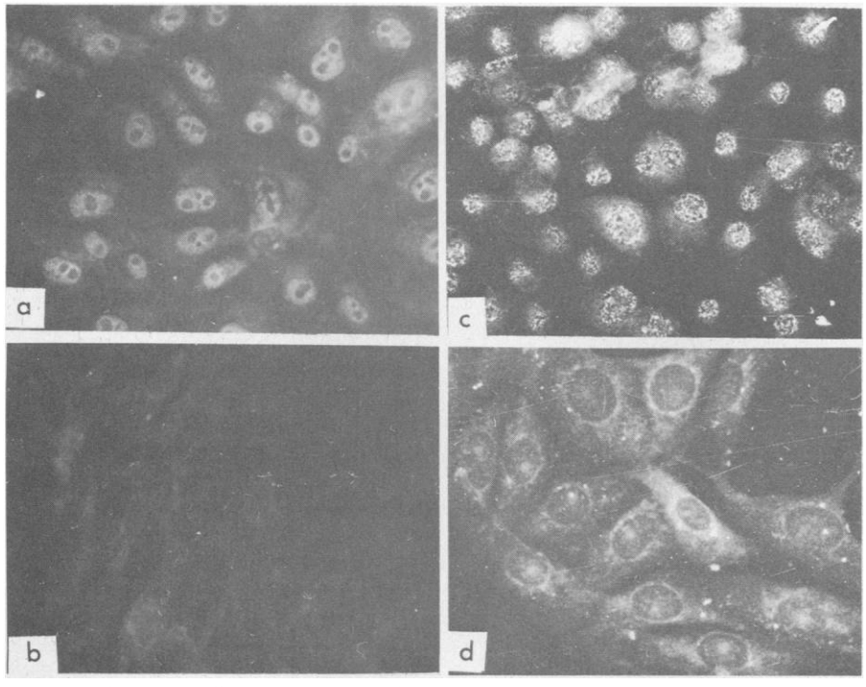
To determine the size of the RNA that contained the reactive dsRNA, we fractionated the total phenol-extracted RNA by sucrose gradient centrifugation. When the gradients were centrifuged for 16 hours, some of the reactive *Aedes albopictus* cell RNA was found in the 28*S* region, as was noted in earlier experiments (*12*), but most was found in the pellet. When centrifugation time was reduced to 9 hours, all of the dsRNA was recovered within the gradient fractions (Fig. 2). Although its distribution was broad and heterogeneous, most of the RNA containing double-stranded reactive material was larger than 28*S* (Fig. 2). The dsRNA made up about 0.5 percent of the total RNA on the gradient, and about 5 percent of the RNA larger than 35*S*. When rapidly sedimenting material from the Vero cell RNA gradient [fraction 16, (Fig. 2b)] was heated to 100°C in dilute buffer (0.014*M* NaCl, 0.001*M* tris, *p*H 7.4) and rapidly chilled, more than 90 percent of the reactive antigen was lost.

The size distribution of the serologically detectable dsRNA in extracted RNA was characteristic of large hnRNA (*16*). The amount of dsRNA we measured was similar to the dsRNA content reported by others; about 3 to 5 percent of the hnRNA, estimated by resistance to ribonuclease, has been reported to be double-stranded (*2, 3, 6, 17*). The double-stranded fragments that remained after ribonuclease digestion included short (20 to 30 base pairs), medium (100 to 200 base pairs), and large regions (300 to 800 base pairs) (*1, 2, 5*). The large fraction did not renature readily after denaturation in low ionic strength solution, and might consist of intermolecular duplexes (*6*). The reactive material we measured serologically also did not renature readily.

It is not clear why the cell lines varied in the availability of dsRNA for reaction in situ. Since no direct relationship existed between the amount of dsRNA in extracted RNA and the nuclear immunofluorescence with anti-dsRNA, most of the extracted dsRNA was probably not involved in nuclear staining. However, the stained material did have the nucleoplasmic distribution of hnRNA (*18*), and probably represented part of that RNA in a ribonucleoprotein complex. The masking of native dsRNA by protein may vary among the cell lines. The ability to detect dsRNA by immunofluorescence in some cells and not in others may also reflect different rates of synthesis and processing of hnRNA in the various cell types. It is also of interest that Vero cells, that do have dsRNA in their nucleoplasm, are unable to produce interferon in response to exogenous dsRNA (*19*).

