Short Title: CONTROL OF NUCLEAR ACTIVITY

IN MOUSE CLEAVAGE EMBRYOS

ABSTRACT

Author: Robert Michael Bernstein

Title: THE CYTOPLASMIC CONTROL OF NUCLEAR ACTIVITY IN PRE-IMPLANTATION MOUSE EMBRYOS

Department: Biology

Degree: Ph.D.

Techniques have been developed for the production of heterokaryons between 2- and 4-cell embryos of mouse and adult somatic cells of various origins, using Sendai virus mediated cell fusion. These techniques were used to study the cytoplasmic control of nuclear activity in 2- and 4-cell blastomeres. It was found that the cytoplasm of 2-cell blastomeres contained factors which inhibited RNA synthesis in previously competent mouse nuclei. These factors could also affect human nuclei and nuclei derived from malignant cells in a similar manner. Inhibitory factors were largely lost by the 4-cell stage. Although RNA synthesis by somatic nuclei in 2-cell cytoplasm was repressed, it was found that the transport of RNA from somatic nuclei was not.

The cytoplasm of 2-cell blastomeres was found to contain components which activated RNA synthesis by hen erythrocyte nuclei which normally synthesize no RNA. The cytoplasm of 2-cell embryos was also found to contain factors which stimulated DNA synthesis in nuclei derived from a contact inhibited culture which normally synthesizes no DNA.

THE CYTOPLASMIC CONTROL OF NUCLEAR ACTIVITY IN PRE-IMPLANTATION MOUSE EMBRYOS

by

Robert Michael Bernstein

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Department of Biology
McGill University
Montreal, Canada

November, 1971.

In keeping with the newly accepted regulations for thesis style which have been authorized by the Graduate Training Committee of the Biology Department at McGill, the main body of this thesis has been written in a form suitable for publication. It will be submitted to DEVELOPMENTAL BIOLOGY. The text includes a more extensive citation of literature than is required for publication and supplementary information is also presented in Materials and Methods.

CLAIM OF ORIGINAL RESEARCH

The studies reported in this dissertation are based on original research and contribute to knowledge in the fields of Cell and Developmental Biology.

ACKNOWLEDGEMENTS

First and foremost I would like to thank Dr. Barid B. Mukherjee without whose ideas, advice, encouragement and criticism this work would never have been accomplished.

In no particular order I would like to thank

Kristine Mann-Kullberg for uncountable discussions, proof reading, and for having an absorbent shoulder;

George Kasupski for helpful discussions and recreational guidance;

Janey Normanton for being a mainstay of the assembly line production of pre-implantation mouse embryos;

My wife Judy for expert phototechnical assistance, proof reading, and for being my muse;

Robert Lamarche for expert phototechnical assistance;

Mrs. Linda Ramaglia for typing this manuscript with a diligence above and beyond the call of duty.

Special thanks to Elsie Fafard, our lab mother, for behaving just as a mother should. Thanks also to all the crazy, happy gang in N/5/10 for a well tested ability to withstand my frenetic activity.

This investigation was supported by a grant from the Medical Resaerch Council of Canada and a postgraduate scholarship from the National Research Council of Canada.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

The changing patterns of RNA synthesis during early amphibian development have been well established. There is little nuclear RNA synthesis at the beginning of cleavage, but before blastula there is a marked increase in the synthesis of RNA which has been shown to be informational, i.e. DNA-like. Transfer RNA synthesis commences soon thereafter, but ribosomal RNA is not synthesized until gastrula, at which time nucleoli first appear (Brown, 1964; Brown and Littna, 1964 and 1966; Bachvarova and Davidson, 1966; Bachvarova et al., 1966; Gurdon and Ford, 1967; Landesman and Gross, 1968 and 1969; Crippa and Gross, 1969).

This basic pattern is remarkably similar in echinoderms even though the course of morphogenesis is very different. A small amount of informational RNA synthesis can be detected between the 4- and 16-cell stages (Kedes and Gross, 1969a and 1969b; Slater and Speigelman, 1970), but the activation of large amounts of DNA-like RNA synthesis occurs at blastula. Transfer RNA synthesis initiates at gastrula, and ribosomal RNA synthesis initiates soon thereafter (Aronson and Wilt, 1969; Hartman and Comb, 1969; Giudice and Vincenzo, 1969; Terman, 1970; Sconzo et al., 1970a and 1970b).

In these organisms, normal development, at least until blastula occurs in the presence of actinomycin D (Gross and Cousineau, 1963; Keifer et al., 1969; Stavy and Gross, 1969; de Vincentiis and Lancieri II, 1970). Although concentrations of actinomycin D sufficient to block

more than 90% of RNA synthesis were used in these experiments, protein synthesis was little affected. Development of embryos obtained from lethal species hybrids in frogs (e.g. Rana pipiens X Rana Sylvatica) arrest at gastrula, and the lethal nuclear transplant hybrids of Rana pipiens X Rana Sylvatica arrest at blastula (Hennen, 1963). These studies of lethal hybrids, and of transcription blocked development, indicate that the onset of functional genomic control of the embryo takes place at or near blastula. Informational RNA synthesis before blastula must be required only for post blastular development.

Emerson and Humphreys (1970) consider the apparent regulation of ribosomal RNA synthesis as artifacts of periodic accumulation and turnover of different species of RNA at different stages of development. Contrary to this point of view, however, is the bulk of experimental evidence cited above, plus the fact that the observed patterns of transcription are not artifacts of differential uptake of precursors into acid soluble pools, or differential pool specific activities (Gurdon and Woodland, 1969; Wilt, 1970). Furthermore, the development of anucleolate mutant embryos of Xenopus, which are characterized by a deletion of the nucleolar organizers and an inability to synthesize new ribosomal RNA, are normally arrested well beyond gastrula (Brown and Gurdon, 1964).

The patterns of RNA synthesis during early development described above appear to be common for all organisms studied so far, with the possible exception of mammals (Davidson, 1968).

The technique of nuclear transplantation in Xenopus has led to an understanding of some cytoplasmic controls of nuclear activity in early development. Nuclei obtained from neurula and swimming tadpole stages, and intestinal epithelia of adult frogs, when transplanted to enucleated frog eggs, are capable of promoting normal development of the embryos to fertile adult frogs (Gurdon and Brown, 1965; Gurdon, 1966; Gurdon and Laskey, 1970; Laskey and Gurdon, 1970), although the frequency of success is rarely more than 35% and generally 1-2% (Gurdon, 1966). RNA synthesis in transplanted nuclei, inferred from sucrose gradient analysis of 32-P ortho-phosphate and 14-CO_2 labelled material (Gurdon and Brown, 1965), followed the normal patterns, i.e. no labelled ribosomal RNA could be detected in early cleavage, whereas DNA-like RNA synthesis was evident. Since the nuclei used for transplantation were previously active in ribosomal RNA synthesis, this obviously represents a suppression of transcription of ribosomal DNA cistrons. This suppression of RNA synthesis was correlated with a 30-fold increase in nuclear volume within 40 minutes, and the disappearance of visible nucleoli. It has been shown by using MAK-chromatography and sephadex G-100 filtration that the sequence of reinitiation of DNA-like, ribosomal and transfer RNA in transplanted nuclei is exactly the same as the temporal sequence in normally fertilized embryos (Gurdon and Woodland, 1969). This implies the existence in the egg cytoplasm of components which are responsible for the repression of synthesis in previously competent nuclei and of other factors which are responsible for the independent sequential reactivation of transcription of the major

classes of RNA in these nuclei.

The pattern of RNA synthesis in pre-implantation mouse embryos is in some respects dissimilar to amphibians and echinoderms. In these embryos RNA synthesis can be detected at all stages of cleavage division (Mintz, 1964a; Monesi and Salfi, 1967; Hillman and Tasca, 1969; Woodland and Graham, 1969; Tasca and Hillman, 1970; Daentl and Epstein, 1971), although little RNA is synthesized by 1- and 2-cell embryos. Cytological and autoradiographic studies have shown that nucleolar RNA synthesis begins during the 4-cell stage (Mintz, 1964a; Hillman and Tasca, 1969). Data obtained from experiments using sucrose gradient centrifugation and MAK chromatography indicate the presence of newly made ribosomal and transfer RNA at this stage (Woodland and Graham, 1969; Piko, 1970; Ellem and Gwatkin, 1968). All of these workers have reported an increase in exogenous precursor incorporation into RNA as mouse cleavage development proceeds. Such observations have been interpreted as an increase in the net rate of synthesis of RNA, even though the final specific activities of the precursor pools have not yet been determined in mouse embryos at any pre-implantation stages. Daentl and Epstein (1971) have shown a parallel increase in uptake of precursor into acid soluble fractions by the embryos, but suggest that increased uptake alone probably does not account for their observed increases in incorporation of H3-uridine into RNA. Woodland and Graham (1969) have demonstrated a 5 to 10-fold increase in H3-uridine incorporation into RNA between the 2- and 4-cell stages in spite of the fact that no great differences exist either in uptake of H3-uridine into acid soluble pools, or its conversion to H3-UTP (see also Daentl and Epstein, 1971).

All the work in mouse embryos reported above correlates well with the data obtained through the use of actinomycin D. Low concentrations of the drug (10^{-8}M) did not inhibit RNA synthesis by 2-cell embryos but this concentration inhibited nucleolar labelling at the 4- and 8-cell stages. Higher concentrations (10^{-5}M) of actinomycin D inhibited the synthesis of all types of RNA (Mintz, 1964a). Similar data were obtained by Tasca and Hillman (1970). A concentration of 0.0008 µg/ml, which inhibited only rRNA synthesis allowed embryos to grow to blastocysts regardless of the stage at which the actinomycin treatment was applied. A higher concentration (0.1µg/ml) which inhibited all RNA synthesis, also inhibited cell division (see also Silagi, 1963; Thomson and Biggers, 1966; Shalko and Morse, 1969; Monesi et al., 1970). Such data indicate that informational RNA synthesis essential for further division may be required at the 2- and 4-cell stages, but that ribosomal RNA synthesis is not. This is in contrast to the situation in amphibia and echinoderms in which no RNA synthesis is required for cleavage.

Thus the temporal sequence of initiation of high molecular weight and ribosomal RNA in mouse pre-implantation embryos is similar to the other organisms discussed, but their extreme sensitivity to actinomycin D and precocious synthesis of ribosomal RNA are major points of difference.

Shiokawa and Yamana (1967) have demonstrated the existence of a ribosomal RNA synthesis suppressor secreted by Xenopus laevis cleaving blastula embryos into their surrounding medium, by co-culturing such embryos with Xenopus neurula cells. In such cultures, ribosomal RNA synthesis was

repressed in the neurula cells, which normally synthesize ribosomal RNA. The inhibitor has the characteristics of a small, non-proteinaceous molecule; is dialysable and heat stable (Shiokawa and Yamana, 1967). It is specific for ribosomal RNA and does not affect 4s RNA synthesis. A later study (Shiokawa and Yamana, 1969) has shown that the action of this factor is not species specific, since Xenopus laevis blastulae are capable of inhibiting rRNA synthesis in co-cultured Rana japonica tailbud embryo cells.

The nature of the cytoplasmic controls of nucleic acid metabolism in preimplantation mammalian embryos was not explored the way it has been done in amphibia and echinoderms. This was primarily due to the difficulties involved in nuclear transplantation in mammalian eggs. The purpose of the present communication is to report for the first time, some studies on the influence of early embryonic cytoplasm on differentiated adult somatic cell nuclei of various origins, using Sendai virus mediated mouse blastomere/somatic cell heterokaryons. The use of this technique is predicated on the fact that the somatic cells used in this study were between 200 and 400 μ^3 (as judged in a Coulter counter) whereas the volume of a single blastomere of a 2-cell embryo is about 75,000 μ^3 (Lewis and Wright, 1935). Thus it was expected that the bulk of the cytoplasmic regulatory factors in the heterokaryons would be embryonic in origin. The techniques for the efficient production of such heterokaryons, which are analogous to nuclear transplantation techniques in amphibia, were developed during the tenure of this study.

II. MATERIALS AND METHODS

A. General Techniques

1. Cell Types

A9 cells (kindly donated by Drs. Asit Mukherjee and O. J. Miller, Department of Obstetrics and Gynecology, Columbia University, New York) were originally derived by Littlefield from the "L" line of mouse fibroblasts by selection for resistance to 8-azaguanine (Littlefield, 1966), and are not highly malignant (Harris, et al., 1969). Growth medium consisted of: Medium 199 supplemented with 10% calf serum and 0.5% extra glutamine (called A9 medium).

Human diploid foreskin cells (kindly donated by Dr. L. Pinsky and E. Powell, Jewish General Hospital, Montreal) were explanted from normal male tissue and were used in the present studies when they were at the 5th to 10th passages. Growth medium consisted of Eagle's minimal essential medium with 10% fetal calf serum.

A31 and SVT2 cells (kindly donated by Dr. G. Todaro, N.I.H., Bethesda, Maryland) are established lines derived from Balb/c mice. A31 is highly sensitive to contact inhibition and is not malignant. SVT2 is an SV40 transformed line derived from A31. It is no longer sensitive to contact inhibition and is highly malignant (Grimes, 1970). Growth medium consisted of Dulbecco's modified Eagle's medium supplemented with 10% calf serum.

2. Isolation of Embryos

Six to 12-week-old Swiss albino female mice were superovulated (Mintz, 1967) by intraperitoneal injections of 5 IU's of pregnant mare serum gonadotrophin (Gestyl, Organon) followed 48 hours later by 7.5 IU's of human chorionic gonadotrophin (Pregnyl, Organon) and mated. Pregnant females were identified by the presence of a copulation plug. Fourty-two hours after the second injection, the pregnant females were sacrificed and the 2-cell embryos were flushed with phosphate buffered saline (Whitten, personal communication) from the oviducts. Four-cell embryos were obtained 55.5 hours after the second injection. Although 40% of these embryos were still at late 2-cell stage, only the 4-cell embryos were selected. After flushing from the oviducts, the embryos were treated with 0.5% pronase in Hanks' balanced salt solution at 37°C to remove the zona pellucida. They were then transferred to Mintz' medium before using for further experimentation (Mintz, 1964b). Approximately 200 embryos from 10 mothers were obtained for each experiment.

3. Techniques for growing and inactivating viruses

Sendai virus (kindly donated by Professor Henry Harris, Oxford University, England) was propagated in the allantoic cavities of 10-day-old embryonated chick eggs according to the method used by Harris (1965a). Half a hemagglutination unit (HAU) of viruses suspended in 0.1ml of Hanks' balanced salt solution (used without glucose, sodium bicarbonate or phenol red) was injected into each egg, which was then incubated at 37°C for 72 hours. After this incubation period the eggs were kept at 0-4°C

overnight, and the allantoic fluid collected. Pooled allantoic fluid from six eggs was centrifuged at 300g to remove debris and the hemagglutination titre of the supernatant was determined. The supernatant was then centrifuged at 30,000g for 30 minutes and the resulting pellet was suspended in sufficient volume of Hanks' solution to produce a concentration of 2000-4000 hemagglutination units per ml. Hemagglutination titre was determined accurately and aliquots of this suspension were stored at -75°C until needed. Hemagglutination titrations were performed in round bottom test tubes with an inside diameter of 0.8cm. Serial 1:1 dilutions of virus were made with Dulbecco's phosphate buffered saline and approximately 2.5×10^7 guinea-pig erythrocytes suspended in 0.05ml of Alsever's solution were added to each tube. One HAU was defined as the smallest amount of virus capable of producing complete hemagglutination after two hours at room temperature. It has been the experience of this investigator, and that of others (Guggenheim, et al., 1968), that virus stored at -75°C loses much of its fusion activity without noticable loss of hemagglutination titre. Accordingly, fresh virus was grown from stock once each month. Virus used for fusion studies with mouse embryos was diluted just before use to a concentration of 200 HAU's per ml in A9 medium, and inactivated by a 5-minute exposure to a 15 watt, 18 inch ultraviolet germicidal light (G.E. #G15T8) at a distance of 3 inches.

4. Isotopes

H3-5-uridine was obtained from Amersham-Searle at a specific

activity of 19.4Ci/Mm and was always used along with a 750 fold excess unlabelled deoxycytidine to prevent its incorporation into DNA (Comings, 1966).

H3-6-thymidine was obtained from New England Nuclear at a specific activity of 20Ci/Mm. Concentrations of these isotopes in the medium varied in different experiments and will be indicated for each experiment.

5. Fusion Techniques

Two fusion techniques were developed for the present studies and the technique used will be indicated separately for each experiment.

a. Fusion of embryos with cells forming a monolayer

Cells were grown in Leighton tubes until just approaching monolayer. Just before fusion, the growth medium in the culture was replaced with inactivated Sendai virus suspended in A9 medium. The embryos were then placed gently on top of the monolayer by a long drawn Pasteur pipette and the Leighton tube containing cells and embryos was transferred to a refrigerator set at 4°C and left for 2 minutes. Following this, the culture was incubated without agitation at 37°C for 30 minutes. This is a modification of the technique of Coon & Weiss (1969). The 10% serum content of the medium used is particularly advantageous because it prevented lysis of the cells, caused by the virus. Since the embryos even after fusion do not remain attached to the monolayer for an extended period, the virus containing medium with suspended embryos was gently pipetted into a cavity slide and the embryos were then picked

up by a finely drawn pipette and transferred to a screw-cap tube containing 0.5ml of Mintz' medium at 37°C (Mintz, 1964b).

b. Fusion of embryos with cells in suspension

Embryos appeared to remain attached to a monolayer of some cell types and when this problem was encountered, a different fusion procedure was employed. Just prior to fusion, cells were trypsinized and an 8% cell suspension (by packed cell volume) in A9 medium was prepared. Embryos were added in a small volume of Mintz' medium to 0.03ml of cell suspension. One ml of virus in A9 medium was added and the fusion process repeated as before. The embryos were then washed in phosphate buffered saline to free them of the majority of suspended cells, and transferred as before to 0.5ml of Mintz' medium in a screw-cap tube at 37°C.

Three types of cells can be obtained from each of these procedures--unfused somatic cells (used as controls), unfused embryos (also used as controls) and somatic cell/embryo heterokaryons.

Mintz' medium was used for hybridization experiments for a variety of reasons. High concentrations of serum sequester the action of the virus (Graham, 1969), and addition of the embryos to a medium containing 50% serum prevents further fusion from occurring. Also, Woodland and Graham (1969) have found increased uptake of H3-uridine into acid soluble fractions of the embryos using this medium as opposed to that of Brinster (1963). Finally, Mintz' medium is exceedingly well buffered and retains physiological pH throughout the open air manipulations.

The present fusion procedures were an improvement on those reported by Graham (1969) and Baranska and Koprowski (1970) in both the yield of heterokaryons and the ease with which large numbers of embryos could be manipulated at one time. In the present experiments fusion frequency ranged from 30 to 70% and the heterokaryons formed by the fusion of A9 cells with 2-cell embryos could be found morphologically normal and synthetically active for up to 72 hours after fusion, although division of the heterokaryons was rare within that period. Graham (1969), and Baranska and Koprowski (1970), however, found heterokaryons formed by the fusion of mouse diploid cells with 1-8 cell embryos to undergo cleavage division. However, the above workers also reported that cells of different somatic origins responded differently as regards nuclear division to embryonic cytoplasm. In the present study, nuclear division in some heterokaryons could be observed (Plate 1).

Somatic nuclei derived from different cells in this study also reacted differently, morphologically, as well as with respect to their ability to cleave in embryonic cytoplasm. For example, the A9 nuclei showed only small changes in morphology after fusion (Plate 2). Occasionally, loss of nucleolar structure could be observed. By contrast, A31, SVT2 and human foreskin nuclei showed marked changes after fusion to 2-cell embryos, characterized by swelling, loss of heterochromatic structure, loss of nucleoli, and rearrangement of chromatin in a more diffuse, embryonic manner (Plates 3-5). Such changes occurred within 2 hours of fusion.

6. Techniques for harvesting cells and autoradiography

At the completion of an experiment, embryos, heterokaryons, and somatic cells were harvested for autoradiography in the following way. Microscope slides were dipped in a solution of 10% albumin fixative and allowed to dry. Coverslips were dipped in a solution of 0.1% silicone in xylene and also allowed to dry. Twenty to 50 embryos were pipetted onto a slide in 2 drops of medium from a micropipette, and a coverslip placed gently over them. This procedure immobilizes the embryos and flattens them slightly without distortion. Freshly filtered acetic orcein (1.2% orcein in 45% acetic acid) was then placed along one edge of the coverslip and drawn underneath with filter paper. The stain causes the embryos to soften and flatten, and application of slight pressure, or drawing the stain through until only a minimum remains, flattens the embryos sufficiently for autoradiography. The embryos on slides were then frozen on dry ice and the coverslips were flipped with a sharp scalpel. The slides were immediately immersed in 70% ethanol for 10 minutes, rinsed in fresh 70% ethanol, and fixed in 1:3 mixture of glacial acetic acid: absolute ethanol, for 15 minutes and dried. The cells were restained with acetic orcein under coverslips and examined and photographed under the microscope. Prior to autoradiography, the coverslips were removed, the slides were washed in 70% ethanol to remove the stain, and the cells were treated for 20 minutes with ice cold 10% trichloroacetic acid to remove all traces of unincorporated precursors. The slides were then washed in running water for 30 minutes, rinsed in

distilled water, and allowed to dry. Autoradiography was performed using Kodak NTB3 liquid emulsion. Autoradiograms were exposed in the dark for variable periods of time, depending on the experiment. At the end of the exposure time, the emulsion was developed in fresh Kodak D19 developer for 2 minutes at 20°C and fixed in Kodak fixer for 5 minutes. The cells were then restained with 0.04% azure B in 1% potassium bipthalate (Taylor, 1960), examined and photographed.

B. Experimental Designs

1. Influence of embryonic cytoplasm on RNA synthesis in synthetically active nuclei

a. Influence on homospecific nuclei

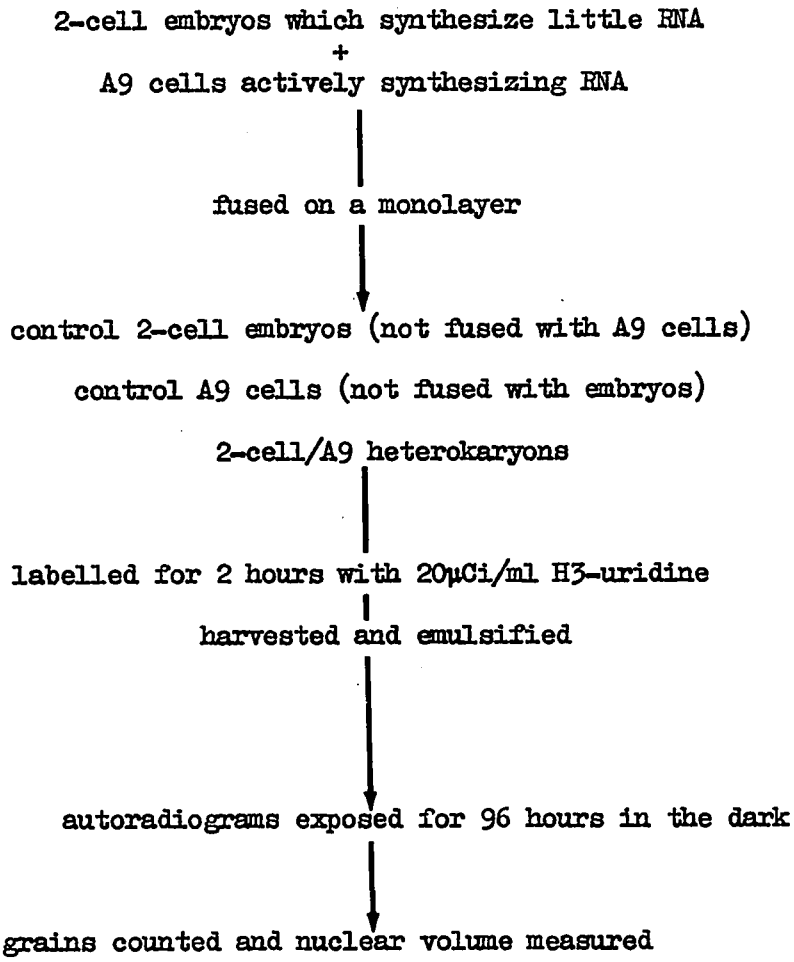
The questions were asked: Why does each cell of the 2-cell mouse embryo synthesize 5-10 fold (Woodland and Graham, 1969; see introduction) less RNA than 4-cell blastomeres? Are inhibitors of RNA synthesis (which may be similar to the inhibitors known in *Xenopus*) present in the 2-cell cytoplasm? These questions were answered in the following way. A9 cells which were actively synthesizing RNA were fused with 2- and 4-cell embryos, and the levels of incorporation of H3-uridine by A9 nuclei in 2-cell cytoplasm were compared with the levels of incorporation by A9 nuclei in 4-cell cytoplasm. At the same time, levels of incorporation of H3-uridine into embryo nuclei of control blastomeres (not fused with A9 cells), and embryo nuclei of heterokaryons, were monitored. Incorporation was determined by grain counts in autoradiograms. A9 cells were chosen because they are convenient to maintain

FIGURE 1

Experimental design for determining the influence of mouse embryonic cytoplasm on RNA synthesis in synthetically active mouse somatic nuclei.

FIGURE 1

Experiment I



Experiment II

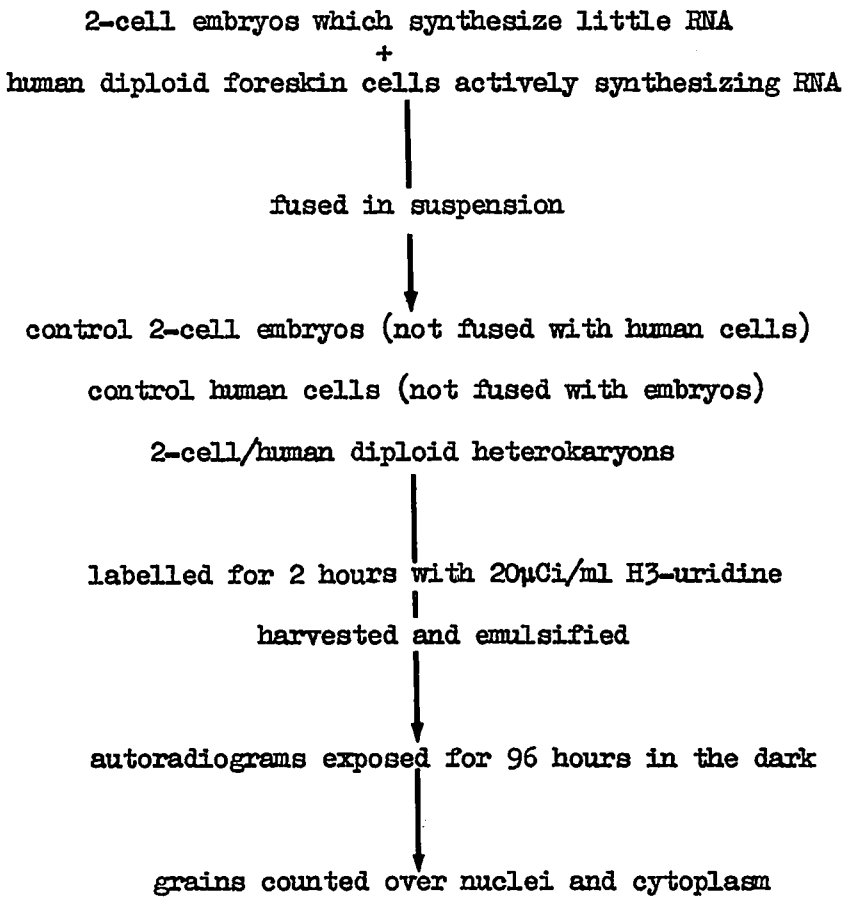
Exactly the same as experiment I, but 4-cell embryos (which synthesize 5-10 times more RNA than 2-cell embryos) were used.

FIGURE 2

Experimental design for determining the influence of mouse embryonic cytoplasm on RNA synthesis in synthetically active heterospecific (human) somatic nuclei.

FIGURE 2

Experiment III



Experiment IV

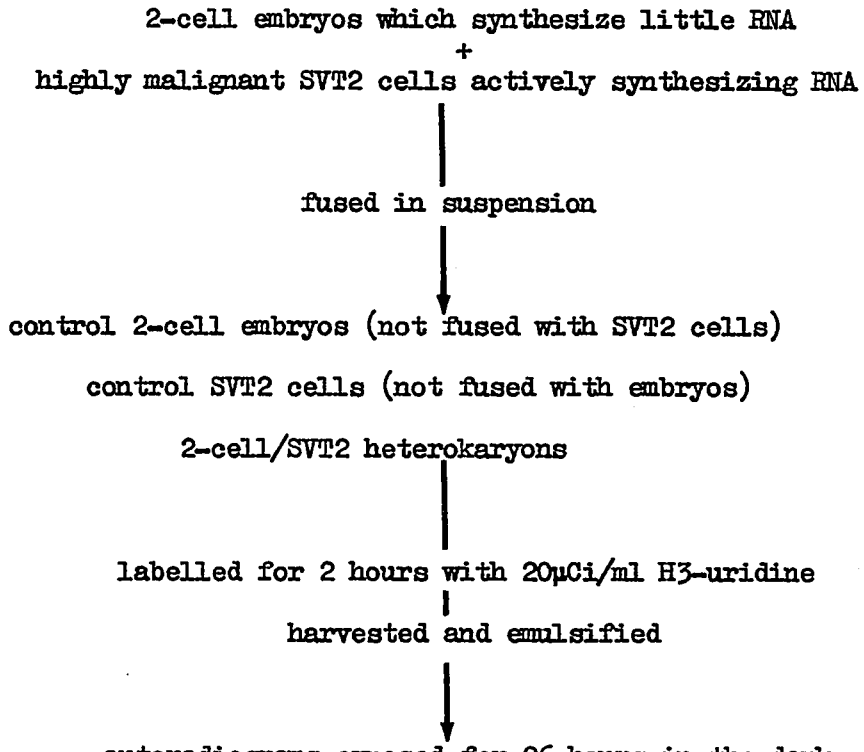
Exactly the same as experiment III, but 4-cell embryos (which synthesize 5-10 times more RNA than 2-cell embryos) were used.

FIGURE 3

Experimental design for determining the influence of mouse embryonic cytoplasm on RNA synthesis in synthetically active mouse malignant somatic nuclei.

FIGURE 3

Experiment V



in culture, are not malignant, and are derived from mouse tissue. Figure 1 demonstrates the exact experimental paradigm including labelling time, concentration of precursor, and exposure time of autoradiograms.

b. Influence on heterospecific nuclei

In order to determine whether embryonic regulatory factors are species specific in their action, the experiments mentioned above were repeated using human diploid foreskin cells instead of mouse A9 cells. Figure 2 outlines the exact experimental paradigm.

c. Influence on homospecific malignant nuclei

It was also considered of interest to know if nuclei from highly malignant cells are capable of responding to embryonic stimuli. Thus SVT2 cells were fused with 2-cell embryos. Figure 3 outlines the exact experimental paradigm.

d. Grain counts

The following methods were used to determine the number of silver grains over cytoplasm and nuclei. Photomicrographs of heterokaryons and cells were magnified to 800 or 2000X. Grains over a nucleus of a cell were counted and where cytoplasmic label was present (in 4-cell embryos and heterokaryons), grain counts over an area of cytoplasm equal to the area of that nucleus were subtracted from the number over that nucleus, and added to the cytoplasmic count. This eliminates both cytoplasmic and background counts from the nuclear counts. Net number of cytoplasmic grains was determined by counting the number of grains over the entire cytoplasm, and adding to it that number of cytoplasmic

grains over the nuclei, determined from the previous step, and finally subtracting the grain counts over a background area equal to the area of the whole cell. In heterokaryons, cytoplasmic grains were divided between the various nuclei in proportion to the number of grains over those nuclei.

e. Measurement of nuclear volume

The average diameter of each nucleus was measured in photographs of identical magnification (800X) and the mean diameters of A9 and embryonic nuclei were determined. Mean cross sectional area was calculated (area=πr^2). It was assumed that the populations of 2- and 4-cell embryos and heterokaryons were flattened equally, in which case, mean nuclear area is a reflection of mean nuclear volume.

In order to avoid errors inherent in quantitation of silver grains and nuclear volume, each of the experiments with 2-cell and 4-cell embryos was repeated 3 times and at least 3 slides were prepared per experiment. The slides from comparable experiments were emulsified and developed together, and grains over a total of at least 100 nuclei of each type were counted. Embryos were obtained for each experiment at exact times after the second superovulatory injection, the somatic cells that were used were at the same stage of growth, the timing and quantities of the superovulatory injections were kept very precise, and the periods of light and dark in the mouse room were uniformly maintained. As a result, data from replica experiments were similar and therefore pooled.

f. RNAase and DNAase digestions and actinomycin D treatment

To determine if H3-uridine was incorporated into RNA, samples of harvested embryos, heterokaryons and somatic cells from each of the experiments were treated with either DNAase or RNAase. RNAase was heated at 60°C for 15 minutes prior to use to free it of residual DNAase activity. Prior to autoradiography RNAase digestion of the cells was carried out at a concentration of 150µg of enzyme per ml of pH6.7 phosphate buffer at 37°C for 2 hours. DNAase digestion was performed at a concentration of 100 µg of enzyme per ml of pH6.7 phosphate buffer in the presence of 5mM $MgSO_4$ for 2 hours at 37°C. (Comings, 1965). Exposure time of autoradiograms in the dark was 96 hours. To determine if H3-uridine was incorporated into DNA-primed RNA, samples of control embryos, somatic cells and heterokaryons from the preceding experiments were labeled with 20µCi/ml of H3-uridine in the presence of 0.3 or 10µg/ml actinomycin D for 2 hours, after which they were harvested, and autoradiograms were prepared. Exposure time of autoradiograms in the dark was 96 hours.

g. Determinations of acid soluble precursors

Since, in the previous experiments, comparisons of 2- and 4-cell materials were made with regard to H3-uridine incorporation into RNA, the questions were asked: Does H3-uridine enter the acid soluble pools of 2- and 4-cell embryos in equivalent amounts, and does H3-uridine become phosphorylated to H3-uridine triphosphate in equivalent amounts? The answers to these questions were provided by the following

experiments.

Two- and 4-cell embryos were isolated and manipulated in the usual manner but they were not subjected to virus treatment. They were labelled with H3-uridine in the same way as in the preceding experiments. Strict sterile technique was used throughout. After the incubation in the isotope containing medium, the embryos were washed 10 times in chilled Mintz' medium to remove all unincorporated labelled precursors adhering to the surface of the embryos. A sample of the medium from the final washing was saved and its radioactivity was determined to check the efficacy of the washing procedure. The embryos were then pipetted into 0.5 ml of 0.6N $HClO_4$ and extracted for 20 minutes at $0^{o}C$ with occasional agitation. This mixture was neutralized with an equal volume of 0.6N KOH, and the resulting precipitate was pelleted by centrifugation at 500g for 10 minutes. The supernatant was counted in 10 ml of Bray's scintillation fluid (Bray, 1970) in a Beckman liquid scintillation spectrometer. This experiment allowed a comparison of 2- and 4-cell embryos to be made with respect to their total amounts of acid soluble radioactive RNA precursors.

Acid soluble fractions were prepared in the same way as above using about 3 times the number of embryos as were used in the previous procedure. An aliquot of the acid soluble material was applied as a narrow band, 3 inches from one end of a 1 X 22.5 inch strip of DEAE cellulose paper (Whatman #DE81) and allowed to dry. This procedure was repeated until all the material was applied. Descending chromatography was carried out in a buffer of 4N formic acid, 0.1M ammonium formate,

without preequilibration of the paper in the vapour phase. Under these conditions, UTP remains at or near the origin and uridine travels with the solvent front (Ives, et al., 1963; Rovera, et al., 1970; Daentl and Epstein, 1971). This experiment allowed a comparison of the relative amounts of H3-UTP in 2- and 4-cell embryos. A similar experiment was performed with A9 cells.

2. Influence of embryonic cytoplasm on RNA synthesis in synthetically inactive nuclei

Two further questions can be asked about the cytoplasmic regulation of RNA synthesis in 2- and 4-cell mouse embryos. Since a small amount of RNA synthesis can be detected at the 2-cell stage (see introduction), is this synthesis maintained by a cytoplasmic activator? Furthermore, is the initiation of large amounts of synthesis at the 4-cell stage induced by cytoplasmic activators? These questions were answered by fusing hen erythrocytes, which synthesize no RNA (Harris, 1965b), to 2- and 4-cell embryos.

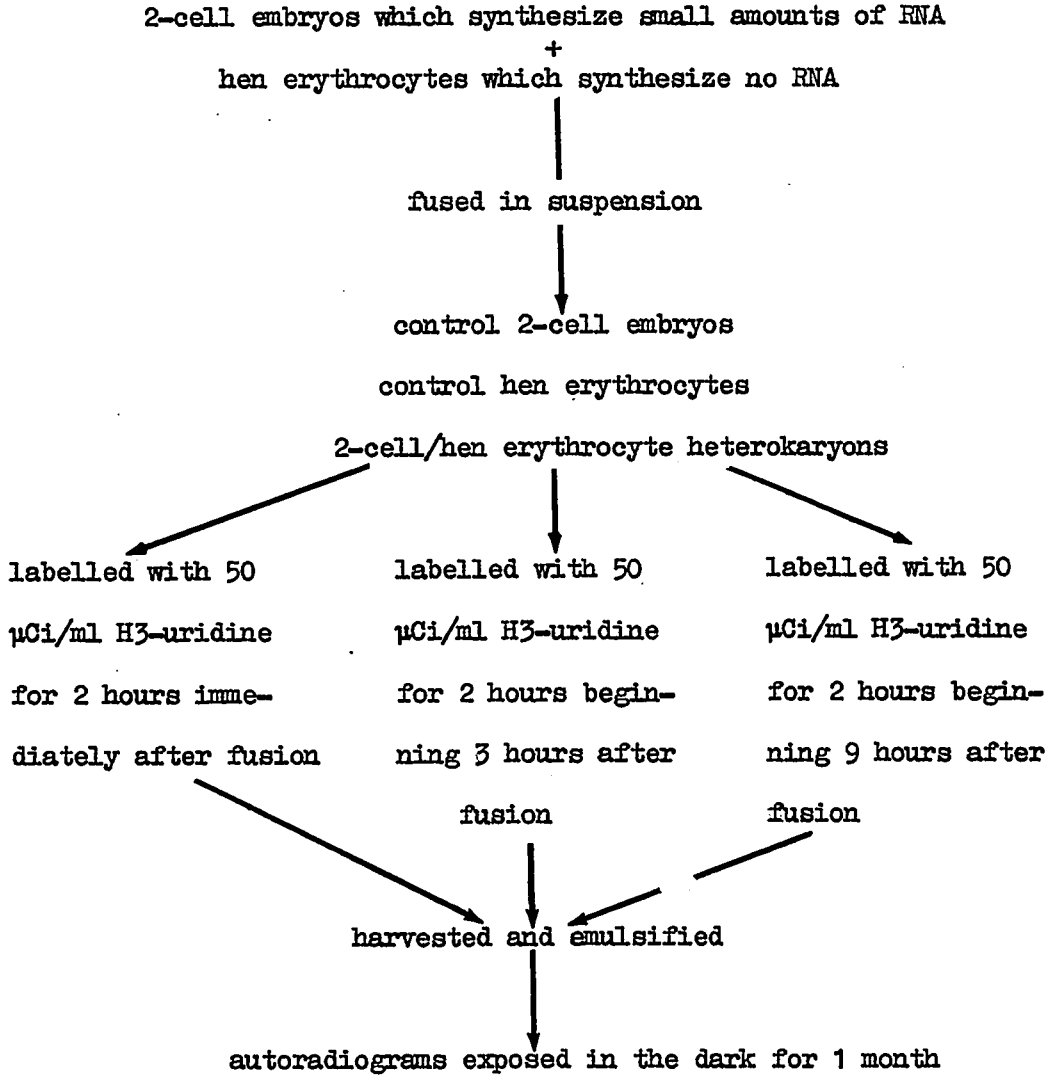
Whole heparinized blood was obtained from the wing vein of a young adult hen and erythrocytes were purified by repeated centrifugation at 300g and washing in fetal calf serum until smears showed no other cell types present (Harris, 1965). Fusion of these erythrocytes to 2- and 4-cell embryos was carried out in suspension. The embryos and heterokaryons were labelled for 2 hours with 50 Ci/ml H3-uridine in Mintz' medium beginning immediately after fusion, 3 hours, and 9 hours after fusion. As a control, hen erythrocytes alone, subjected to the same fusion and labelling procedures were pelleted, washed in fetal calf serum, smeared

FIGURE 4

Experimental design for determining the influence of mouse embryonic cytoplasm on RNA synthesis in synthetically dormant nuclei.

FIGURE 4

Experiment VI



Experiment VII

Exactly the same as experiment VI but 4-cell embryos (which had initiated 5–10 times the amount of RNA synthesis as 2-cell embryos) were used.

FIGURE 5

Experimental design for determining the influence of mouse embryonic cytoplasm on the transport of RNA from nucleus to cytoplasm.

FIGURE 5

Experiment VIII

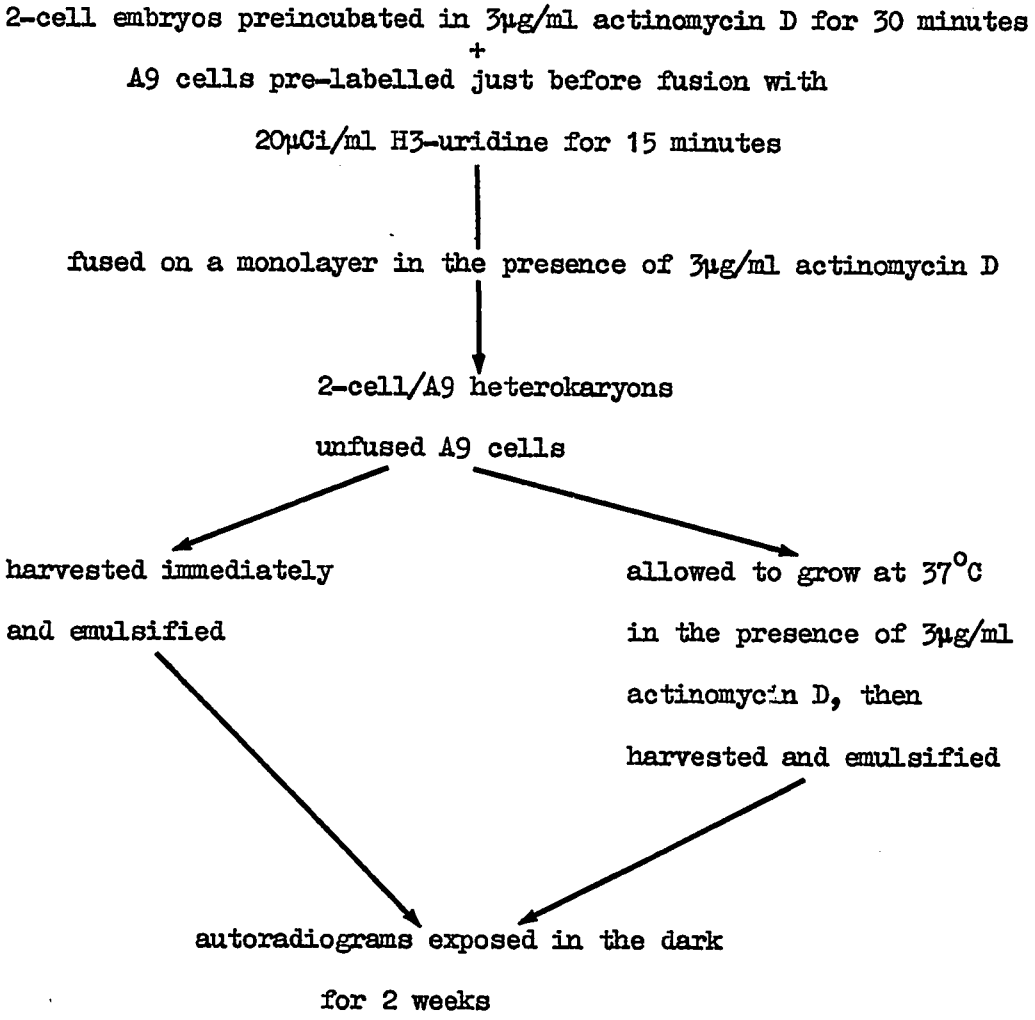
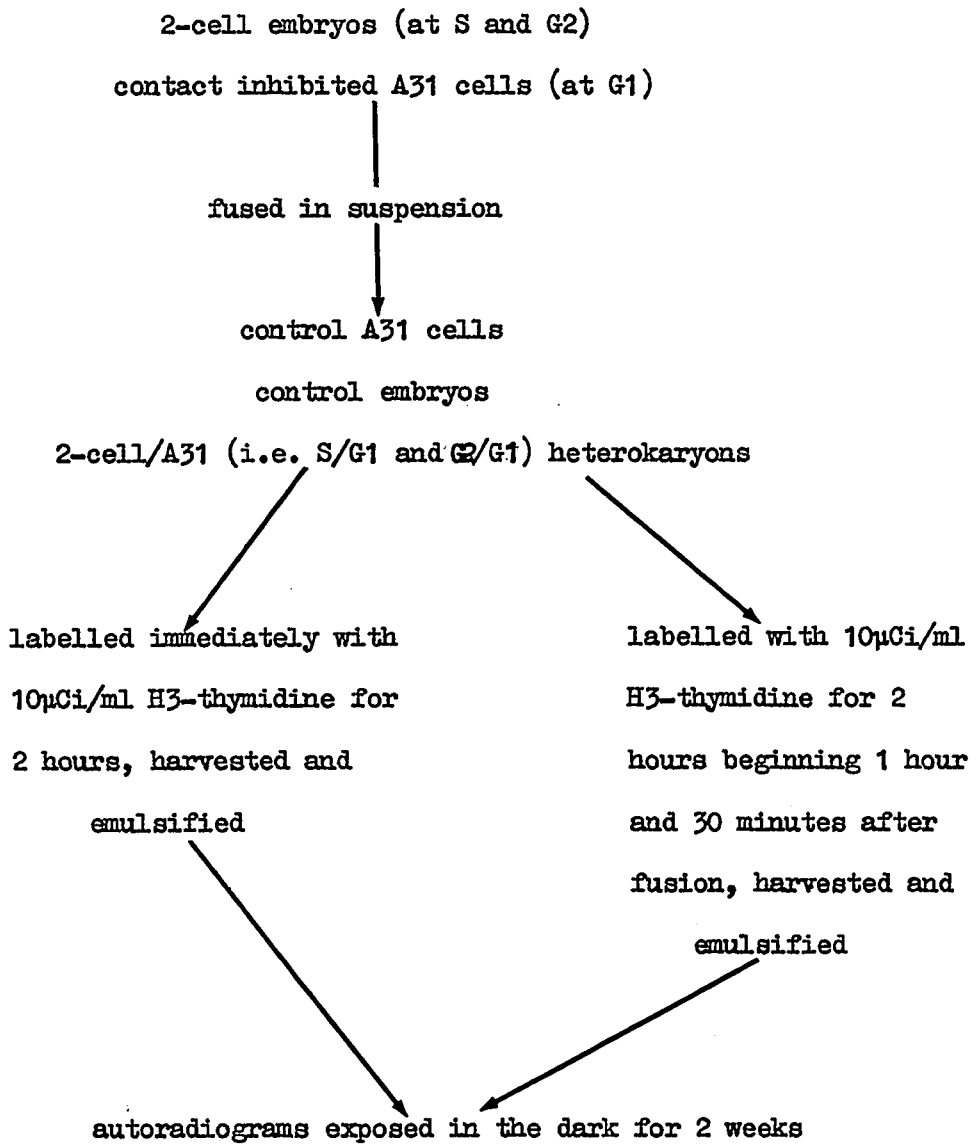


FIGURE 6

Experimental design for determining the influence of mouse embryonic cytoplasm on DNA synthesis in synthetically inactive nuclei.

FIGURE 6

Experiment IX



in serum on slides and allowed to dry. The smears were then fixed in absolute methanol and stained with geimsa prior to autoradiography. Embryos were harvested after labelling as before and autoradiograms were exposed in the dark for 1 month. The exact experimental paradigm is described in Figure 4.

3. Influence of embryonic cytoplasm on migration of RNA from a somatic nucleus to the cytoplasm of the heterokaryon

A9 cells with prelabelled nuclear RNA were fused with 2-cell embryos. Figure 5 outlines the exact experimental paradigm. Actinomycin D was used to prevent de novo RNA synthesis and further incorporation of H3-uridine into RNA after fusion. Therefore, the migration of only the prelabelled A9 RNA was studied.

4. Influence of embryonic cytoplasm on DNA synthesis in non-DNA synthesizing (G1) nuclei

Since it has been shown that embryonic cytoplasm of Xenopus contains components capable of initiating DNA synthesis in synthetically dormant brain nuclei and in purified DNA (Graham et al., 1966; Gurdon, 1967; Gurdon et al., 1969), it would be of considerable interest to know whether or not similar factors are present in mouse embryos. Two-cell embryos were fused with contact inhibited A31 cells and initiation of DNA synthesis was determined by culturing the unfused A31 cells and heterokaryons in Mintz' medium containing H3-thymidine. Figure 6 describes the exact experimental paradigm.

III. RESULTS AND DISCUSSION

1. Influence of embryonic cytoplasm on RNA synthesis in synthetically active nuclei

It was first established that the 2-cell to 4-cell difference of 5-10 fold the amount of H3-uridine incorporated into RNA per cell (observed by Woodland and Graham, 1969) was manifested in the embryos used in this laboratory (Plates 6 and 7)

a. Influence on homospecific nuclei; 2- and 4-cell embryos fused with A9 cells

An examination of control embryos (which did not fuse with A9 cells) obtained from these experiments indicates that a sizable difference in H3-uridine incorporation existed between 2- and 4-cell embryos after treatment with Sendai virus (Plates 8-11). Similarly, when 2-cell/A9 heterokaryons were compared with 4-cell/A9 heterokaryons, a sizable difference existed in H3-uridine incorporation into RNA in both the embryonic and the A9 nuclei (Plates 12-18). Concomitant with these quantitative changes, there was a qualitative change in synthesis. In no case was nucleolar labelling evident in 2-cell material, even when the number of grains was relatively large. Grains were scattered randomly throughout the nucleoplasm. However, in 4-cell material, even with relatively few grains, nucleolar synthesis was evident in both somatic and embryonic nuclei (Plate 19a & b). This is in agreement with the findings of Woodland and Graham (1969), Mintz (1964a) and others (see introduction).

Table I demonstrates the quantitative changes in H3-uridine

TABLE I

Mean numbers of grains per nucleus for at least 100 nuclei ± standard error of the mean.

Type of nucleus	EXPERIMENT		Ratio of 4-cell:2-cell
	2-cell X A9	4-cell X A9	
Embryo nuclei of control embryos	38.2 ± 2.9	383.8 ± 36.5	10.0:1
Embryo nuclei of heterokaryons	84.5 ± 5.3	718.0 ± 32.7	8.5:1
A9 nuclei of heterokaryons	18.2 ± 1.1	189.7 ± 9.0	10.4:1

incorporation into RNA in experiments in which 2-cell and 4-cell embryos were fused with A9 cells. The average number of grains per A9 nucleus in 4-cell cytoplasm was 8.5 times the average number of grains per A9 nucleus in 2-cell cytoplasm. Similarly, the average number of grains per embryo nucleus in 4-cell heterokaryons was 10.4 times the average number of grains per embryo nucleus in 2-cell heterokaryons. The average number of grains per embryo nucleus in 4-cell control embryos (not fused with A9 cells) was 10.0 times the average number of grains per embryo nucleus in 2-cell control embryos (not fused with A9 cells). The ratios of 8.5:1 and 10.4:1 are not significantly different either from each other or from the control ratio of 10.0:1.* These results are in accord with those reported by Woodland and Graham (1969) using mouse embryos which were not virus treated and which had an intact zona pellucida.

The results indicate that A9 nuclei in embryonic cytoplasm are regulated in the same way as embryo nuclei in embryonic cytoplasm. Since there was little H3-uridine incorporation into RNA in A9 nuclei in 2-cell cytoplasm, and A9 cells were synthesizing large amounts of RNA before fusion (Plate 20), this represents a suppression of synthesis in previously active nuclei.

---

* If the 95% confidence limits of the means are calculated (1.96 X SE), then the ratio of 8.5:1 may range from a minimum of 6.9:1 to a maximum of 10.6:1. Similarly, the ratio of 10.4:1 may range from 8.4:1 to 13.0:1. Therefore within the 95% confidence limits, no 2 ratios are statistically different from each other. This test is valid only if a small number of comparisons is made, and if all ratios overlap. If either of these criteria is not met, an analysis of variance becomes necessary.