The lack of nucleolar and cytoplasmic staining indicated that the reactive antigen was not ribosomal RNA or its precursor. Ribosomal RNA, despite its considerable secondary structure, also failed to react with these antibodies in immunodiffusion and counterimmunoelectrophoresis assays with undiluted serum, or in complement-fixation assays with diluted serum. The regions of secondary structure may differ in conformation from elongated helical rods, or higher orders of folding of the RNA may mask its helical structures.

The native dsRNA structure could involve oligo(U)·poly(A) regions that may



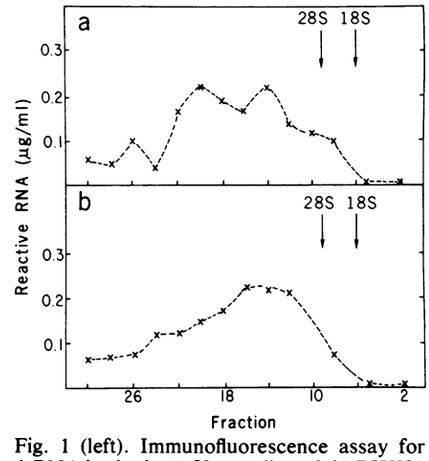


Fig. 1 (left). Immunofluorescence assay for dsRNA in situ in (a) Vero cells and (b) BHK21 cells, tested with purified antibodies to dsRNA; (c) *Aedes albopictus* cells tested with γ-globulin from antiserum to dsRNA; and (d) Vero cells, tested with γ-globulin from antiserum to dsRNA that had been absorbed with poly(I)·poly(C). The Vero and BHK21 cells were obtained from the American Type Culture Collection. The *A. albopictus* cells, clone LT C-7, have been described (*22*). Cells, grown on glass cover slips, were washed with phosphate-buffered saline (PBS), fixed with cold (−20° or −80°C) acetone, and dried in air. After rehydration for 5 minutes in PBS, the fixed cells were covered with either purified antibody or the γ-globulin fraction (at a 1:20 dilution) from antiserum against poly(A)·poly(U) for 30 minutes at 37°C. Cover slips were rinsed in PBS, drained, and covered with fluorescein-labeled goat antiserum to rabbit IgG at a 1:50 dilution (Miles Laboratories) for 30 minutes at 37°C. The cover slips were rinsed, mounted on a glass slide on a drop of 60 percent glycerol in PBS, and examined with a Zeiss fluorescence microscope. For absorption, the gamma globulin from antiserum against poly(A)·poly(U) was incubated with poly(I)·poly(C) (5 μg per microliter of serum) at 4°C for 24 hours. The mixtures were centrifuged at 6000 rev/min for 10 minutes, and the supernatant was tested for immunofluorescent staining of Vero cells. Similar incubation of serum with calf DNA or total RNA from yeast did not remove any staining reactivity. Fig. 2 (right). Size analysis by sucrose gradient centrifugation of serologically reactive dsRNA in RNA from (a) *Aedes albopictus* cells and (b) Vero cells. RNA extraction in 0.1 percent SDS and 35 percent phenol at room temperature, and the sucrose gradient centrifugation for 9 hours at 26,000 rev/min at 4°C in SW27 rotor were performed as in (*12*). Ribosomal RNA's, measured by absorption at 260 nm, were size markers. Varying dilutions of individual fractions were tested for dsRNA with 0.5 μg of purified antibody to dsRNA in each tube in a quantitative complement-fixation assay (*21*).

be associated with specific proteins (*17*). Calvet and Pederson have detected small amounts of dsRNA associated with ribonucleoprotein particles that were isolated from HeLa cells by sonic disruption of nuclei (*8*). These structures may have more accessible dsRNA in Vero and mosquito cells than in HeLa cells.