A further extrapolation of these data (Table I) is that the addition of A9 nuclei along with A9 cytoplasm, to either 2-cell or 4-cell blastomeres, stimulates the embryo nuclei in the heterokaryons to incorporate approximately twice the amount of H3-uridine into RNA per nucleus as embryo nuclei in control embryos. This relationship is further demonstrated in Figures 7 and 8. Figure 7 shows the changes in H3-uridine incorporation into RNA per nucleus in the embryo and A9 nuclei of 2-cell heterokaryons, with increasing proportions of A9 nuclei. As the proportion of A9 nuclei in 2-cell heterokaryons increases, there is a continuous and linear increase in the amounts of H3-uridine incorporated into RNA per nucleus in both embryo and A9 nuclei. In contrast, in 4-cell heterokaryons, increasing the proportion of A9 nuclei does not result in a continuous increase in H3-uridine incorporation into RNA per nucleus in either embryonic or A9 nuclei (Figure 8). Here, incorporation in both nuclei reaches a maximum in heterokaryons with a ratio of 1 embryo nucleus: 1 A9 nucleus, and remains at this level even with the addition of further stimulatory A9 cytoplasm. At this point, it appears as if the limit of the capacity of 4-cell cytoplasm to support RNA synthesis has been reached. Since Daentl and Epstein (1971) have shown that 4-cell embryos contain uridine kinase in excess of the amount required to phosphorylate the complete acid soluble radioactive pool, such a limitation may be imposed by insufficient supply of acid soluble precursors, but not by insufficient levels of uridine kinase.

FIGURE 7

Changes in H3-uridine incorporation into RNA with increasing proportions of A9 nuclei in 2-cell/A9 heterokaryons.

Ordinate: mean number of grains per nucleus

Abcissa: ratio of A9 nuclei: embryo nuclei in 4 different types of 2-cell/A9 heterokaryons.

Small circles = A9 nuclei

Large circles = embryo nuclei

As the ratio of A9 nuclei: embryo nuclei in 2-cell/A9 heterokaryons increases, the amount of H3-uridine incorporated into RNA per nucleus increases linearly for both types of nuclei.

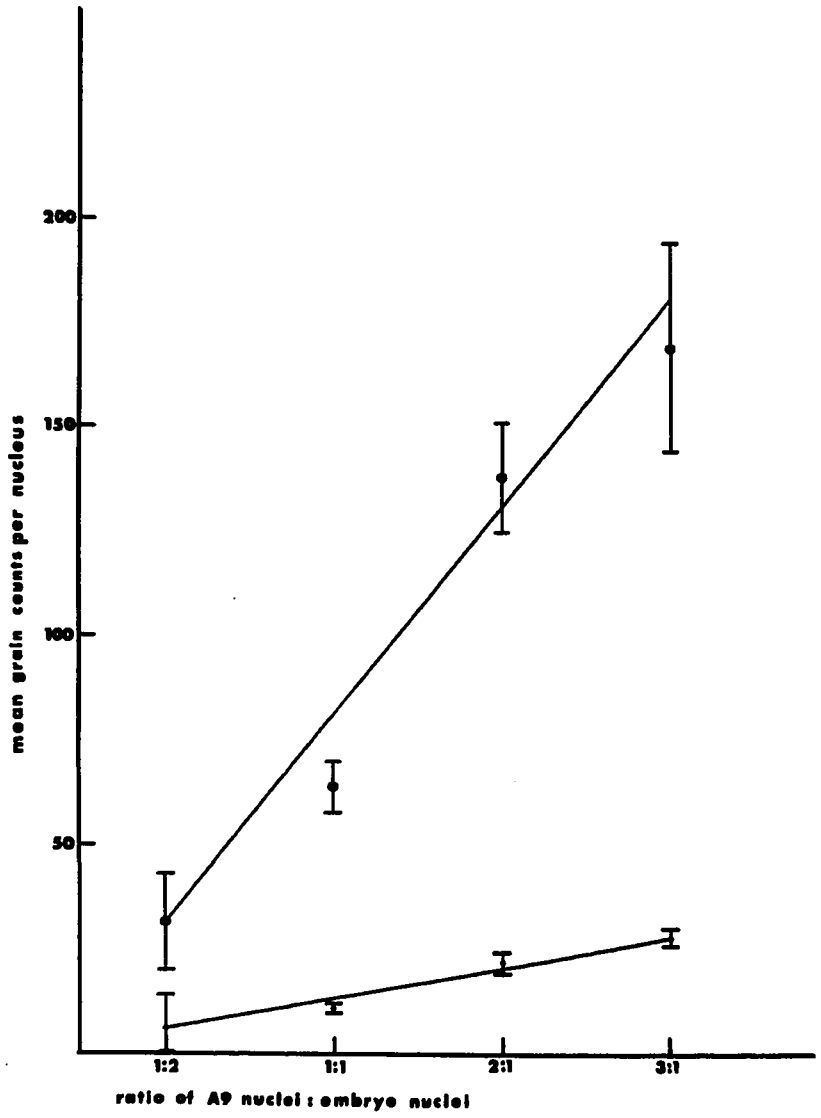
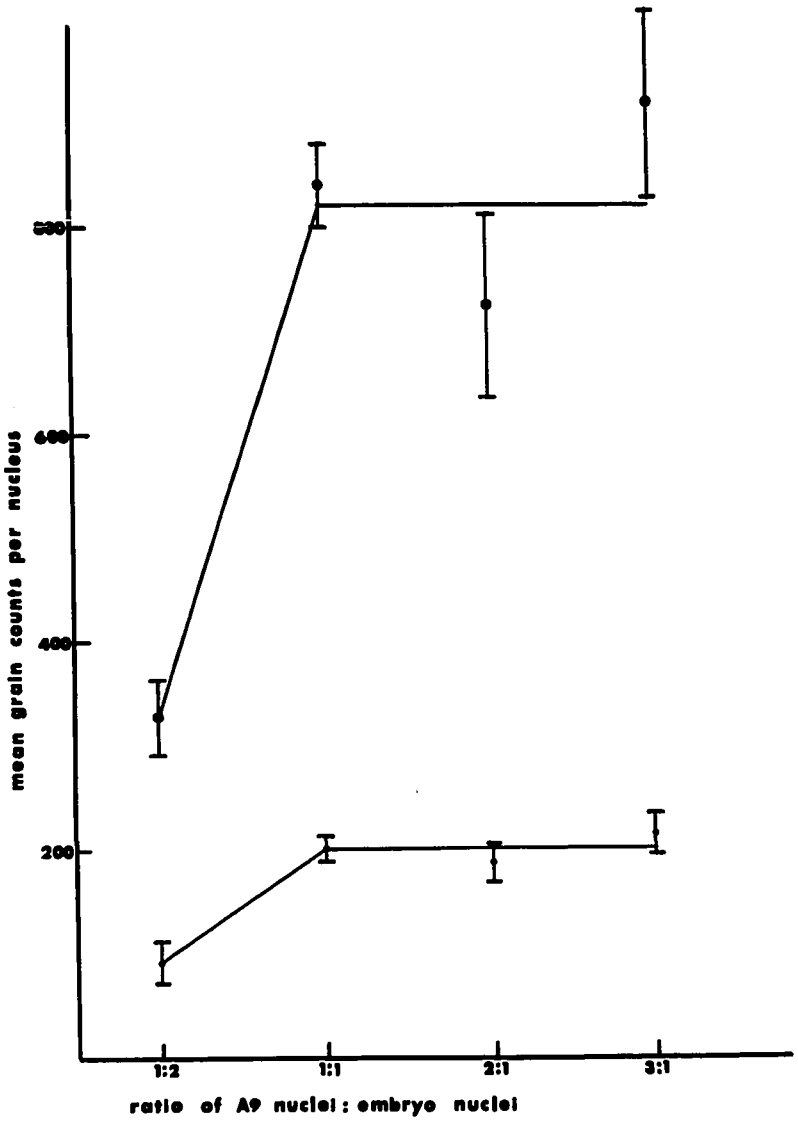

200
150
100
50
mean grain counts per nucleus
1:2
1:1
2:1
3:1
ratio of A9 nuclei : embryo nuclei

FIGURE 8

Exactly the same as figure 7, but in 4-cell/A9 heterokaryons.

Incorporation of H3-uridine into RNA per nucleus reaches a maximum in heterokaryons with a ratio of 1 A9 nucleus: 1 embryo nucleus.


mean grain counts per nucleus
800
600
400
200
1:2
1:1
2:1
3:1
ratio of A9 nuclei : embryo nuclei

Since the changes in H3-uridine incorporation into RNA per nucleus with increasing proportions of A9 nuclei are similar for both types of nuclei in 2-cell cytoplasm and similar for both types of nuclei in 4-cell cytoplasm, this provides further strong evidence that RNA synthesis in 2- and 4-cell embryos is influenced by cytoplasmic controls.

In heterokaryons involving both 2- and 4-cell blastomeres, the magnitude of the increase in H3-uridine incorporation into RNA with increasing proportions of A9 nuclei is greater in embryo nuclei than in A9 nuclei (Figures 7 and 8). This may indicate a fundamental difference between these nuclei in responding to changes in cytoplasmic stimuli. The structure of A9 nuclei before fusion and in heterokaryons is greatly different from embryo nuclei (Plate 2). A9 nuclei are more compact and contain more heterochromatic areas than the embryonic nuclei. It is possible, therefore, that more template material is readily and quickly available in embryo nuclei which are known to contain little heterochromatin as judged by amount of late replicating DNA (Fraccaro et al., 1969).

Since nuclear volume has been shown to be intimately related to the amount of RNA synthesis in HeLa/hen erythrocyte heterokaryons (Harris, 1967), and in developing Xenopus laevis embryos (Gurdon, 1968), nuclear sizes were measured in 2- and 4-cell control embryos and in 2- and 4-cell/A9 heterokaryons (Tables II & III). It can be seen that embryo nuclei in 4-cell cytoplasm are approximately two-thirds

TABLE II

Measurements of nuclear size in 2- and 4-cell/A9 heterokaryons.

Type of nucleus	Mean diameter ± standard error	Mean area
2-cell control	5.1* ± .38	20.44*
Embryo nuclei in 2- cell heterokaryons	5.3 ± .39	22.07
A9 nuclei in 2-cell heterokaryons	1.0 ± .09	0.7857
4-cell control	2.8 ± .25	6.16
Embryo nuclei in 4-cell heterokaryons	2.9 ± .30	6.61
A9 nuclei in 4-cell heterokaryons	1.1 ± .11	0.9507

* arbitrary units

TABLE III

Ratio of mean nuclear area in 2-cell:mean nuclear area in 4-cell.

Type of nucleus	Ratio	Probability of null hypothesis (that there is no change)
Control	3.3:1	$p < .01$
Embryo in heterokaryons	3.3:1	$p < .01$
A9 in heterokaryons	0.8:1	$p > .05$

smaller in size than embryo nuclei in 2-cell cytoplasm. Such a difference in size in A9 nuclei was not observed in 2- and 4-cell/A9 heterokaryons. Furthermore, there was an insignificant change in the size of embryonic nuclei after fusion, even though H3-uridine incorporation into RNA in these nuclei was stimulated 2-fold after fusion. Therefore, changes in RNA synthesis in A9 nuclei in 2- and 4-cell heterokaryons are independent of nuclear volume, and changes in RNA synthesis in embryo nuclei may be only casually and not causally related to volume. The relationship of nuclear size to RNA synthesis is therefore different in mouse embryos than in Xenopus embryos.

b. Influence on heterospecific nuclei; 2- and 4-cell embryos fused with human cells

An examination of autoradiograms of 2- and 4-cell/human diploid heterokaryons indicates that human nuclei in mouse embryonic cytoplasm are regulated in the same way as A9 nuclei in mouse embryonic cytoplasm (Plates 21 - 23). Little RNA synthesis could be seen in human and embryonic nuclei of heterokaryons involving 2-cell blastomeres, whereas RNA synthesis in heterokaryons with 4-cell blastomeres was heavy. As has been observed in the previous experiment, nucleolar labelling could be seen in 4-cell heterokaryons even in the human nuclei, which indicates that human ribosomal RNA was being synthesized. This is in contrast to the findings of Eliceiri and Green (1969) and Bramwell and Handmaker (1971) in human/mouse somatic cell hybrids. Although such hybrids contained up to 35 human chromosomes and human cell surface antigens, and therefore certainly contained human ribosomal RNA genes, human 28s

RNA was not synthesized. Since the 28s and 18s components of ribosomal RNA are derived from the same 45s precursor molecule (Darnell, 1968), it is likely that the human 28s ribosomal component was not suppressed in the mouse 4-cell embryo/human diploid heterokaryons examined in the present experiment.

Table IV indicates that the quantitative patterns of H3-uridine incorporation into RNA in 2- and 4-cell embryos and 2- and 4-cell/human diploid heterokaryons were virtually identical to those observed in the previous experiments with A9 cells. The average number of grains per embryo nucleus of control (not fused with human cells) 4-cell blastomeres was 10.3 times the average number of grains per embryo nucleus of control 2-cell blastomeres. The average number of grains per human nucleus in 4-cell heterokaryons was 11.3 times the average number of grains per human nucleus in 2-cell heterokaryons. Similarly the average number of grains per embryo nucleus in 4-cell heterokaryons was 10.8 times the average number of grains per embryo nucleus in 2-cell heterokaryons. The ratios of 11.3:1 and 10.8:1 are not significantly different either from each other, or from the control ratio of 10.3:1.

The addition of human cytoplasm through hybridization to either 2- or 4-cell blastomeres did not increase H3-uridine incorporation into RNA in the embryo nuclei, even in heterokaryons with a ratio of 3 human nuclei:1 embryo nucleus. It is evident that fusion per se does not result in increased incorporation of H3-uridine. Therefore the stimulation of RNA synthesis in embryonic nuclei of A9/embryo heterokaryons

TABLE IV

Mean numbers of grains per nucleus for at least 100 nuclei ± standard error of the mean.

Type of nucleus	EXPERIMENT		Ratio of 4-cell:2-cell
	2-cell X human diploid	4-cell X human diploid	
Embryo nuclei of control embryos	35.3 ± 2.7	362.1 ± 35.2	10.3:1
Embryo nuclei of heterokaryons	32.5 ± 3.5	350.7 ± 32.1	10.8:1
Human diploid nuclei of heterokaryons	9.6 ± 0.7	108.4 ± 5.3	11.3:1

must be due to factors contained in the A9 cytoplasm which are not present in the human cell cytoplasm. This may represent either a heterospecific difference or a heteroploid - diploid cell difference.

Since the human cells used in these experiments were synthesizing large amounts of RNA prior to fusion (Plate 24) the results described above demonstrate again a suppression of RNA synthesis in previously active nuclei.

c. Influence on malignant (SVT2) nuclei

When highly malignant SVT2 cells were fused with 2-cell blastomeres, H3-uridine incorporation into RNA in the SVT2 nuclei was repressed in the same way as was observed in 2-cell/A9 and 2-cell/human diploid heterokaryons (Plates 25 & 26). Thus, these malignant cells have not lost the capacity to respond to the regulatory factors present in the early embryonic cytoplasm. This is in accord with the fact that nuclei from the frog renal adenocarcinoma are capable of supporting development of enucleated ova of Rana pipiens to normal blastulae in 1-5% of cases (DiBerardino and King, 1965; King and DiBerardino, 1965).

d. Conclusion

The experiments described above, in which mouse 2- and 4-cell embryo blastomeres were fused with mouse A9 cells, human diploid cells, and highly malignant SVT_2 cells, indicate that any somatic nucleus in 2-cell cytoplasm is severely repressed with regard to H3-uridine incorporation into RNA by comparison to incorporation levels in the same types of nuclei in 4-cell cytoplasm. This indicates the presence in

the 2-cell cytoplasm of factors responsible for the repression of RNA synthesis which are not species specific in their action, and which have largely disappeared or become inactivated in the 4-cell cytoplasm. The fact that nuclei in 4-cell cytoplasm incorporate 10 times the amount of H3-uridine into RNA than the same nuclei in 2-cell cytoplasm, irrespective of the origins of such nuclei, indicates that the RNA synthetic behavior of somatic nuclei in embryonic cytoplasm is identical to the RNA synthetic behavior of normal embryonic nuclei in embryonic cytoplasm and that such behavior must be cytoplasmically controlled. These results are directly analogous to those obtained from nuclear transplantation experiments in Xenopus laevis (see introduction).

2. Validity of Results

In order to establish the validity of the above conclusions, it is necessary to show that the embryos and heterokaryons were alive; that heterokaryons were indeed formed; that incorporation of H3-uridine occurred primarily in RNA; and that uridine entered the cells and became converted to UTP in similar quantities in 2- and 4-cell embryos. It would also be of importance to know the specific activities of the H3-UTP pools in 2- and 4-cell blastomeres.

a. Viability of the embryos

Since both embryonic and somatic nuclei in heterokaryons in culture incorporated H3-uridine into their RNA, and the patterns of their H3-uridine incorporation remained unchanged from the patterns

observed by other workers (Woodland and Graham, 1969), it is beyond question that the heterokaryons remain alive and synthetically active after virus mediated fusion. However, to ensure that spurious results would not be obtained, the following precautions were taken. (i) The capacity of the culture medium to support and sustain growth of unfused embryos for at least one division was checked before use; (ii) minimal temperature shock to the cells (2 minutes) was allowed during the fusion procedure; (iii) experiments in which more than one-third of the embryos appeared morphologically unhealthy (cytoplasm of unhealthy embryos becomes grannular) were discarded and only morphologically healthy embryos in the remaining experiments were harvested. Furthermore, all combinations of fusion such as 6 somatic cells fused with 2 blastomeres, produced synthetically active cells. Since some blastomeres reached metaphase during the tenure of an experiment it can be assumed that the experimental procedures do not lead to immediate developmental arrest.

b. Proof of heterokaryon formation

That the cells examined in this study were indeed heterokaryons and not artifacts resulting from adsorption of cells on the blastomere surface was apparent for the following reasons. When no virus, or low concentrations of virus were used, no cells with more than one nucleus were found, using otherwise identical procedures. A change in the RNA synthetic activity of all somatic nuclei and structure of most somatic nuclei was observed after fusion with embryos. Furthermore, there was no indication of somatic nuclei being regulated, as regards their H3-

uridine incorporation, independently of embryonic cytoplasm, and in no case did a somatic nucleus incorporate H3-uridine into RNA without parallel incorporation into the blastomere nucleus of the same heterokaryon. For these reasons, other methods to check the validity of heterokaryon formation, such as serial sectioning of the cells, were not considered.

c. RNAase and DNAase digestions and actinomycin D treatment

Evidence that H3-uridine was incorporated into DNA-primed RNA comes from two sources. First, the isotopic labelling in all control cells and in all heterokaryons was RNAase sensitive but not DNAase sensitive. Second, incorporation of H3-uridine into RNA was sensitive to actinomycin D in a dosage dependent manner. Without the antibiotic, 61% of control 2-cell embryos, 81% of 2-cell/A9 heterokaryons, all 4-cell embryos and 4-cell/A9 heterokaryons incorporated H3-uridine into RNA. At a concentration of 0.3 µg/ml of actinomycin D, only 20% of the control 2-cell embryos, 28% of the 2-cell/A9 heterokaryons and 50% of the material involving 4-cell blastomeres were labelled. At a concentration of 10 µgm/ml no incorporation could be detected in any cells. Furthermore, this concentration of actinomycin D had a severe effect on the morphology of the embryo and somatic nuclei in heterokaryons (Plate 27). The inhibition of further cleavage division of 2-cell embryos may therefore be due to other effects of the drug unrelated to DNA-primed RNA synthesis. Heterokaryons involving human diploid, SVT2 cells and 2- and 4-cell embryos were sensitive to actinomycin D to the same extent as the control

embryos.

d. Determination of acid soluble precursors

Table V shows the uptake of H3-uridine into the acid soluble fractions of blastomeres from 2- and 4-cell embryos. There was a 1.28 fold increase in uptake by the blastomeres at the 4-cell stage over that of the 2-cell stage. Therefore a slight increase in H3-uridine incorporation into RNA by blastomeres from 4-cell embryos may be expected but such differences in uptake can by no means account for a 10-fold difference in H3-uridine incorporation into RNA between 2- and 4-cell blastomeres observed in the present experiments. Tasca and Hillman (1970) have also demonstrated little change in H3-uridine uptake at these stages.

Woodland and Graham (1969) have shown that approximately 25% of the radioactive acid soluble fraction in both 2- and 8-cell embryos is UTP, but Daentl and Epstein (1971) have shown that 79% of the radioactive acid soluble fraction of 2-cell embryos is UTP and this proportion was reduced to 50% by the time embryos reached the 8-16 cell stage. An attempt was made, therefore, to repeat these latter experiments, but in order to allow comparisons of this data with the autoradiographic work, it was necessary to use the same labelling times and concentrations of isotopes as were used during the present experiments for quantitative autoradiography. As a result, it was difficult to obtain enough label in a chromatogram to be detected after fractionation. The only fractions in which significant label (3x background) could be detected were those of UTP, and although it

TABLE V

Uptake of H3-uridine into acid soluble fractions of 2- and 4-cell embryos.

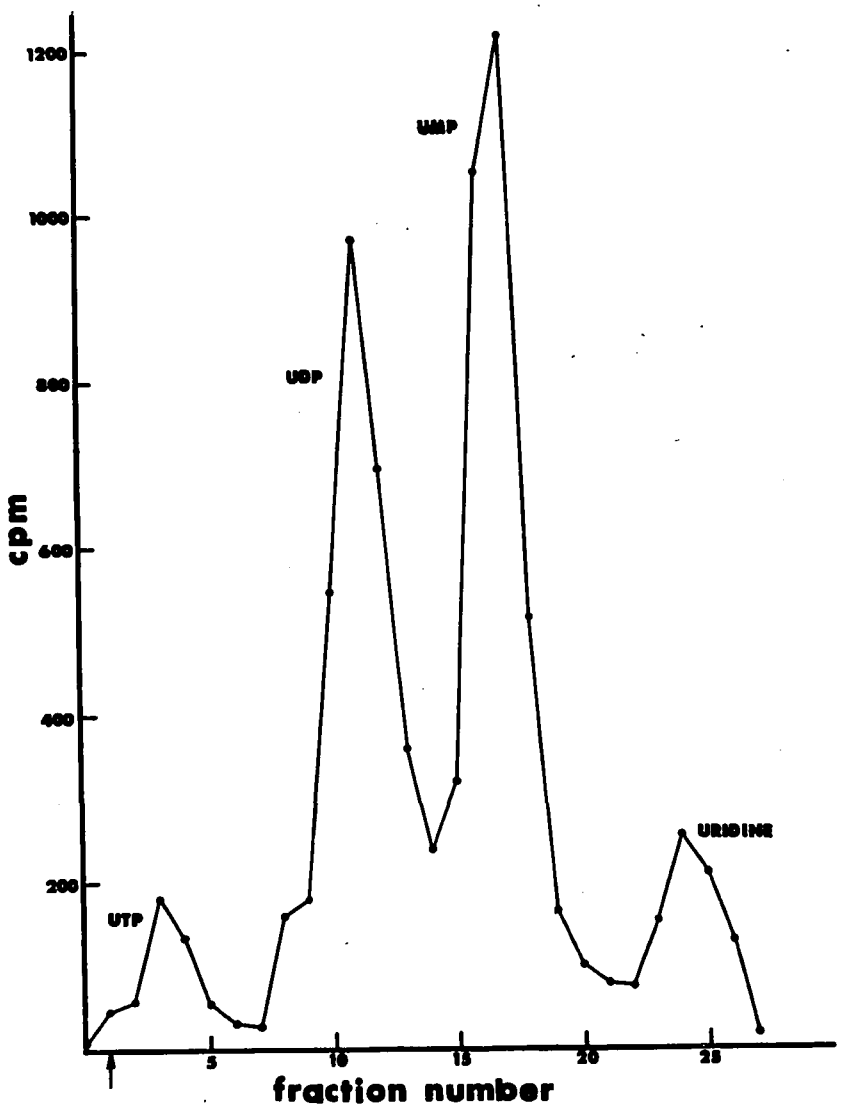
	Number of embryos	Number of cells	Total CPM above background	CPM/100 cells	Ratio of CPM/100 cells in 4-cell:CPM/100 cells in 2-cell
2-cell embryos	250	500	329.8	65.96	1.28:1
4-cell embryos	150	600	506.6	84.43	

Experiment was repeated once with similar results.

Quenching (as determined by comparisons to an external standard) was equivalent for all samples.

FIGURE 9

Fractionation of intracellular acid soluble radioactivity of A9 cells which were labelled with H3-uridine, by chromatography on DEAE-cellulose paper.


1200
1000
800
600
400
200
cpm
UMP
UDP
UTP
URIDINE
5
10
15
20
25
fraction number

was impossible to determine the exact proportion of soluble precursors present as UTP, it represented a majority of the total counts in materials from both 2- and 4-cell embryos. These results agree well with those of Daentl and Epstein (1971). In contrast to the blastomere cells, the proportion of H3-UTP in the acid soluble material obtained from A9 cells, after identical labelling conditions was only 6% (Figure 9).

The specific activities of the UTP pools have not been estimated at any stage of mouse cleavage development, and until they are determined, the results reported herein can only be expressed as an estimation of incorporation of uridine into RNA and not as the net rate of RNA synthesis in mouse embryos. The results obtained from the present experiment are, however, reflections and approximations of the true rates of RNA synthesis for the following reasons: (i) As labelling time is increased, the effects of the unlabelled endogenous UTP pool on the specific activity of H3-uridine in the acid soluble pool decreases. When the labelling time becomes sufficiently long, the specific activity of the UTP pool approaches that of the label in the medium. Such reasoning was employed by Ellem & Gwatkin (1968). These workers decided that a 2 hour labelling time was sufficiently long to lead to an intracellular pool specific activity which approached that of the medium. It follows that if the difference in H3-uridine incorporation into RNA between cells from 2- and 4-cell embryos remains 10 fold with different labelling times, then the specific activities of the intracellular pools are not the determinant factors in the kinetics of H3-uridine incorporation into RNA

at those times. Since Woodland and Graham (1969) found a 5-10 fold difference with a 6 hour labelling time, which is in agreement with a 10 fold difference observed in this study with a 2 hour labelling time, it follows that the specific activities of the H3-uridine pools are not responsible for the observed patterns of RNA synthesis in cells from 2- and 4-cell embryos. (ii) It was observed in this study that embryo nuclei in 2- and 4-cell/A9 heterokaryons were stimulated to incorporate more H3-uridine into RNA than embryo nuclei of control cells. The endogenous unlabelled uridine pool in these heterokaryons is a composite of the pools from embryonic and A9 cytoplasms. A priori, a decrease in H3-uridine incorporation into RNA would be expected in these heterokaryons. Since, in actual fact, an increase was observed, the specific activity of the H3-uridine pools cannot be the limiting factor in this system. (iii) Daentl and Epstein (1971) have reported stage specific differences in the incorporation of H3-guanine into RNA in the face of different uptake parameters. (iv) The uptake of label into acid soluble pools in this study was equivalent at both stages studied, as was phosphorylation of H3-uridine to H3-UTP.

e. Conclusion

The incorporation of H3-uridine in these experiments was based on DNA dependent RNA synthesis. The patterns of such incorporation in 2- and 4-cell embryos and heterokaryons are not due to pathogenic effects of the experimental procedures, nor are they due to changes in

uptake of precursors into acid soluble fractions or their phosphorylation to immediate RNA precursors. Changes in specific activity of the radioactive precursor pools are probably not responsible for the observed changes in H3-uridine incorporation into RNA.

3. Influence of embryonic cytoplasm on RNA synthesis in synthetically inactive nuclei

a. Observations and discussion

Hen erythrocytes are inactive in RNA synthesis but it has been shown that they are capable of being reactivated in HeLa cytoplasm (Harris, 1965). Therefore, in the present experiment erythrocytes were fused with 2-cell blastomeres to determine if embryonic cytoplasm contains similar activating factors. Care was taken to obtain a pure population of hen erythrocytes for this experiment (Plate 28a), free of other cell types which are normally capable of synthesizing RNA *in vivo*. Table VI shows that approximately 9% of the 2-cell embryo/hen erythrocyte heterokaryons (many of these heterokaryons contained more than one hen erythrocyte nuclei) had at least one synthesizing erythrocyte nucleus. In these cells, 50% of the erythrocyte nuclei could be seen to be synthesizing RNA (Plate 29). When unfused hen erythrocytes alone were subjected to the same experimental procedures as was applied to the heterokaryons, autoradiograms showed no detectable incorporation in any erythrocyte nucleus (Plate 28b). Thus the experimental procedure was not responsible for erythrocyte reactivation. These observations suggest, therefore, that the embryonic cytoplasm is capable of inducing

TABLE VI

Reactivation of hen erythrocyte nuclei by 2-cell cytoplasm

Time after fusion including labelling time	2 hours	5 hours	11 hours	TOTALS
a. Number of heterokaryons	38	37	17	92
b. Number of heterokaryons with at least 1 reactivated nucleus	3	3	2	8
c. Number of erythrocyte nuclei in "b"	5	7	8	20
d. Number of reactivated erythrocyte nuclei in "b"	4	3	3	10

Proportion of heterokaryons with at least 1 reactivated erythrocyte nucleus $= \frac{8}{92} = 9\%$

Proportion of reactivated nuclei in these heterokaryons $= \frac{10}{20} = 50\%$

Experiment was repeated once with similar results.

RNA synthesis in hen erythrocyte nuclei. A number of reasons, however, can be advanced to explain the low frequency of reactivation of hen erythrocyte nuclei in these heterokaryons. (i) The thickness of the overlying cytoplasm may have prevented some beta rays from being recorded on the photographic emulsion, (ii) not all hen erythrocyte nuclei may be capable of resuming RNA synthesis, and (iii) the length of time that the erythrocyte nuclei were exposed to the actions of embryonic cytoplasm (11 hours including labelling time) in the present experiment may not have been long enough for reactivation of more erythrocyte nuclei to have occurred. Harris (1967) has shown that 20 to 40% of all the erythrocyte nuclei in HeLa/hen erythrocyte heterokaryons showed some enlargement after 24 hours in HeLa cytoplasm and only a proportion of these had initiated RNA synthesis. It is therefore possible that reactivation of a greater proportion of erythrocytes in 2-cell/hen erythrocyte heterokaryons would be seen after longer times in 2-cell cytoplasm. The only barrier to performing such experiments is that two-cell embryos reach the 4-cell stage within 12 hours and therefore by allowing the erythrocytes to be exposed to the embryonic cytoplasm for a longer time, no inferences could be made about the control of RNA synthesis by cytoplasmic factors in 2-cell embryos.

Plates 31 & 32 demonstrate the increase in size of hen erythrocyte nuclei after fusion to embryonic cytoplasm. The numbers of synthesizing nuclei (Table VI) were too small to allow a correlation study to be made between RNA synthesis and increased nuclear volume.

Immediately after fusion there was no detectable change in nuclear volume. By 2 hours many stages of nuclear enlargement could be seen, and still greater enlargement was evident 5 and 11 hours after fusion.

Similar experiments were performed with 4-cell embryos. However, the presence of large amounts of cytoplasmic label after a two hour labelling time effectively obscured any labelling which may have been present over the erythrocyte nuclei. A one hour labelling time was not sufficient for the detection of reactivation. Out of more than 150 heterokaryons that were examined after one hour of labelling, only one cell contained a reactivated erythrocyte nucleus (Plate 30).

Enlargement of hen erythrocyte nuclei in 4-cell cytoplasm proceded in the same general way as in 2-cell cytoplasm. Two hours after fusion, a majority of erythrocyte nuclei had begun enlargement.

b. Conclusion

The results stated above imply that factors which are responsible for the initiation of RNA synthesis in inactive nuclei coexist in the 2-cell cytoplasm with the previously demonstrated inhibitory factors. Such factors are probably responsible for the initiation and perhaps maintenance of RNA synthesis in normal unfused 2-cell embryos. These factors are analogous to those found in Xenopus laevis which are responsible for the independent sequential activation of the different classes of transcription (see introduction).

If a great increase in RNA stimulating factors had occurred at the 4-cell stage, it is probable that significant reactivation of erythrocyte nuclei would have been detected at this time. These experiments, however, do not rule out the possibility that some of the increase in RNA synthesis at the 4-cell stage may be attributable to a small increase in the amount or concentration of activating factors.

It is evident that embryonic nuclei, particularly at the 2-cell stage are precisely balanced as regards their regulation of RNA synthesis. They are influenced by both inhibitory and stimulatory factors at the same time.

4. Influence of embryonic cytoplasm on migration of RNA from a somatic nucleus to the cytoplasm of the heterokaryon

a. Observations and discussion

Previous studies have demonstrated that cytoplasmic label of presumably nuclear origin is detectable in mouse 2-cell embryos after a 4 hour incubation with H3-uridine, or after a long chase with unlabelled uridine (Mintz, 1964a). Since 2-cell embryos synthesize only high molecular weight RNA (Woodland and Graham, 1969), migration of only that RNA was studied. The synthesis of ribosomal RNA is normally impaired in 2-cell embryos (see introduction and this study) and *a priori*, one might expect that transport of ribosomal RNA would also be impaired. This was studied by introducing somatic (A9) nuclei containing labelled nuclear RNA into 2-cell blastomeres.

It has been shown that the majority of RNA synthesized by mammalian cells in culture is ribosomal RNA precursor, i.e. 45s (Warner, et al., 1966, cited by Darnell, 1968). Accordingly A9 cells were pulsed with H3-uridine just before fusion for 15 minutes. An examination of autoradiograms of these cells just after fusion revealed that most of the label was nucleolar (Plate 35a).

Immediately after the fusion of 2-cell blastomeres to A9 cells containing prelabelled nuclear RNA, there was little labelled RNA detectable in the cytoplasm of the heterokaryons. Two hours after fusion, however, much of the label which was present previously in the A9 nuclei had migrated into the cytoplasm (Plates 33 & 34). As a control, A9 cells which had not fused with embryos were also harvested 2 hours after fusion. An examination of autoradiograms of such cells revealed that labelled RNA migrated to the A9 cell cytoplasm (Plate 35b). The conclusion, therefore, may be drawn that there is no immediate impairment of transport functions specifically of the nucleoli of somatic nuclei in 2-cell cytoplasm. Alternatively, the migration of RNA into the cytoplasm observed in these heterokaryons may represent a non-specific spillage of nuclear contents into the cytoplasm concomitant with the migration of cytoplasmic components into the nucleus (Merriam, 1969). Such activity may be necessary for changes in nuclear function, i.e. RNA synthesis, to occur.

b. Conclusion

These observations suggest strongly that the transportation

of RNA from nucleus to cytoplasm is regulated independently of RNA biosynthesis. This is particularly interesting in view of the immediate and gross impairment of both nuclear RNA synthesis and nucleolar RNA synthesis in 2-cell/A9 heterokaryons.

5. Influence of embryonic cytoplasm on DNA synthesis in non-DNA synthesizing (G1) nuclei

a. Observations and discussion

When mouse embryos are obtained 42 hours after the second superovulatory injection, most of them are in the latter half of the 2-cell stage, which has been shown to be a long G2 period (Graham, 1969; Gamow and Prescott, 1970). In subsequent development from the 4-cell stage to blastocyst a G1 phase is undetectable and DNA synthesis is virtually continuous except for mitosis and a brief G2 (Gamow and Prescott, 1970). It might be expected, therefore, that factors capable of initiating DNA synthesis would be present in 2-cell embryos preparatory for continuous DNA synthesis at the 4-cell stage. Accordingly, contact inhibited A31 cells were fused with 2-cell blastomeres (obtained 42 hours after the second superovulatory injection). When the heterokaryons, unfused cells and embryos were labelled with H3-thymidine for 2 hours beginning 1 hour and 30 minutes after fusion, only 11% of the unfused control blastomeres and 2.5% of the control A31 cells in the culture initiated DNA synthesis. By contrast, 30% of the A31 nuclei in heterokaryons from the same culture had initiated DNA synthesis. This represents a 12-fold increase in the proportion of nuclei initiating

TABLE VII

Activation of DNA synthesis in G1 nuclei by 2-cell cytoplasm

Type of nucleus	Number of nuclei examined	Number of nuclei synthesizing DNA	% nuclei synthesizing DNA
A31 nuclei in control A31 cells	241	6	2.5%
Embryo nuclei in control embryos	63	7	11.0%
A31 nuclei in embryo cytoplasm	183	55	30.0%

DNA synthesis over control levels (Table VII). In most of the heterokaryons obtained, the embryo nuclei were not synthesizing DNA but the A31 nuclei were (Plate 37). Such cells were probably composites of G2 blastomeres and G1 A31 cells.

When this experiment was performed by labelling the cells for 2 hours beginning immediately after fusion, no activation of DNA synthesis in A31 nuclei could be observed above the control levels. The length of time required to detect activation in the present experiments is greater than that found by Gurdon (1967) in mature, unfertilized Xenopus laevis oocytes but it is consistent with that found by Rao and Johnson (1970) in G1/S HeLa homokaryons. However, these workers were unable to demonstrate a stimulatory effect of G2 HeLa cytoplasm on G1 HeLa nuclei. In this respect, mouse embryonic cytoplasm is greatly different from that of somatic cells. The DNA stimulatory factors in the 2-cell cytoplasm may be similar to those found in unfertilized Xenopus laevis eggs (Graham, et al., 1966; Gurdon, 1967; Gurdon et al., 1969).

b. Conclusion

The cytoplasm of 2-cell mouse embryos contains factors responsible for the activation of DNA synthesis along with factors that regulate RNA synthesis. Such DNA stimulatory factors remain in the cytoplasm even after the embryonic DNA has been replicated. The presence of these factors in late 2-cell stage embryos is probably preparatory for continuous DNA synthesis in the embryos from the 4-cell

stage to blastocyst in the absence of a detectable G1 period. Nuclei in which DNA synthesis has already occurred must be insensitive to these factors, but nuclei containing unreplicated DNA are not.

SUMMARY AND CONCLUSIONS

The technique of Sendai virus mediated cell fusion has been employed to elucidate some cytoplasmic controls of nuclear activity in 2- and 4-cell mouse embryos. Such embryos were fused with

a. mouse cells actively synthesizing RNA

b. human cells actively synthesizing RNA

c. malignant cells actively synthesizing RNA

d. hen erythrocytes which were synthesizing no RNA

RNA synthesis in the heterokaryons was monitored by grain counts in autoradiograms. A comparison of acid soluble radioactivity in 2- and 4-cell embryos was made after labelling with H3-uridine

Two-cell embryos were fused with cells which contained radioactive nuclear RNA and transport of this RNA to the cytoplasm of the heterokaryons was studied by autoradiography. Two-cell embryos in the G2 period of their cell cycle were also fused with cells in the G1 period of the cell cycle and initiation of DNA synthesis was monitored by autoradiography.

The following conclusions were obtained from these experiments:

1. The cytoplasm of 2-cell embryos appears to contain factors which severely inhibit RNA synthesis in competent adult somatic nuclei. Inhibitory factors are largely lost by the 4-cell stage.

2. Such inhibitory factors are not species specific as far as has been tested.

3. Malignant cells are capable of responding to the cytoplasmic inhibitory factors and they also respond to embryonic stimuli which bring about morphological changes in the nuclei.

4. The cytoplasm of 2-cell embryos contains components capable of initiating RNA synthesis in previously inactive nuclei.

5. No nucleolar RNA synthesis can be seen in any nucleus in 2-cell cytoplasm.

6. Although RNA synthesis is repressed, migration of RNA from a somatic nucleus in 2-cell cytoplasm is not immediately impaired.

7. Differences between nuclei in 4-cell cytoplasm and the same nuclei in 2-cell cytoplasm cannot be accounted for by differences in uptake or phosphorylation of precursor.

8. RNA synthesis may not be causally related to nuclear volume although it seems to be in other systems.

9. The cytoplasm of 2-cell embryos in the G2 period of their cell cycle contains factors capable of initiating DNA synthesis in nuclei derived from contact inhibited cells.

The cytoplasmic factors shown in this study to be present in 2- and 4-cell mouse embryos have all been demonstrated in Xenopus laevis embryos by Gurdon and his co-workers (see introduction).

The regulation of RNA synthesis in 2- and 4-cell mouse embryos can be understood in terms of an interaction of stimulatory and inhibitory factors. The following model may be presented on the basis of the results of this paper and others. Inhibitory factors are present in

the 2-cell cytoplasm and are not specific in their action; given the opportunity, they would affect all cistrons in DNA. The small amounts of synthesis seen in 2-cell embryos are stimulated and probably maintained by factors capable of cistron recognition. Initiation of large amounts of mainly ribosomal RNA synthesis at the 4-cell stage is accomplished mainly by the loss or inactivation of the inhibitors present in 2-cell although some further specific stimulation may occur. The embryonic nucleus, therefore, is in a state of precisely balanced regulation.

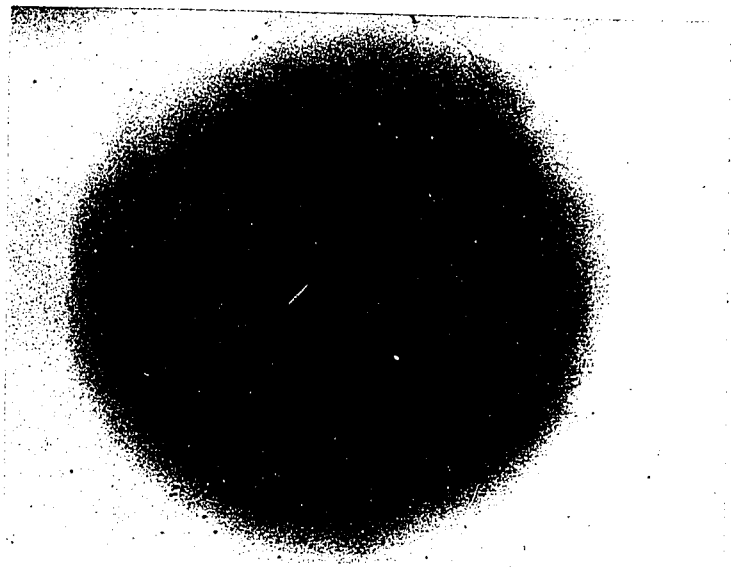
The basic scheme of regulation of RNA synthesis in preimplantation mouse embryos is very similar to that of amphibians and other organisms. In all cases, the regulation of nuclear activity in cleavage development is cytoplasmic in nature.

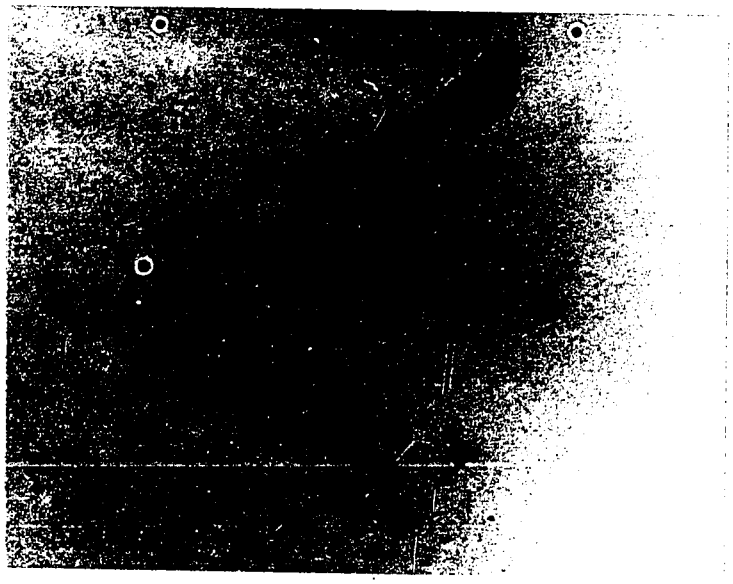
PLATE 1

a. Mitoses in 2 nuclei of a 2-cell heterokaryon obtained from an experiment using human diploid cells. a. 400X.

b. Enlargement of one mitotic nucleus of above. Arrow points to putative metacentric (possibly human) chromosome. b. 2000X.

c. Induction of abortive mitosis in a 4-cell/ human diploid heterokaryon labelled with H3-uridine. Embryo nucleus is in well defined metaphase. Note lack of RNA synthesis by all but one of the four human nuclei. c. 640X.





72

PLATE 2

a. 400X.

a & b. Two-cell/A9 heterokaryons obtained 2 hours after fusion.

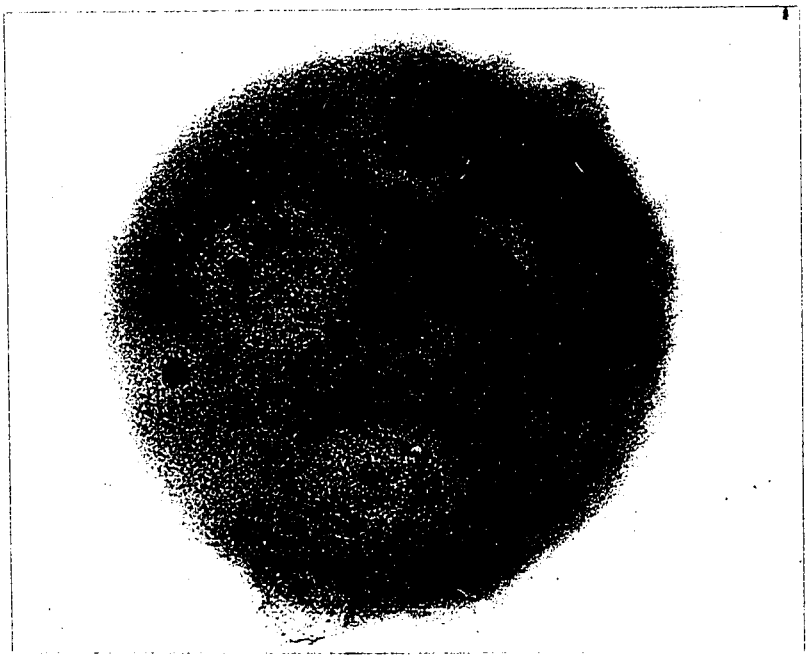
c. A9 cells before fusion.

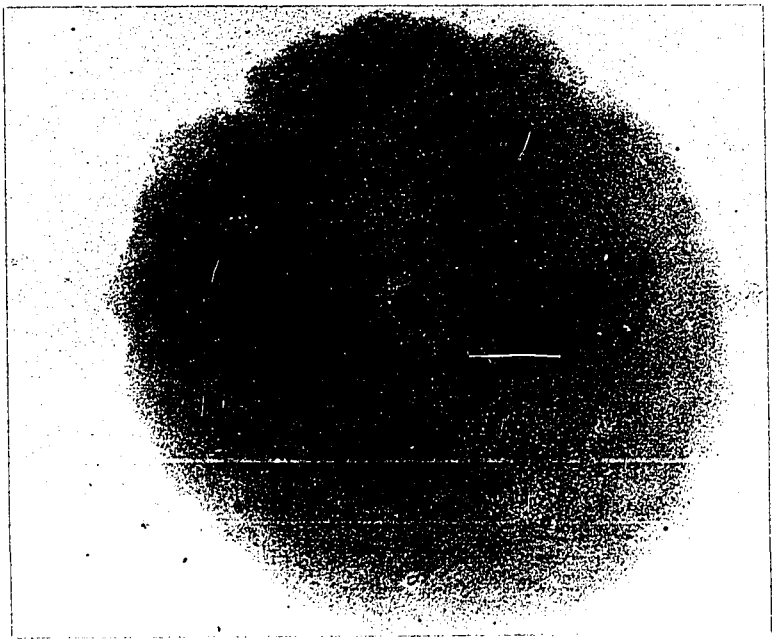
Note the characteristic diffuse structure of the embryo nuclei, and that little morphological change occurred in the A9 nuclei after fusion to embryonic cytoplasm.

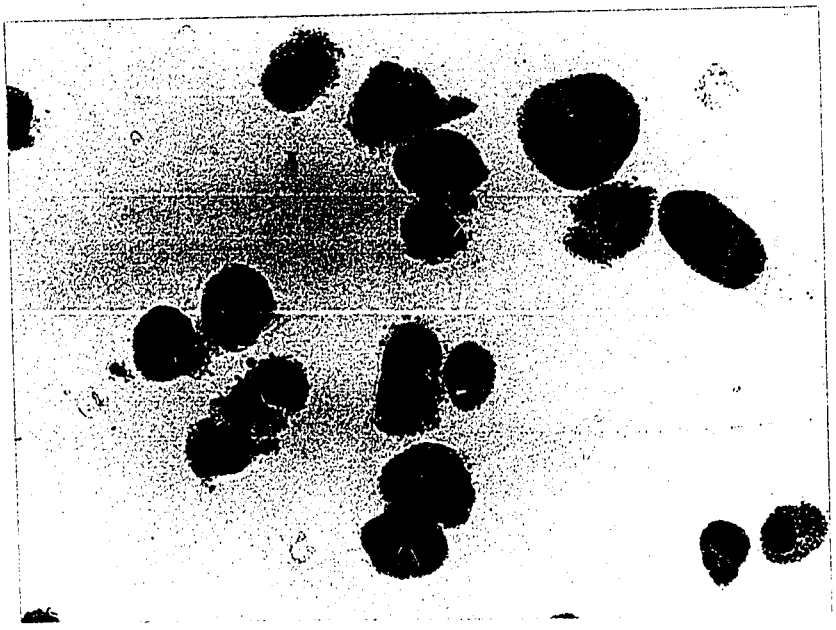
b. 400X.

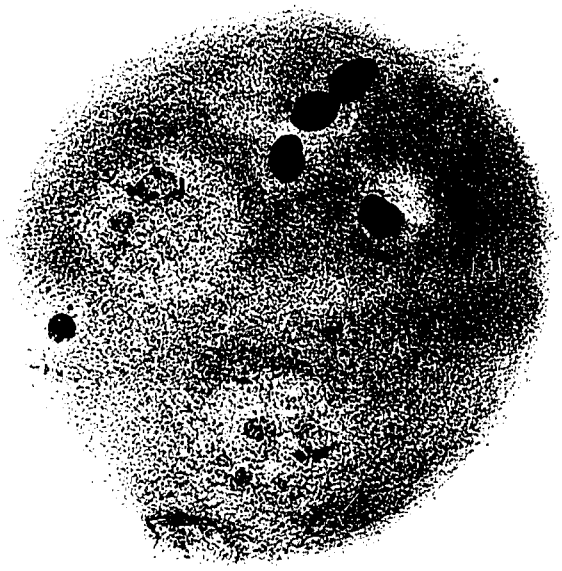
Small nucleus on the left of "a" is a polar body nucleus.

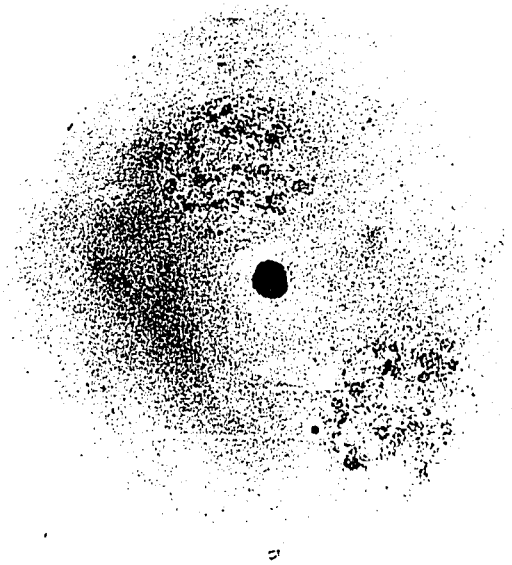
c. 400X.











74

PLATE 3

a. 400X.

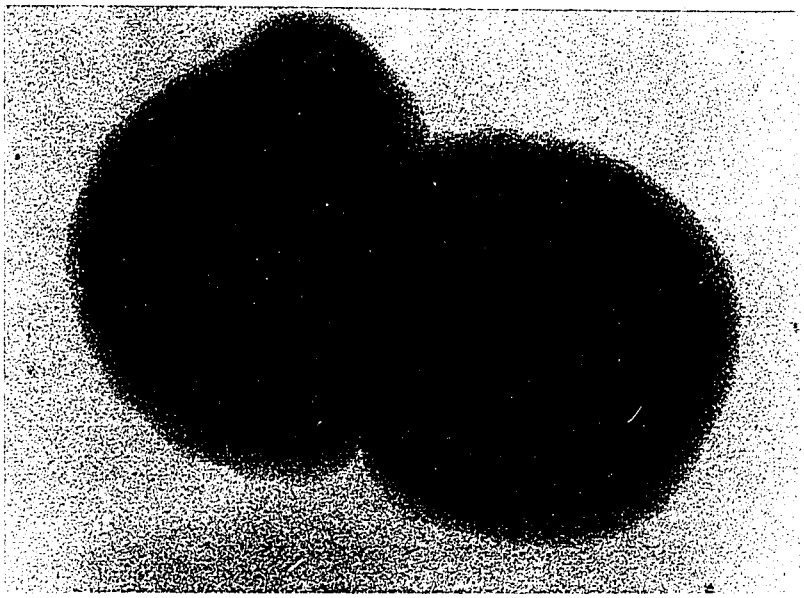
a & b. Two-cell/human diploid foreskin heterokaryons obtained 2 hours after fusion.