In the immunofluorescence method, cell disruption is not required; the only treatment before antibody exposure is fixation with cold acetone. Since little if any protein would be removed by this procedure, no opportunity exists for in vitro annealing or shearing of native structures. This assay, based on specific structural recognition, provides evidence that dsRNA does occur in some cells in their native state.

Discrete ribonucleoprotein particles may be derived from newly transcribed, nascent RNA fibrils that are associated with characteristic proteins in repeating subunits (*8, 20*). Regions of dsRNA in these particles could be important in further processing of the transcription product. Immunochemical studies with antibodies specific for dsRNA should be useful for the characterization of such regions, and for relating in situ observations to those made with isolated particles.

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Evidence of Paleozoic Chromosomes from Lycopod Microgametophytes

Abstract. *A Pennsylvanian arborescent lycopod cone,* Lepidostrobus schopfii, *has microspores that have been found to have intracellular features that are interpreted as nuclei and mitotic chromosomes. The cellularized gametophytes conform to the early stages of growth that occur in modern* Selaginella *microgametophytes. Since the megagametophyte of* L. schopfii *is similar in development to extant species of* Isoetes, *the fossil now is known to have portions of its life cycle in common with both* Selaginella *and* Isoetes.

Little is known of the internal contents of fossil plant cells, although there have been studies of fossil plant cell walls (*1*) and identification of residues such as chlorophyll and amino acids (*2*). Precambrian rocks have been discovered which contain fossil blue-green algae that have granular contents (*3*) and spherical green algal cells that have densely granular objects interpreted as nuclei (*4*). Cellular structures of vascular plants that have been reported include distinctive nuclei (*5*) and starch grains (*6*). Some Jurassic fern spores are reported to contain granular chromatin and chromosomes (*7*).

The opportunity to study gametophytes and nucleated spores in fossil vascular plants has been rare as a result of vagaries of preservation and random sampling of fossil floras. Recently, however, Paleozoic rocks have provided several well-preserved, structurally intact specimens from groups that include early Devonian plants (*8*), sphenopsids (*9*), lycopods (*10*), pteridophytes (*11*), and seed plants (*12*). Stewart (*13*) identified a monolete prepollen grain with sperm cells within a medullosan ovule. A germinated saccate gymnospermous grain discovered within a Pennsylvanian ovule has been observed to have a branched pollen tube similar to the ones produced by living species of *Ginkgo*, cycads, and some conifers (*14*). In other Pennsylvanian saccate pollen grains, Millay and Eggert (*5*) found three prothallial cells with nuclei and an antheridial initial. These features also occur in pollen of some extant conifers. Well-preserved gametophytes have been reported in megaspores of the arborescent lycopod fructification, *Lepidostrobus* (*15*). In one species (*L. schopfii*) rhizoids and archegonia in several developmental stages occur in abundance and compare closely to the same stages in modern *Isoetes* megagametophytes (*16*). Further examination of microspores from this same cone species now provides evidence for microgametophytic development complete with intracellular features that are interpreted to represent nuclei and mitotic chromosomes.

The most consistently well-preserved microgametophytes have come from in situ spores of Paleozoic plant fructifications. In the case of *L. schopfii*, cone specimens were discovered in coal balls (*17*) that were formed in a Pennsylvanian coal swamp, probably in an acidic environment. Cytochemical killing and fixing procedures for plant and animal tissues often prescribe the use of acidic reagents to preserve chromosomal material, because nucleic acids precipitate at low *p*H. During the early stages of fossilization, the highly acidic environment would minimize decomposition of submerged plant debris by bacteria and fungi. This environment was undoubtedly important in early stabilization of the structural details described here.

Within the microspores of *L. schopfii* there occur what appear to be nuclei and distinct mitotic figures in several stages of division. Nucleus-like bodies about one-fifth of the cell diameter are consistently observed. Mitotic figures were observed with differential interference contrast optics, which allow optical sectioning of intact spores, and by transmission and scanning electron microscopy. There appears to be an undetermined but small number (6 to 12) of chromosomes (1 to 2 μm long) in the fossil spores.

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