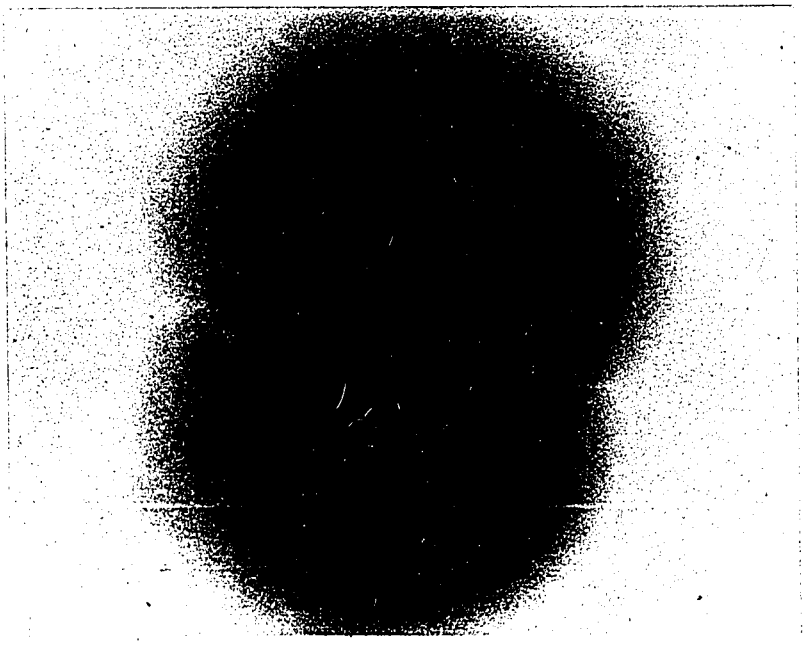
c. Human diploid foreskin cells before fusion.

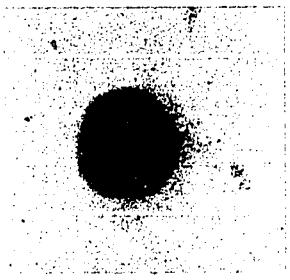
Note changes in human nuclei after fusion, particularily loss of heterochromatin.

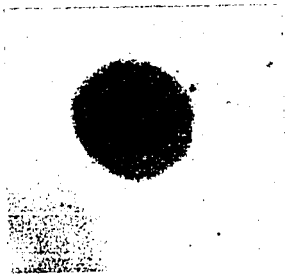
b. 400X.

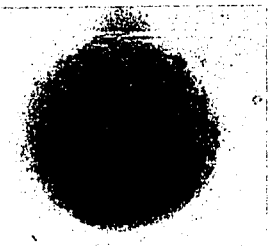
c. 400X.

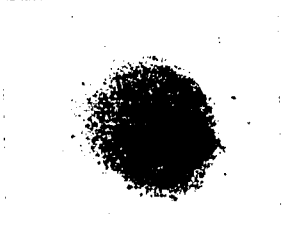












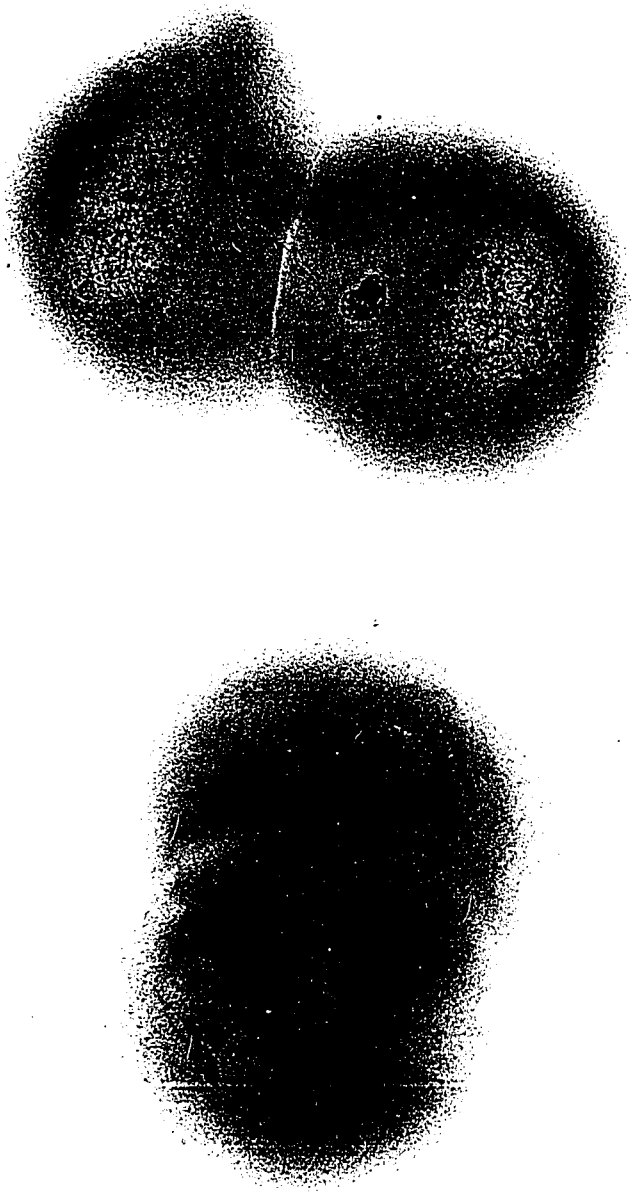


PLATE 4

a. 400X.

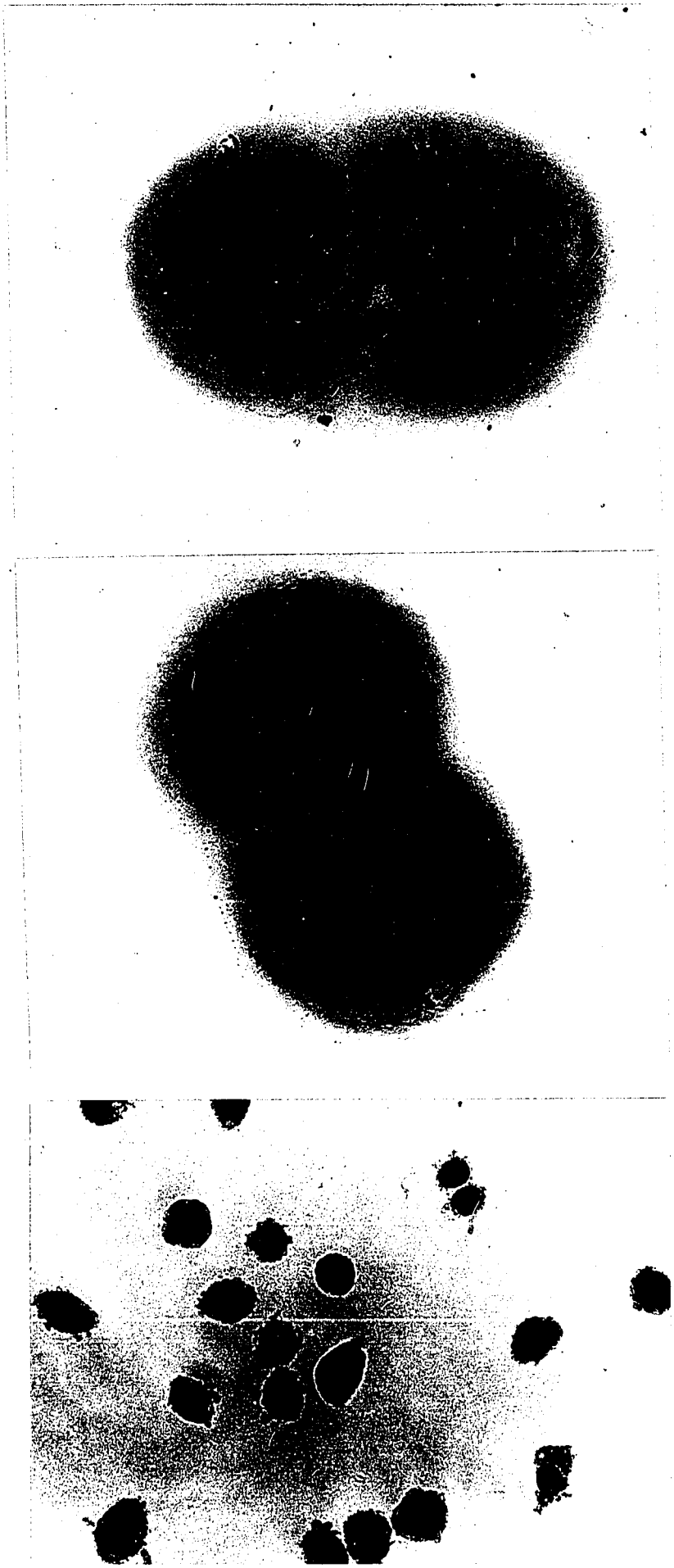
a & b. Two-cell/SVT2 heterokaryons obtained 2 hours after fusion.

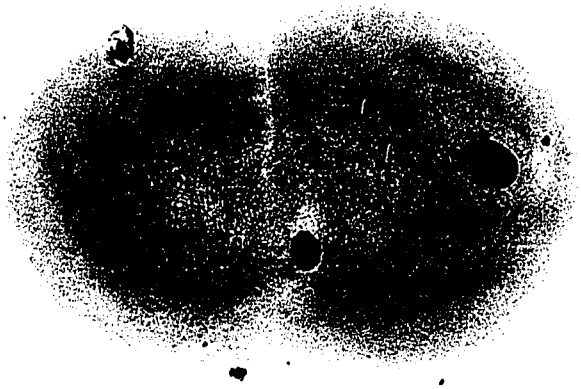
c. SVT2 cells before fusion.

Note changes in SVT2 nuclei of heterokaryons characterized by "loosening" of chromatin, and poorly defined nucleoli.

b. 400X.

c. 400X.





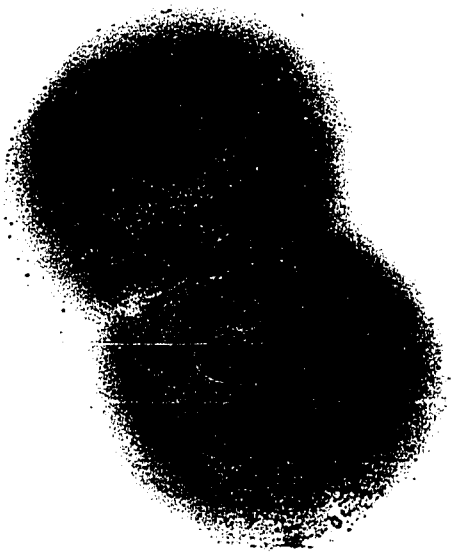


PLATE 5

a. 400X.

a & b. Two-cell/A31 heterokaryons obtained 2 hours after fusion.

c. A31 cells before fusion.

Note changes in A31 nuclei after fusion characterized by swelling, loss of nucleoli, and rearrangement of chromatin in a diffuse, embryonic manner.

b. 400X.

c. 400X.

PLATE 6

Autoradiogram of a 2-cell control embryo labelled with H3-uridine.

This embryo was obtained from a culture in which the zona pellucida was removed prior to labelling, but the culture was not exposed to virus. Labelling was performed using an H3-uridine concentration of 20μCi/ml (specific activity, 19.4 Ci/mM) with 750 fold excess unlabeled deoxycitidine for 2 hours. Exposure time of autoradiograms in the dark was 96 hours.

640X.

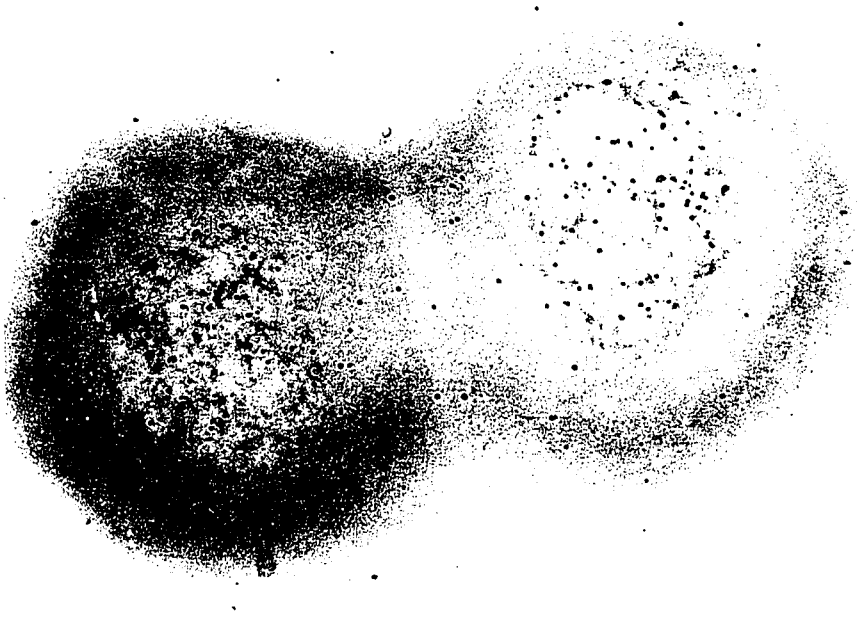


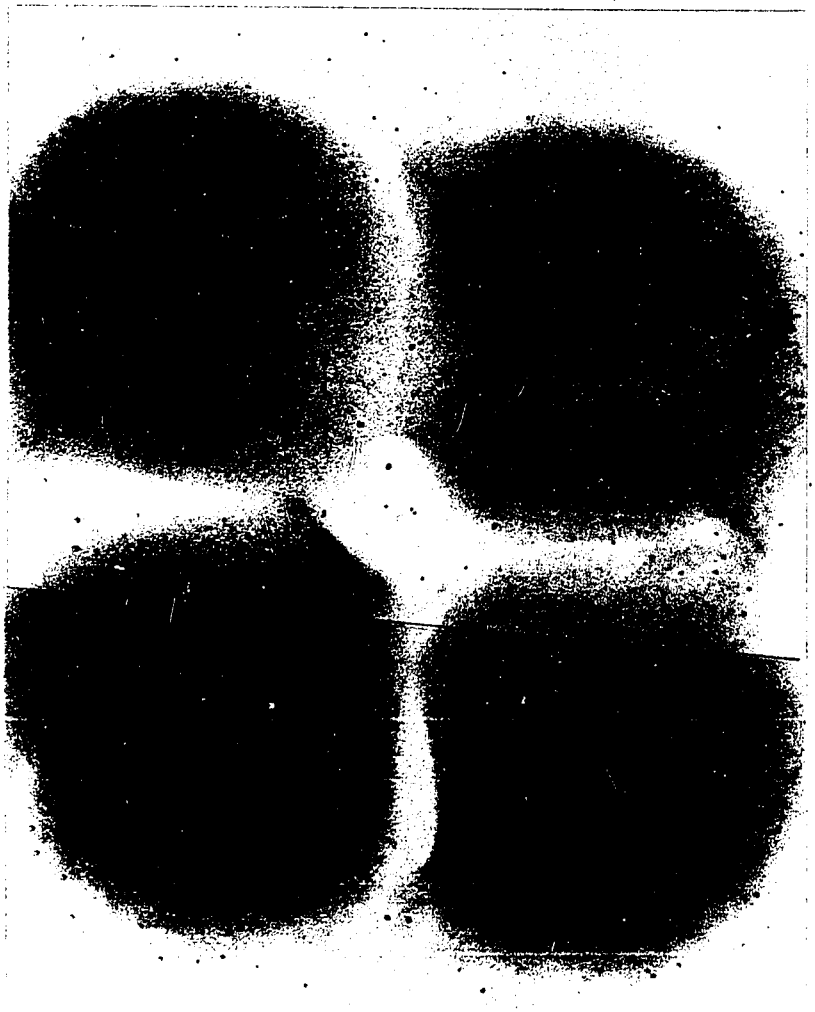
PLATE 7

Autoradiogram of a 4-cell control embryo labelled with H3-uridine.

640X.

The zona pellucida was removed prior to labelling but the embryo was not exposed to virus. Details of labelling and autoradiography are the same as for plate 6.

Note the level of labelling and the nucleolar concentration of grains.



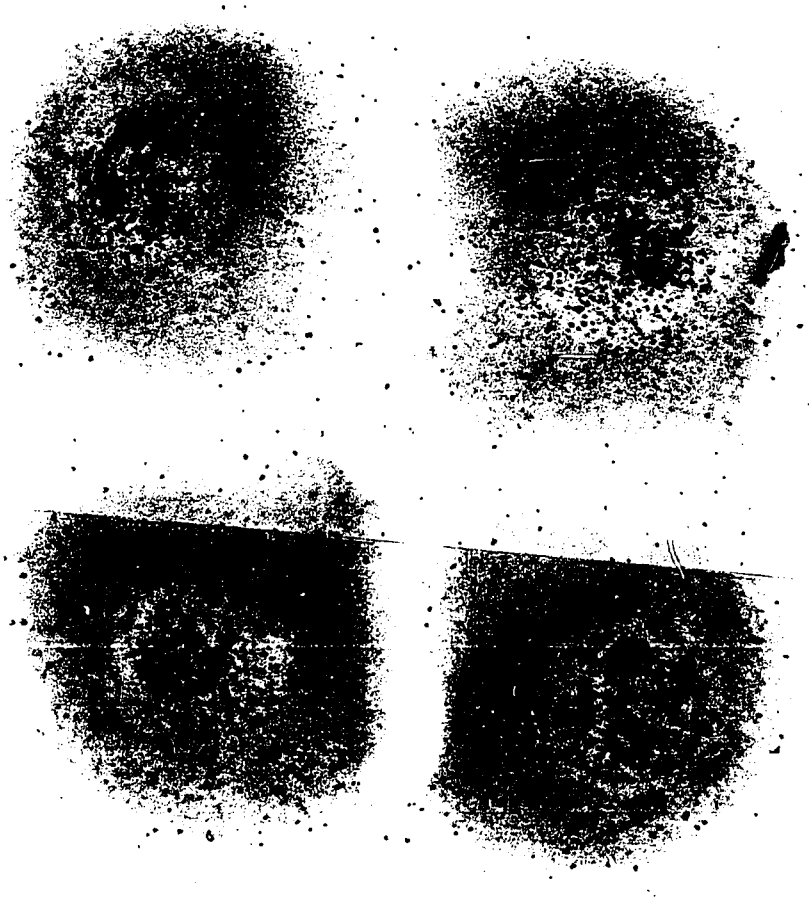


PLATE 8

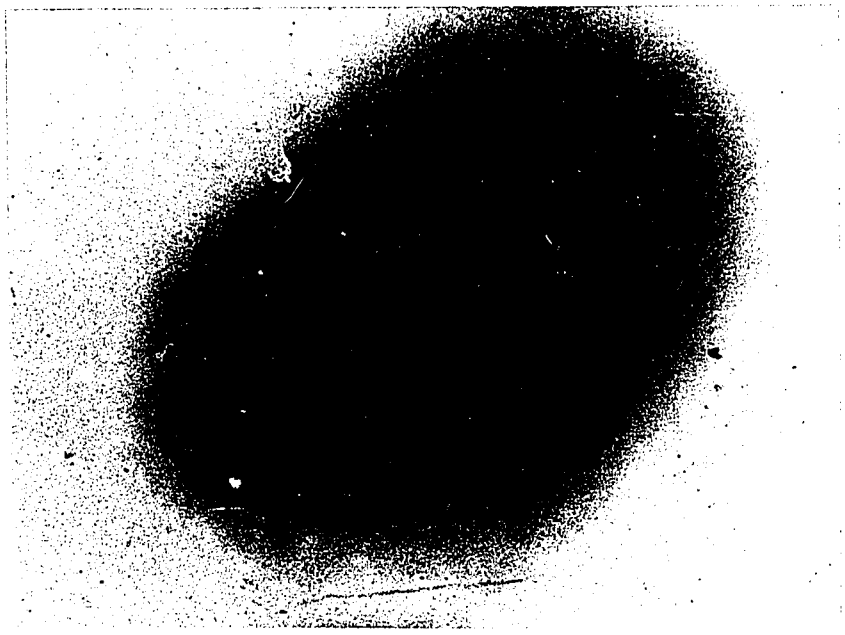
a. 400X

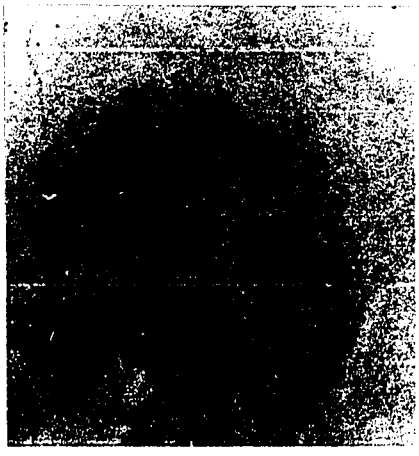
a. Autoradiogram of virus treated 2-cell control embryo labelled with H3-uridine.

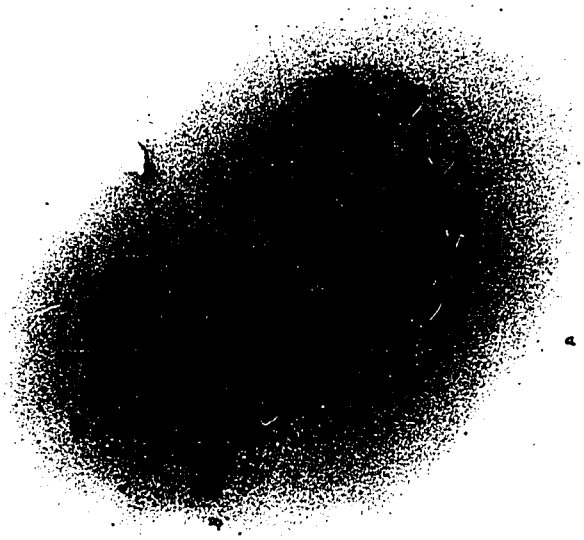
b. Enlargement of nuclei of "a".

See explanation plate 9.

b. 640X.







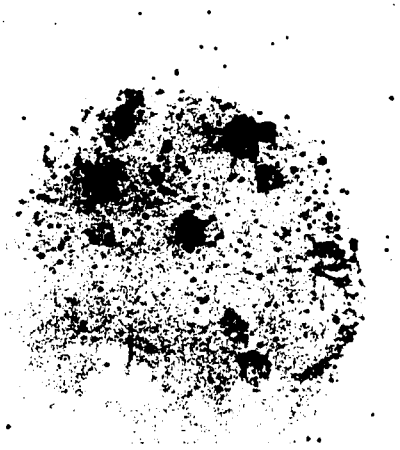


PLATE 9

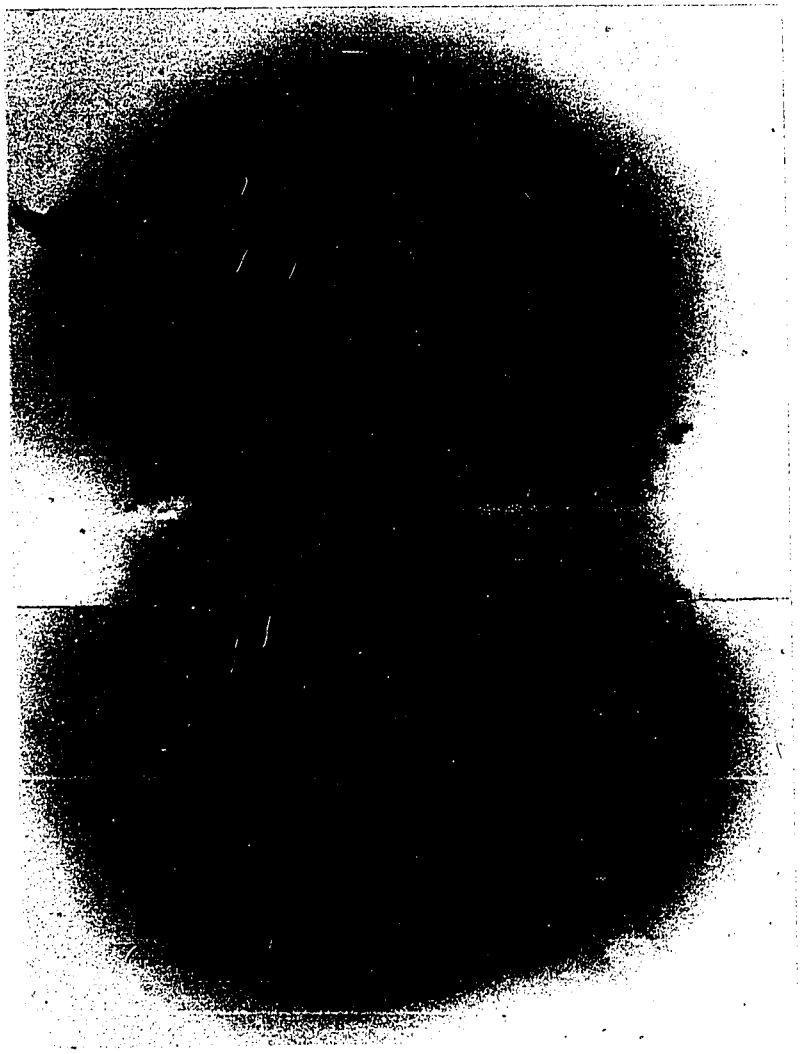
Autoradiogram of virus treated 2-cell control embryo labelled with H3-uridine.

Plates 8 and 9. Details of labelling and autoradiography are the same as for plate 6.

640X.

These embryos were obtained from experiments designed to determine RNA synthesis in 2-cell/A9 heterokaryons. They were treated with virus but did not fuse with A9 cells. From 30 to 70% of the embryos in the same cultures did fuse with A9 cells. Note the variability in the number of grains over the different nuclei.

Control embryos obtained in the same way from experiments using human diploid or SVT2 cells had similar mean numbers of of grains and similar variability.



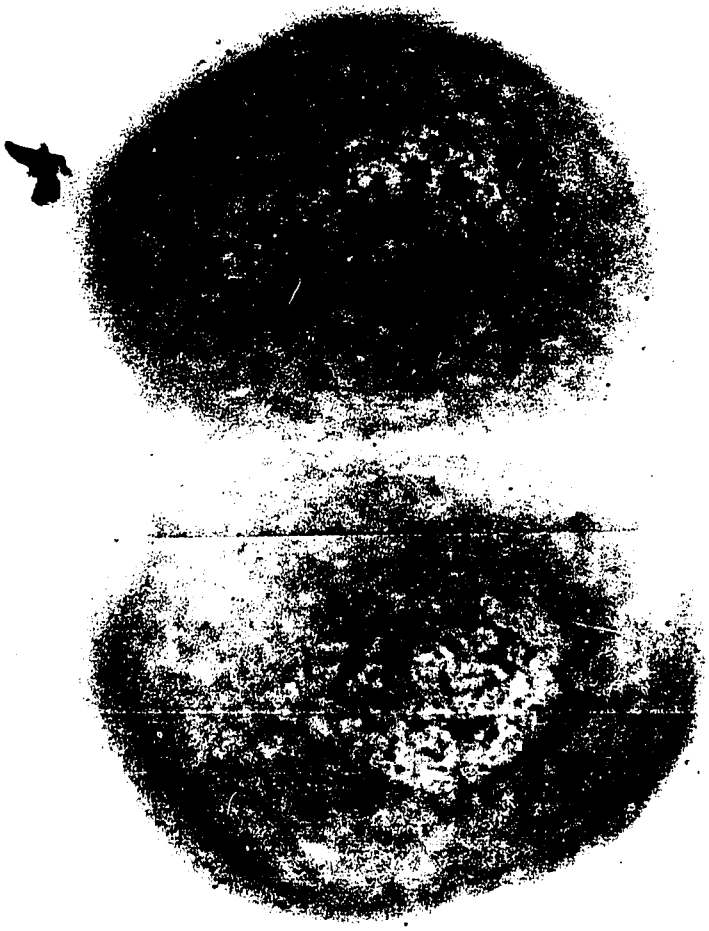


PLATE 10

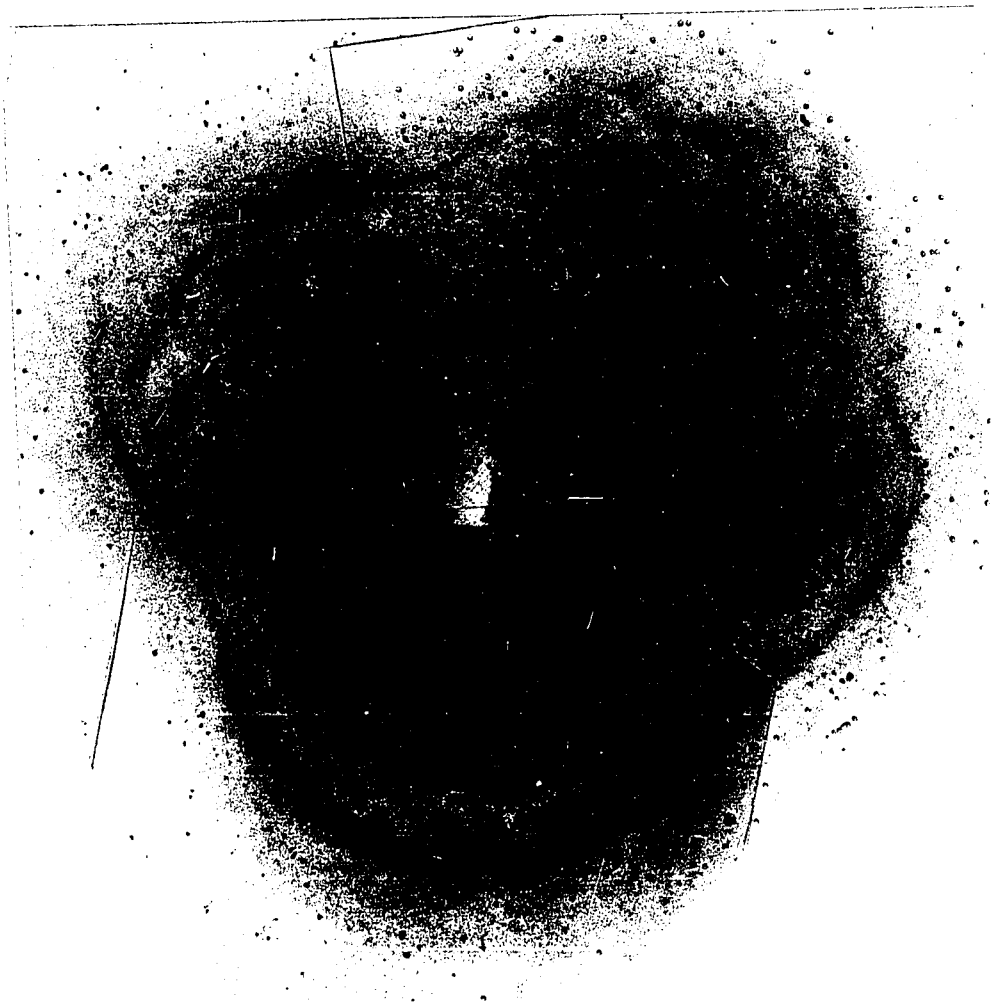
Autoradiogram of virus treated 4-cell control embryo labelled with H3-uridine.

Plates 10 and 11. Details of labelling and autoradiography are the same as for plate 6.

640X.

These embryos were obtained from experiments designed to determine RNA synthesis in 4-cell/A9 heterokaryons. They were treated with virus but did not fuse with A9 cells. From 30 to 70% of the embryos in the same cultures did fuse with A9 cells. Note the variability in the number of grains over the different nuclei.

Control embryos obtained in the same way from experiments using human diploid cells had similar mean numbers of grains and similar variability.



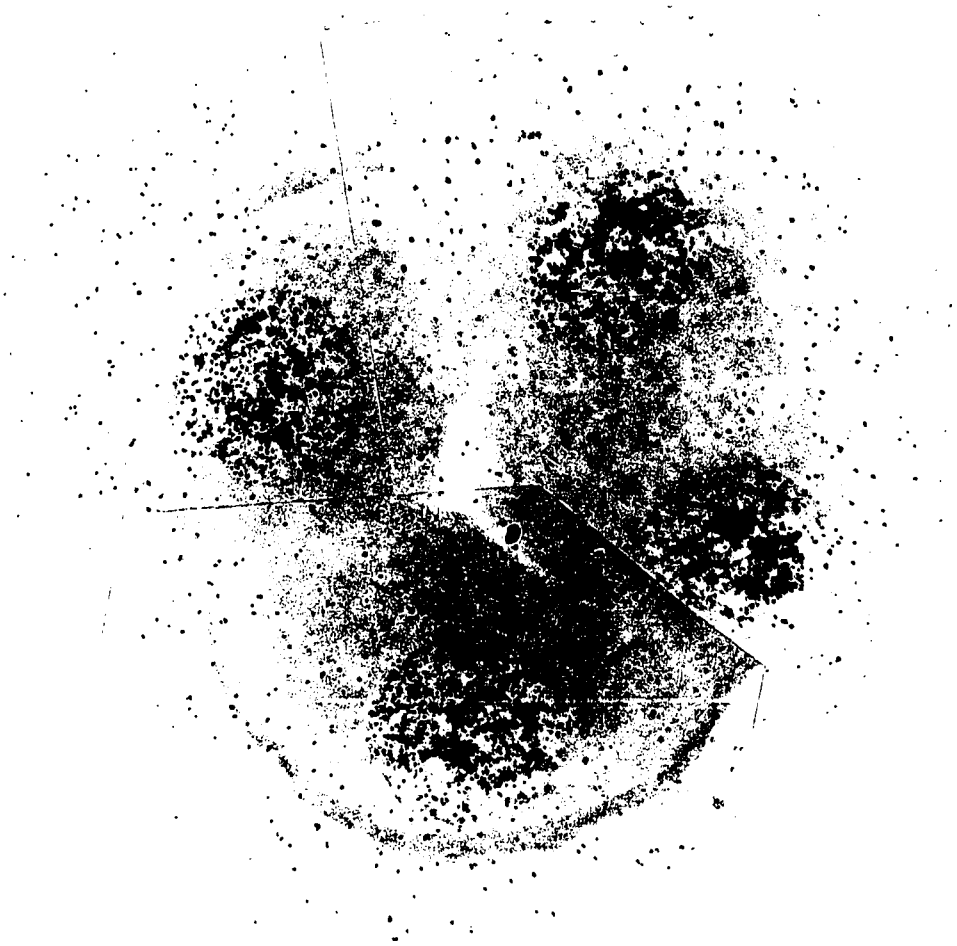
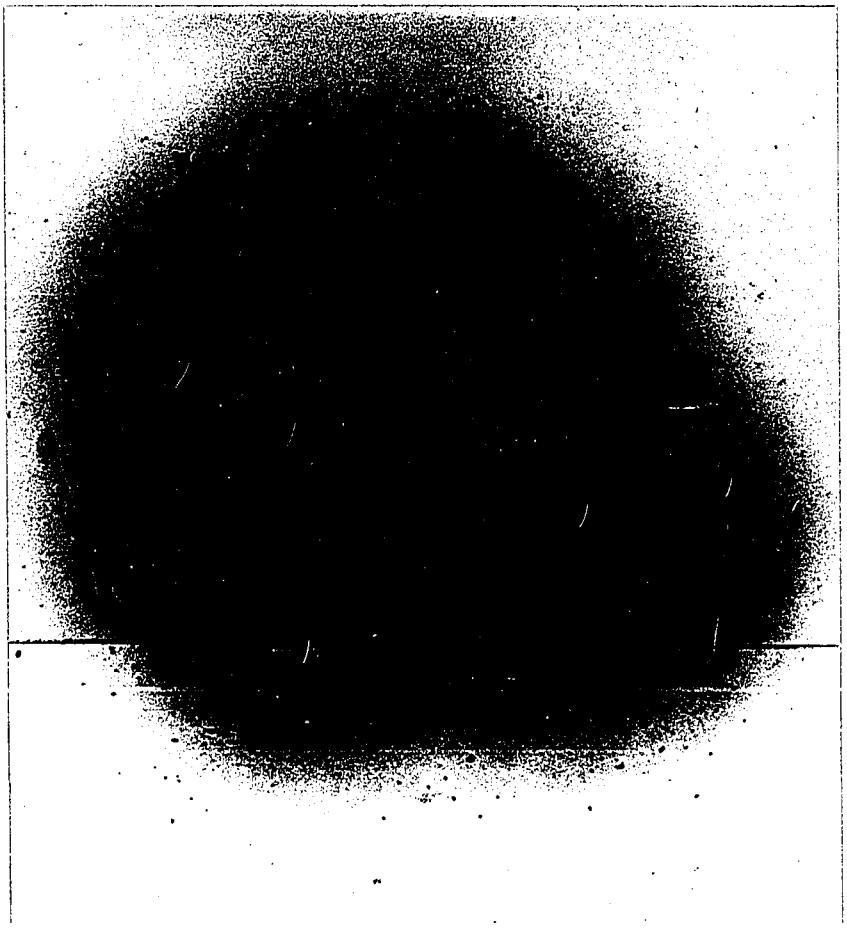


PLATE 11

Autoradiogram of virus treated 4-cell control embryo labelled with H3-uridine.

See explanation plate 10. 640X.



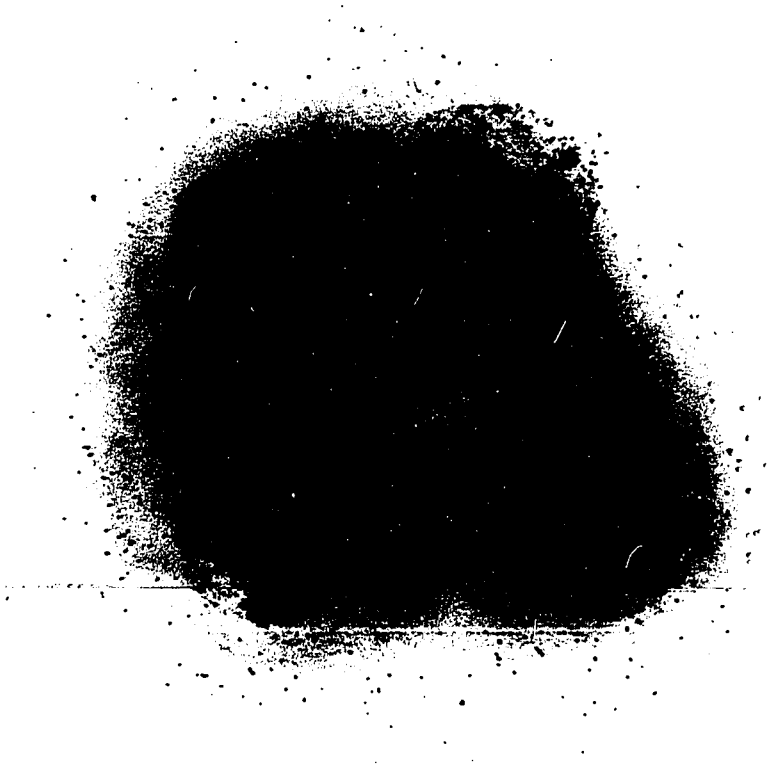


PLATE 12

Autoradiogram of 2-cell/A9 heterokaryons labelled with H3-uridine. Details of labelling and autoradiography are the same as for plate 6. 640X.

Note the virtual absence of grains over the A9 nuclei and the small number of grains over the embryo nuclei.

PLATE 13

a. 400X.

a. Autoradiogram of 2-cell/A9 heterokaryon labelled with H3-uridine.

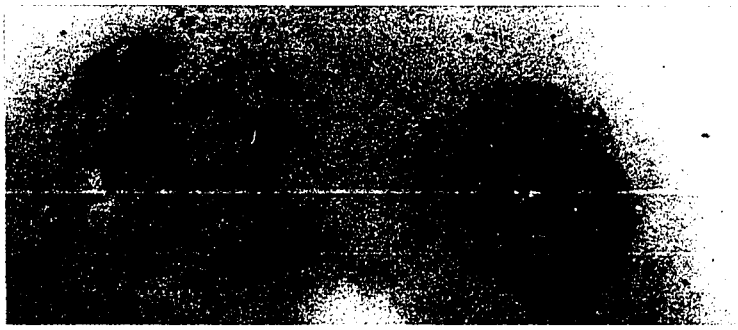
b. Enlargement of embryo nuclei of "a".

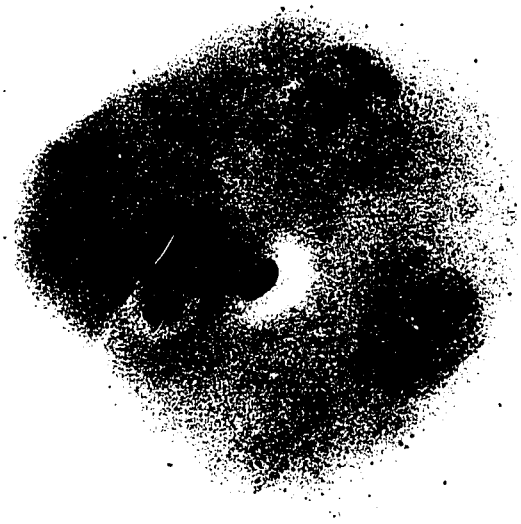
c. Enlargement of A9 nuclei of "a".

Details of labelling and autoradiography are the same as for plate 6.
Note levels of RNA synthesis.

b. 640X.

c. 1600X.





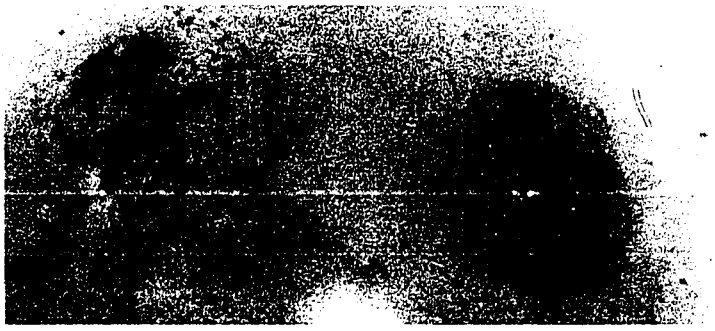


PLATE 14

a. 400X.

a. Autoradiogram of 2-cell/A9 heterokaryon labelled with H3-uridine.

b. Enlargement of embryo nucleus of "a".

c. Enlargement of A9 nuclei of "a".

b. 640X.

Details of labelling and autoradiography are the same as for plate 6.

Note the levels of RNA synthesis, and that grains are scattered randomly throughout the nucleoplasms.

c. 1600X.

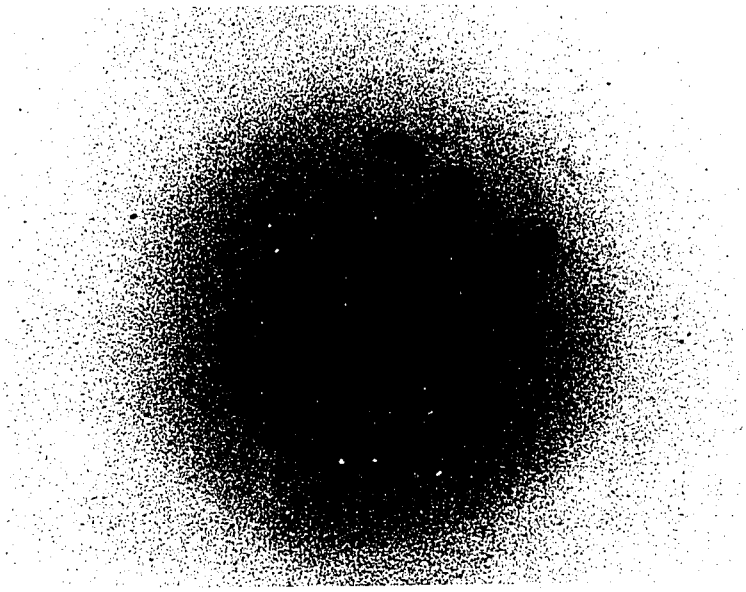


PLATE 15

a. 400X.

a. Autoradiogram of 2-cell/A9 heterokaryon labelled with H3-uridine.

b. Enlargement of nuclei of "a".

Note levels of RNA synthesis.

b. 640X.

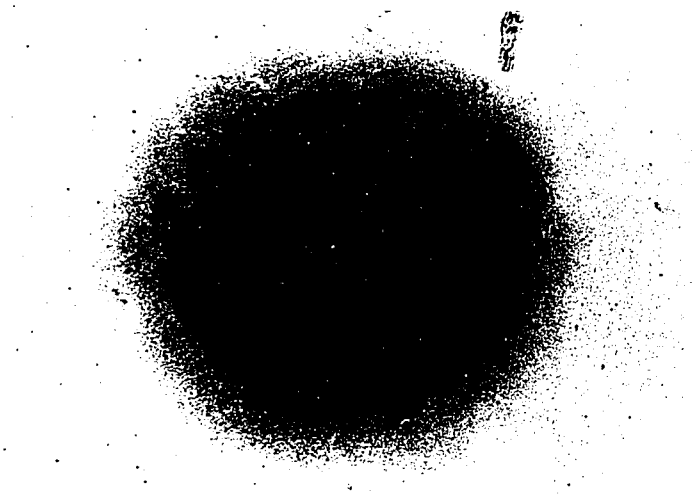


PLATE 16

Autoradiogram of 4-cell/A9 heterokaryons labelled with H3-uridine.

Details of labelling and autoradiography are the same as for plate 6.
Note heavy synthesis and cytoplasmic label.

640X.

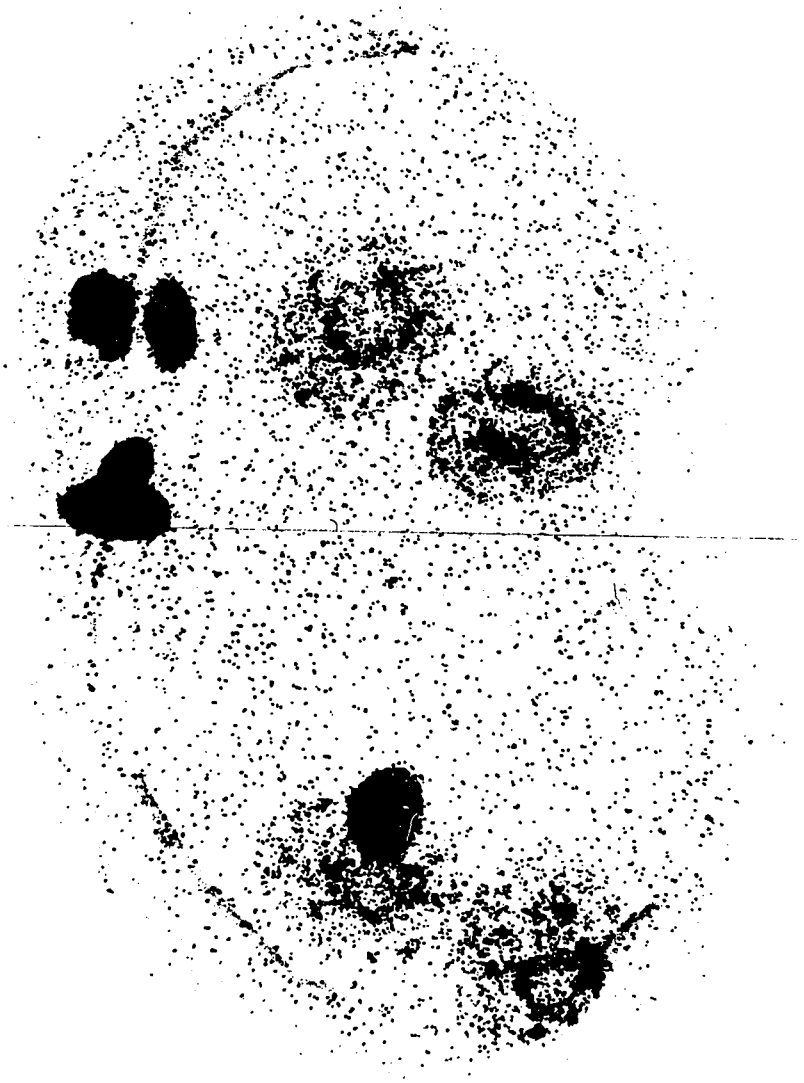


PLATE 17

Autoradiogram of two 4-cell/A9 heterokaryons and two unfused 4-cell blastomeres labelled with H3-uridine.
Details of labelling and autoradiography are the same as for plate 6. 640X.

Note that one of the unfused blastomeres is in mitosis.

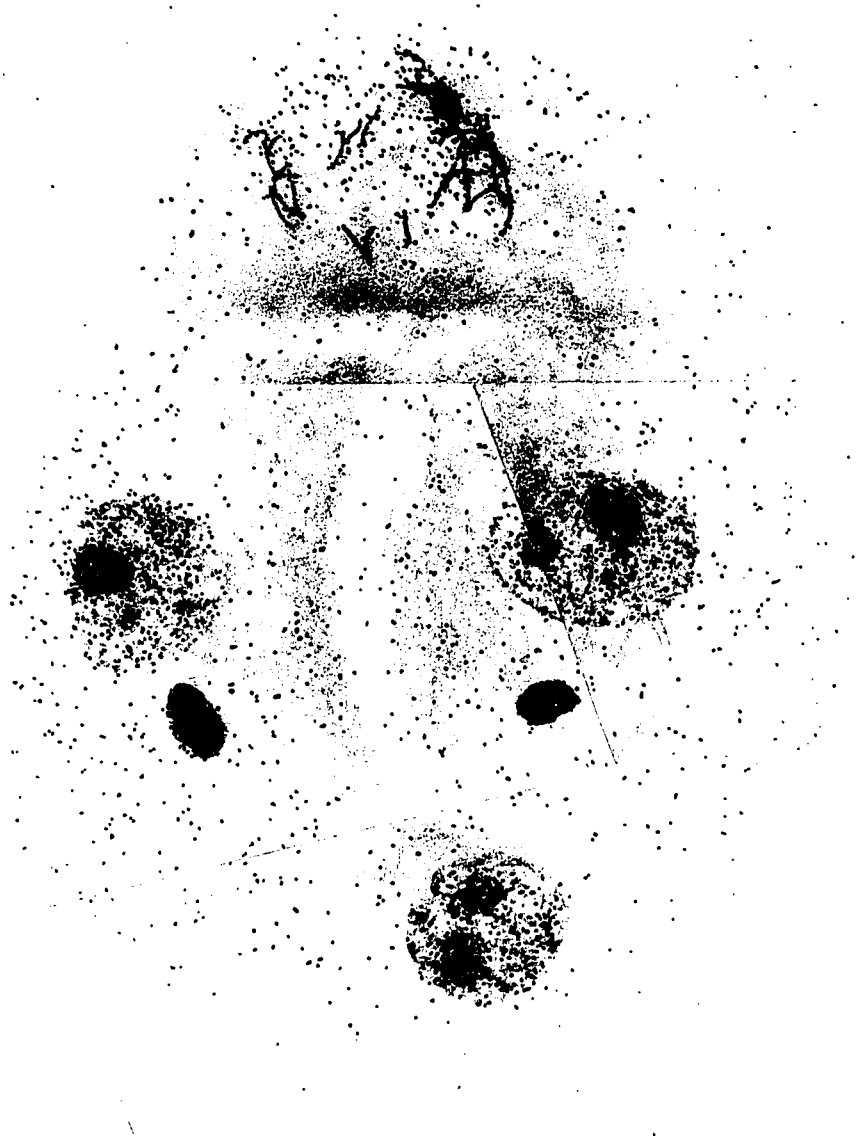


PLATE 18

Autoradiogram of a 4-cell/A9 heterokaryon and an unfused 4-cell blastomere labelled with H3-uridine. 640X.

Details of labelling and autoradiography are the same as for plate 6.

Note levels of RNA synthesis.

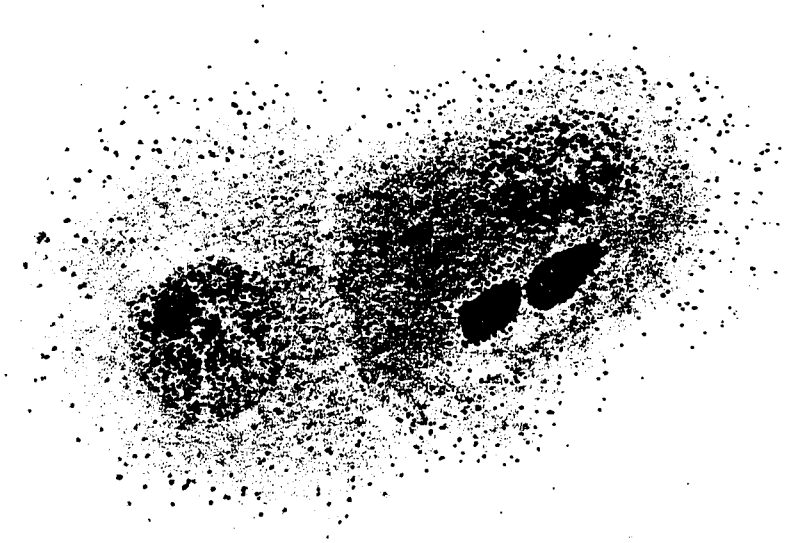


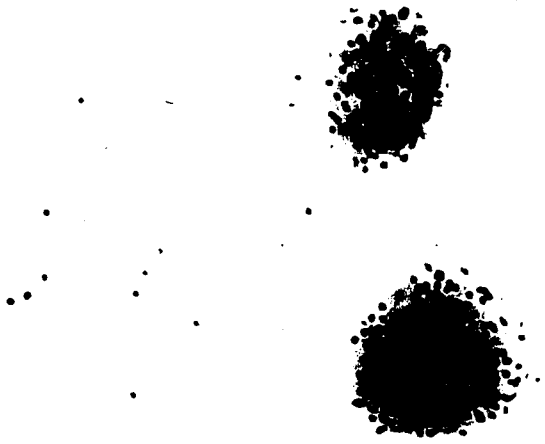
PLATE 19

a. Autoradiogram of two A9 nuclei of an exceptionaly heavily labelled 2-cell/A9 heterokaryon. Details of labelling and autoradiography are the same as for plate 6.
Note random scattering of grains throughout the nucleoplasms. There is no nucleolar concentration of grains.

a. 1600X.

b. Autoradiogram of an A9 nucleus from a relatively lightly labelled 4-cell/A9 heterokaryon. Details of labelling and autoradiography are the same as for plate 6.
Note non-random clustering of grains.

b. 1600X.



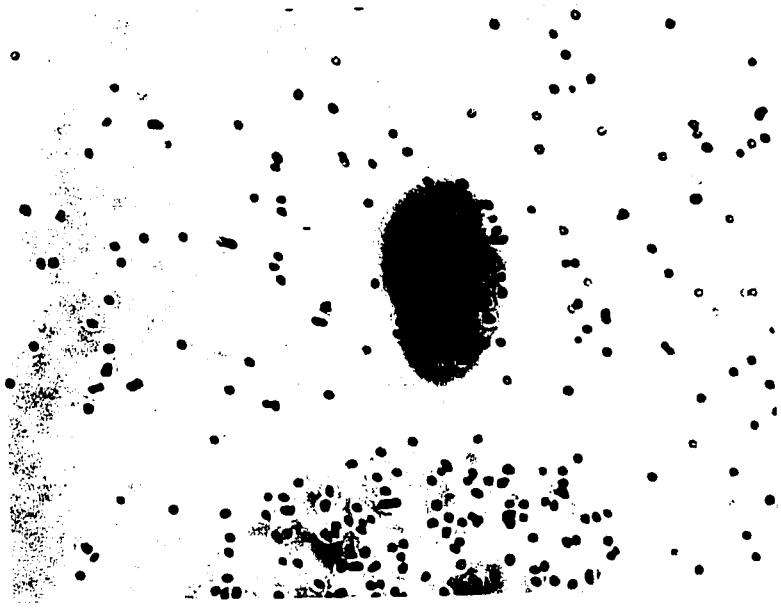


PLATE 20

a. Autoradiogram of A9 cells labelled with H3-uridine in A9 medium. Details of labelling and autoradiography are the same as for plate 6. Note that A9 cells normally synthesize large amounts of RNA.

a. 640X.

b. Autoradiogram of A9 cells labelled with H3-uridine in Mintz' medium. Details of labelling and autoradiography are the same as for plate 6. These cells were obtained from a culture in which some A9 cells had fused with embryos. Note that the experimental procedure did not alter the pattern of RNA synthesis demonstrated in "a".

b. 640X.

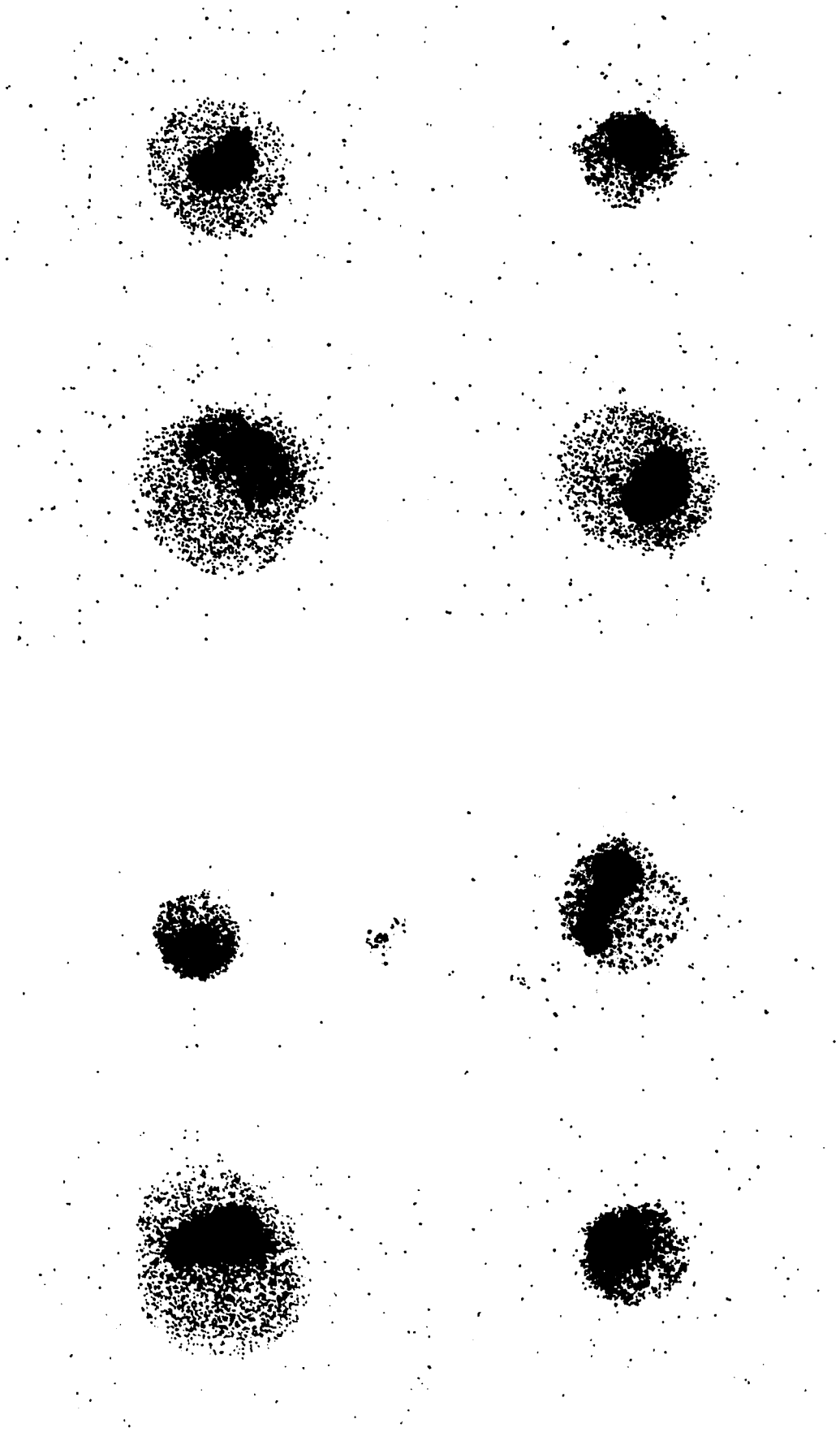


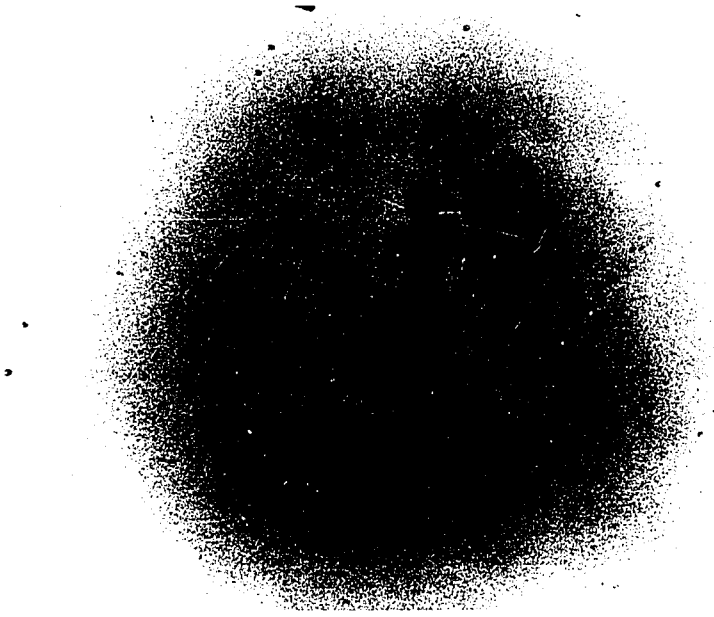
PLATE 21

a. 640X.

a & b. Autoradiograms of 2-cell/human diploid foreskin heterokaryons labelled with H3-uridine. Details of labelling and autoradiography are the same as for plate 6.

Note virtual absence of RNA synthesis by the human nuclei.

b. 640X.



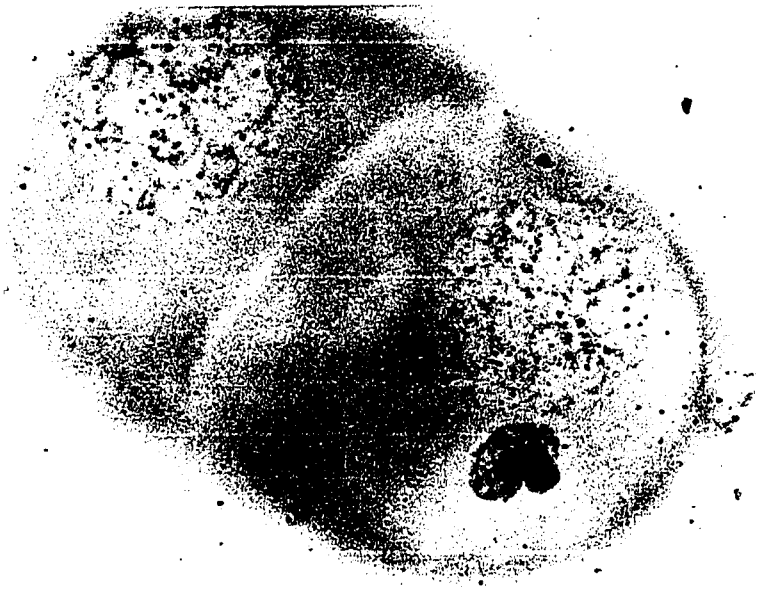


PLATE 22

a. Autoradiogram of 4-cell/human diploid foreskin heterokaryon labelled with H3-uridine. Details of labelling and autoradiography are the same as for plate 6. Two embryo nuclei are in the process of fusion.

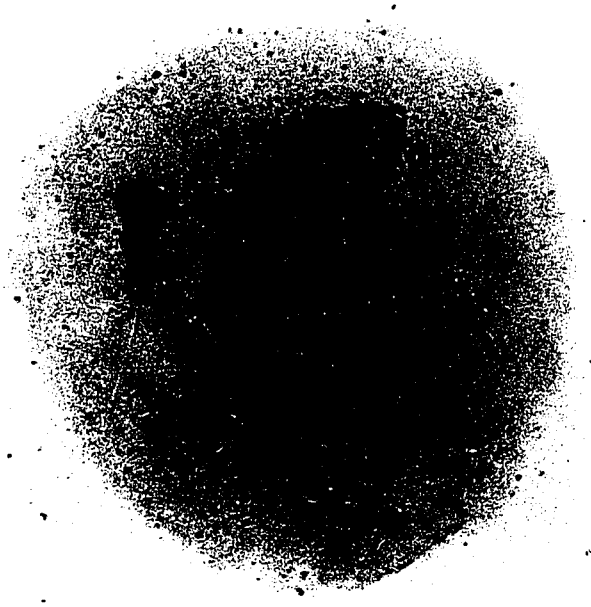
Note levels of RNA synthesis.

a. 640X.

b. Autoradiogram of 4-cell/human diploid foreskin heterokaryon labelled with H3-uridine. Details of labelling and autoradiography are the same as for plate 6.

Note levels of RNA synthesis.

b. 640X.



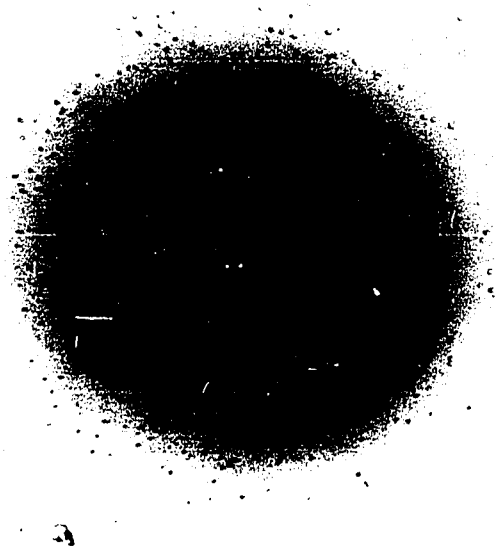


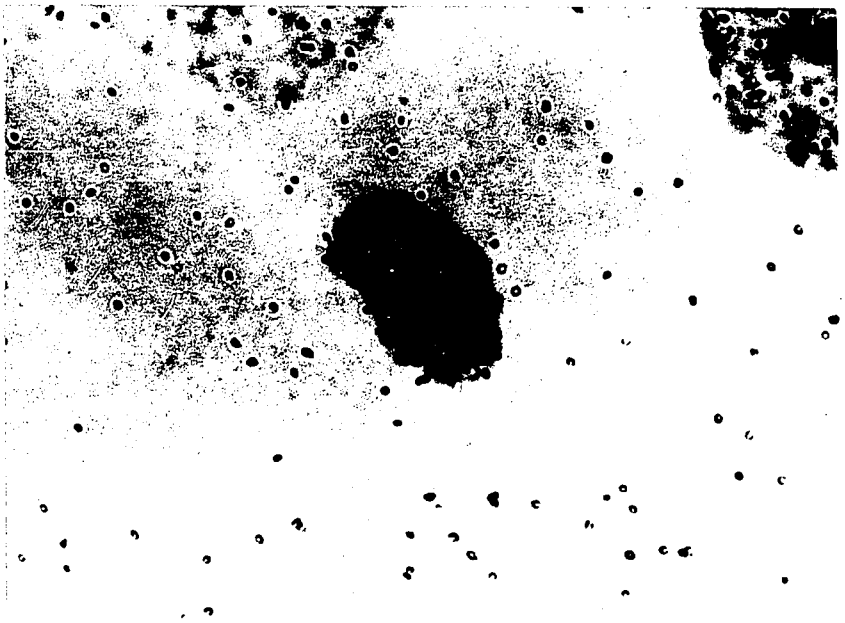
PLATE 23

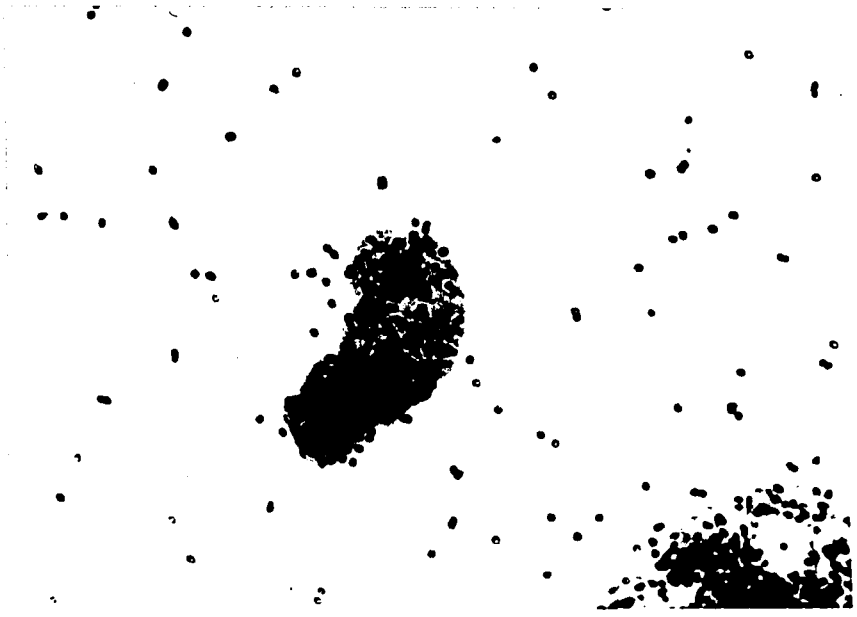
a. 1600X.

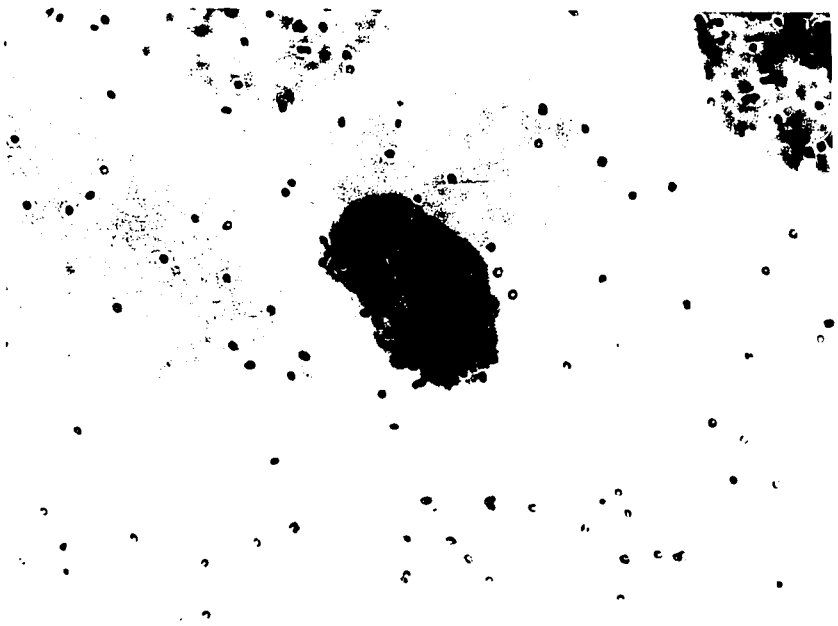
a & b. Autoradiograms of human nuclei in mouse 4-cell embryo cytoplasm. Details of labelling and autoradiography are the same as for plate 6.

Note that nucleolar label is evident, i.e. that human ribosomal RNA is being synthesized.

b. 1600X.







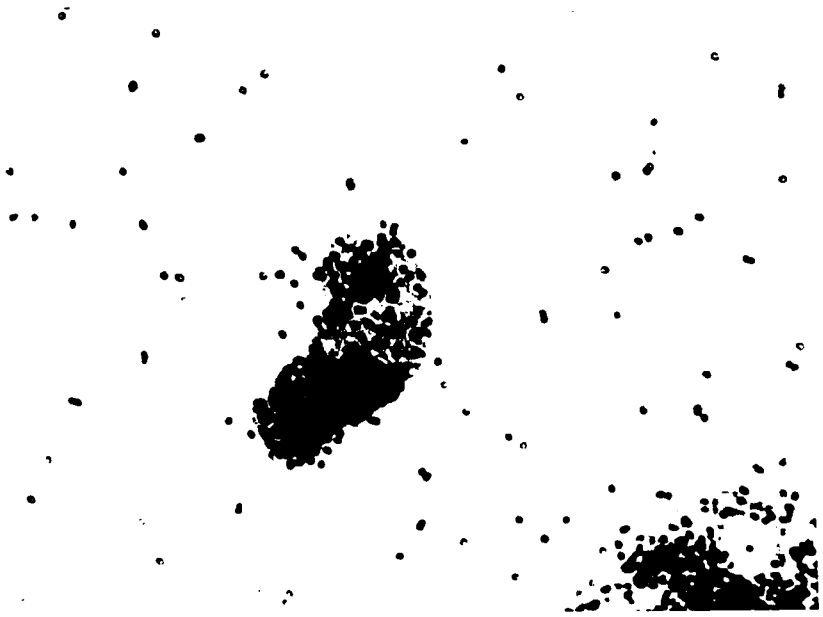


PLATE 24

Autoradiogram of human diploid foreskin cells labelled before fusion with H3-uridine. Details of labelling and autoradiography are the same as for plate 6.

640X.

Note that human diploid foreskin cells normally synthesize large amounts of RNA

This pattern of RNA synthesis was not altered by the experimental procedures.

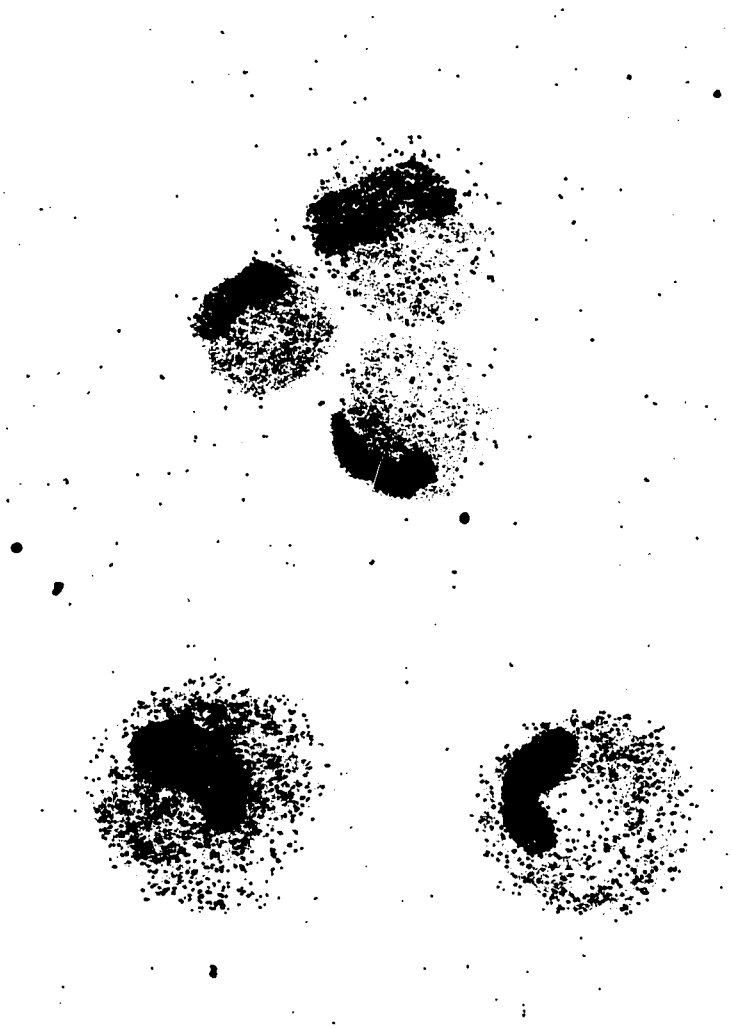


PLATE 25

Autoradiogram of 2-cell/SVT2 hetero-karyons labelled with H3-uridine. Details of labelling and autoradio-graphy are the same as for plate 6. 640X.

Note the virtual absence of RNA synthesis by the SVT2 nuclei.

PLATE 26

a. Autoradiogram of 2-cell/SVT2 heterokaryon labelled with H3-uridine. Details of labelling and autoradiography are the same as for plate 6.

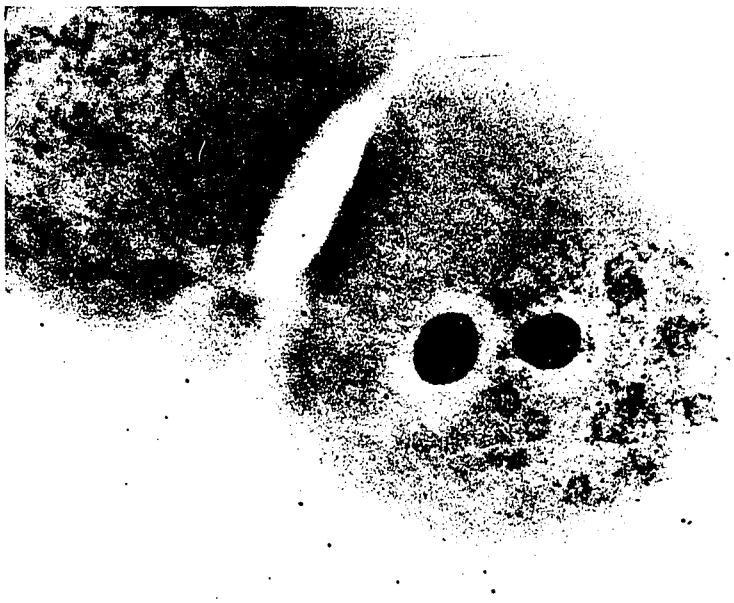
a. 640X.

Note the virtual absence of RNA synthesis by the SVT2 nuclei.

b. Autoradiogram of SVT2 cells labelled with H3-uridine before fusion. Details of labelling and autoradiography are the same as for plate 6.

b. 640X.

Note that SVT2 cells normally synthesize large amounts of RNA.



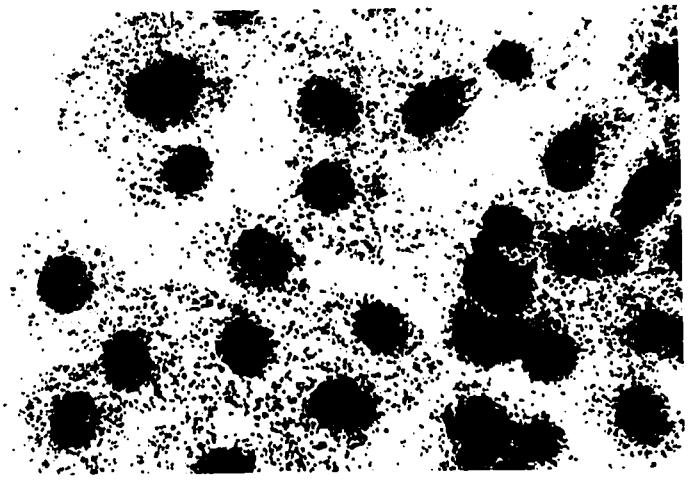


PLATE 27

Two-cell/A9 heterokaryon containing two A9 nuclei.

400X.

This cell was treated with 10µgm/ml actinomycin D for 2 hours. Note gross morphological abnormalities of both embryonic and A9 nuclei as compared with plate 2.

PLATE 28

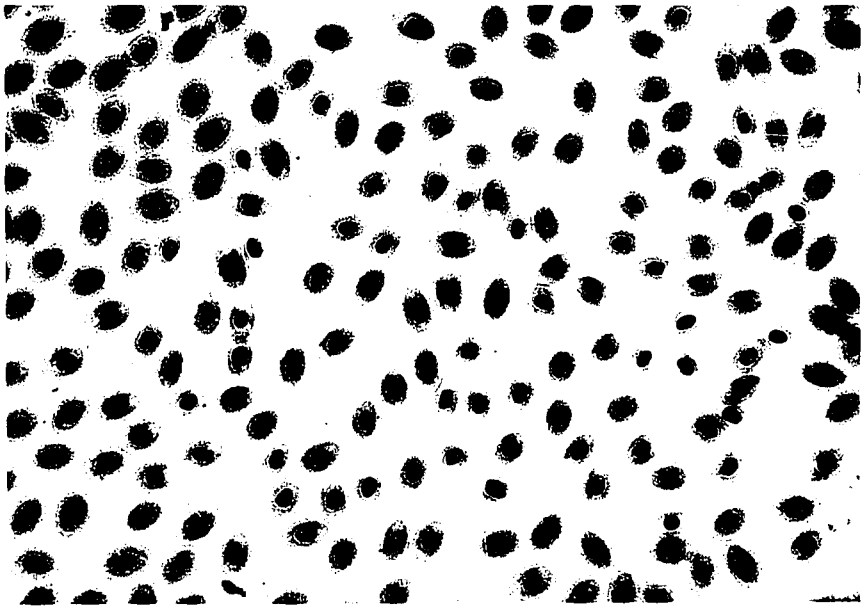
a. Smear of hen erythrocytes after purification by centrifugation. Only nucleated erythrocytes are present. Such a preparation is suitable for fusion studies.

a. 640X.

b. Autoradiogram of hen erythrocytes labelled with H3-uridine (50µCi/ml) for 2 hours. Exposure time of autoradiograms in the dark was 1 month. These cells were obtained from a population in which some erythrocytes had fused with embryos. Note that the experimental procedure did not stimulate RNA synthesis by any erythrocyte.

1600X.

Erythrocytes labelled in the same way before fusion did not synthesize any RNA.



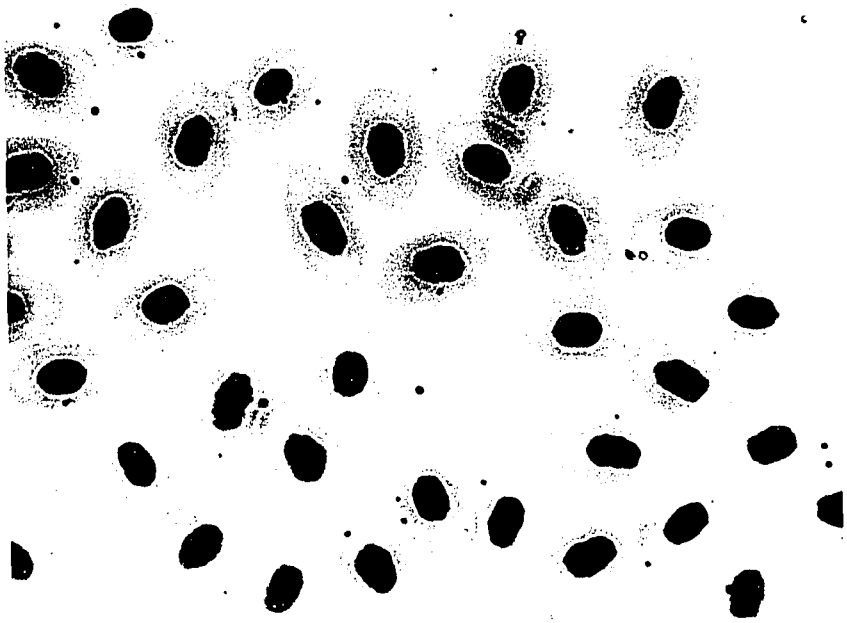


PLATE 29

a. Autoradiogram of 2-cell/hen erythrocyte heterokaryons labelled with H3-uridine. a. 400X.

b. Enlargement of some erythrocyte nuclei of "a".

Details of labelling and autoradiography are the same as for plate 28b. This embryo was obtained 2 hours after fusion (including labelling time). Note the incorporation of H3-uridine into RNA by 2 of the 5 erythrocyte nuclei.

b. 1600X.

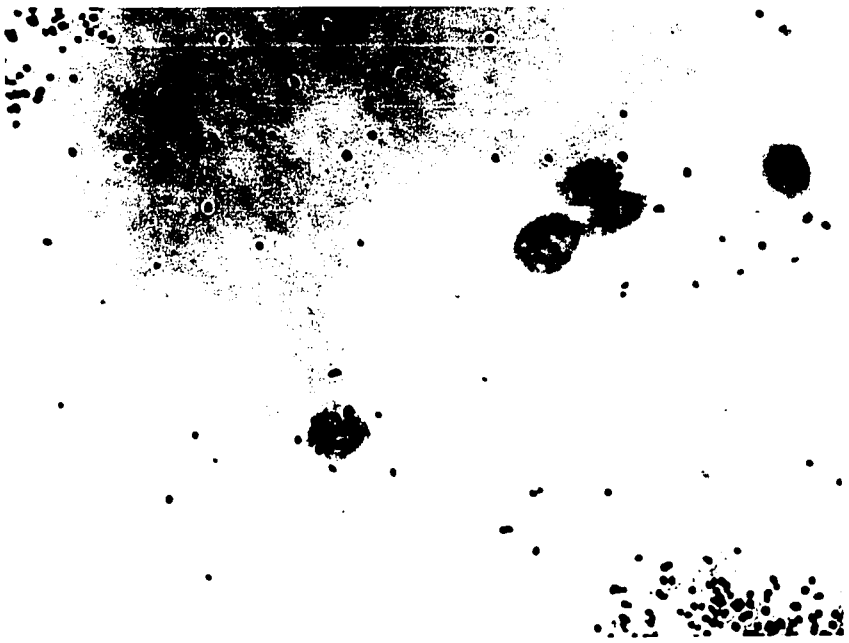


PLATE 30

a. Autoradiogram of 4-cell/hen erythrocyte heterokaryons labelled with H3-uridine. a. 400X.

b. Enlargement of some erythrocyte nuclei of "a".

These cells were labelled for 1 hour with H3-uridine (50μCi/ml) beginning 1 hour after fusion. Note RNA synthesis by one of the 5 erythrocyte nuclei. Note also the heavy incorporation by the embryo nuclei, and the presence of large amounts of cytoplasmic label.

b. 1600X.

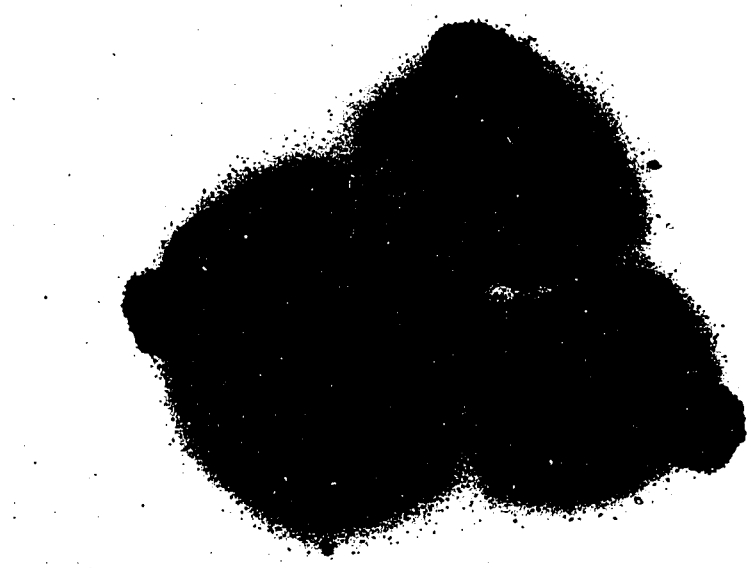


PLATE 31

a. Hen erythrocyte nucleus after 2 hours in 2-cell cytoplasm. Nucleus has not enlargrd.

a. 1600X.

b. Hen erythrocyte nucleus after 2 hours in 2-cell cytoplasm. Some enlargement has occurred.

b. 1600X.

c. Hen erythrocyte nucleus after 2 hours in 2-cell cytoplasm. Still greater enlargement has occurred.

c. 1600X.

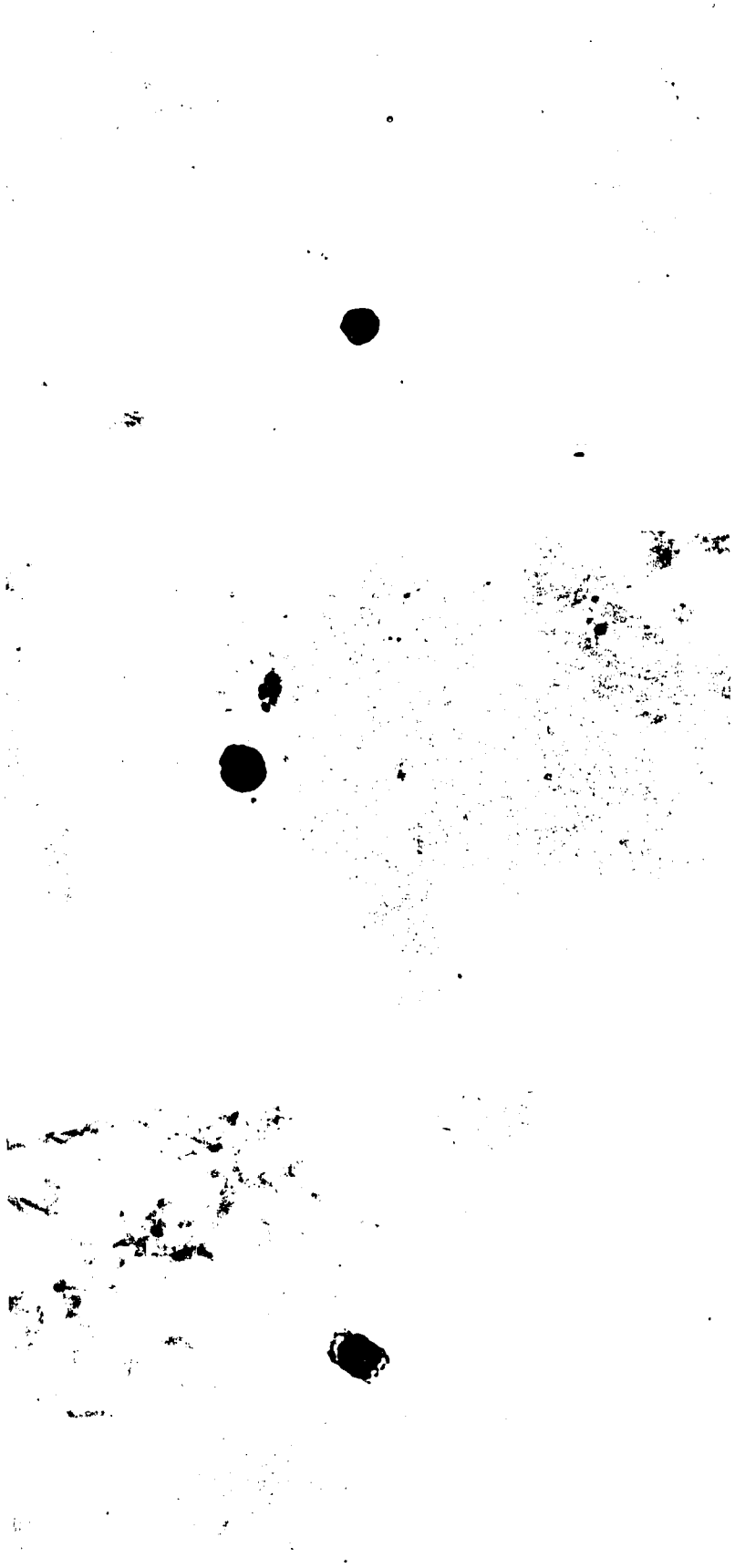


PLATE 32

a. Hen erythrocyte nuclei after 5 hours in 2-cell cytoplasm. Some nuclei are perpendicular to the plane of the photograph. Note nuclear enlargement greater than in plate 31c.

a. 1600X.

b. Hen erythrocyte nuclei after 11 hours in 2-cell cytoplasm (phase contrast). Compare nuclear enlargement with "a".

b. 1600X.

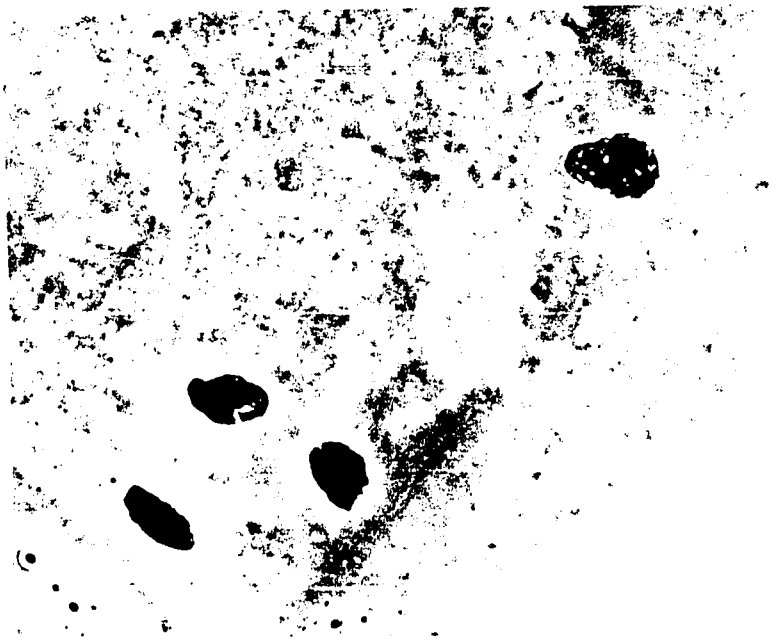


PLATE 33

Autoradiogram of a 2-cell/A9 heterokaryon. The A9 nucleus was labelled with H3-uridine immediately before fusion. This heterokaryon was harvested immediately after fusion. Note that very little RNA has left the A9 nucleus and entered the cytoplasm of the heterokaryon.

640X.

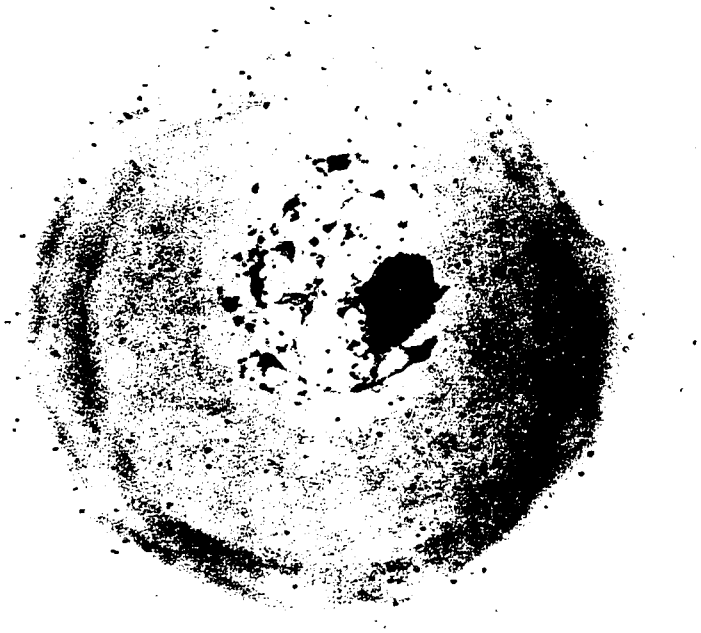


PLATE 34

a. 640X.

a, b & c. Autoradiograms of 2-cell/A9 heterokaryons. A9 nuclei were labelled with H3-uridine immediately before fusion and heterokaryons were harvested 2 hours after fusion. Note that much RNA has migrated from the A9 nuclei into the cytoplasms of the heterokaryons.

b. 640X.

c. 640X.

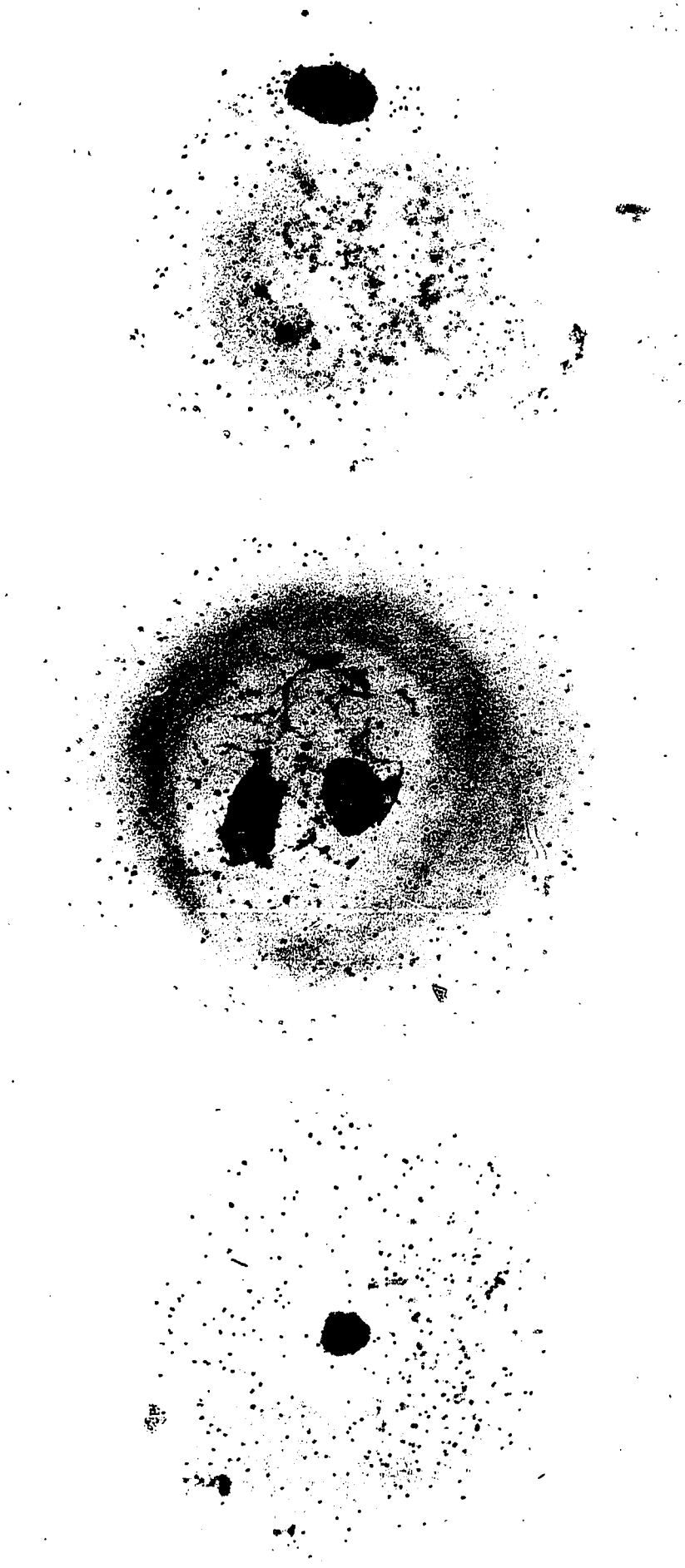


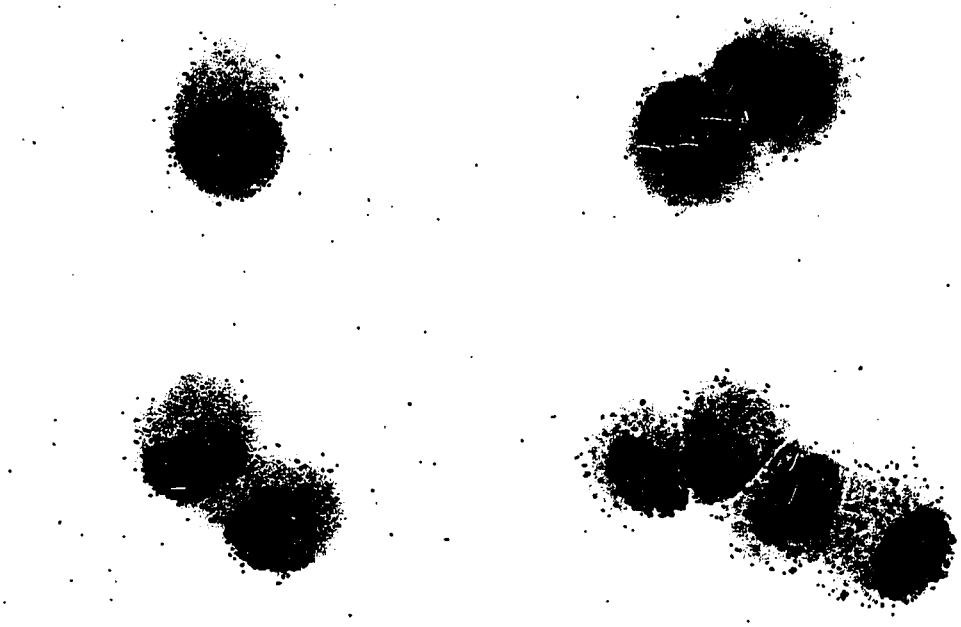
PLATE 35

a. Autoradiogram of A9 cells labelled with H3-uridine immediately prior to fusion. Note that very little labelled RNA is present in the cytoplasm, and that much of the labelled RNA is nucleolar.

a. 640X.

b. Autoradiogram of A9 cells from the same culture as "a". These cells participated in the fusion technique but did not fuse with embryos. They were harvested 2 hours after fusion. Note that much labelled RNA has migrated from nucleus to cytoplasm.

b. 640X.



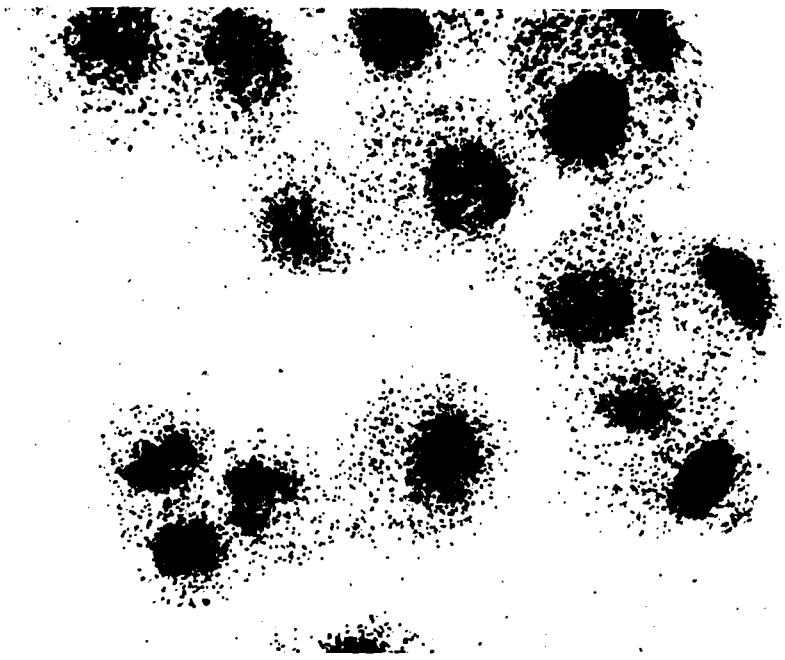


PLATE 36

Autoradiogram of A31 cells labelled with H3-thymidine. These cells were obtained from a contact-inhibited culture and were labelled for 2 hours beginning 2 hours after trypsinization of the culture. Most cells (97.5%) did not initiate DNA synthesis during this time.

640X.

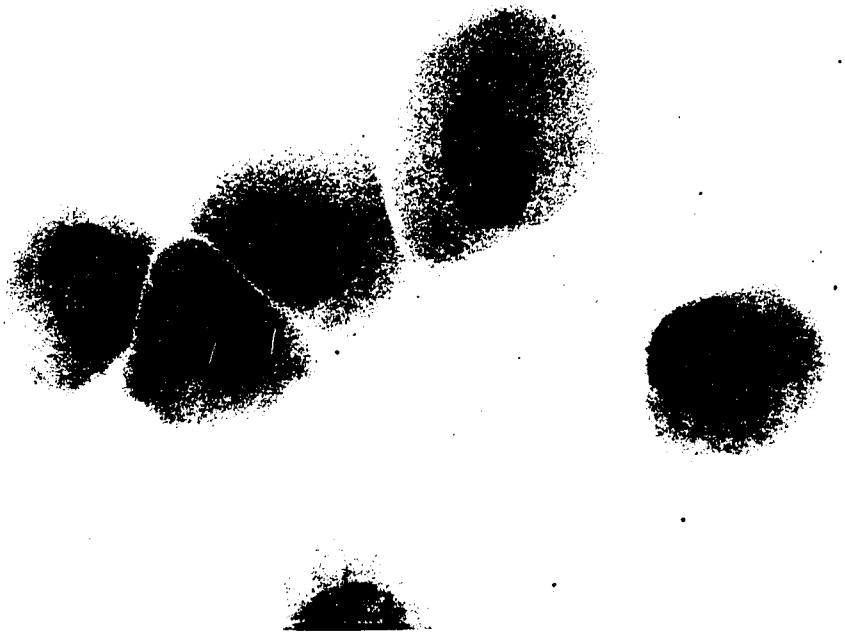


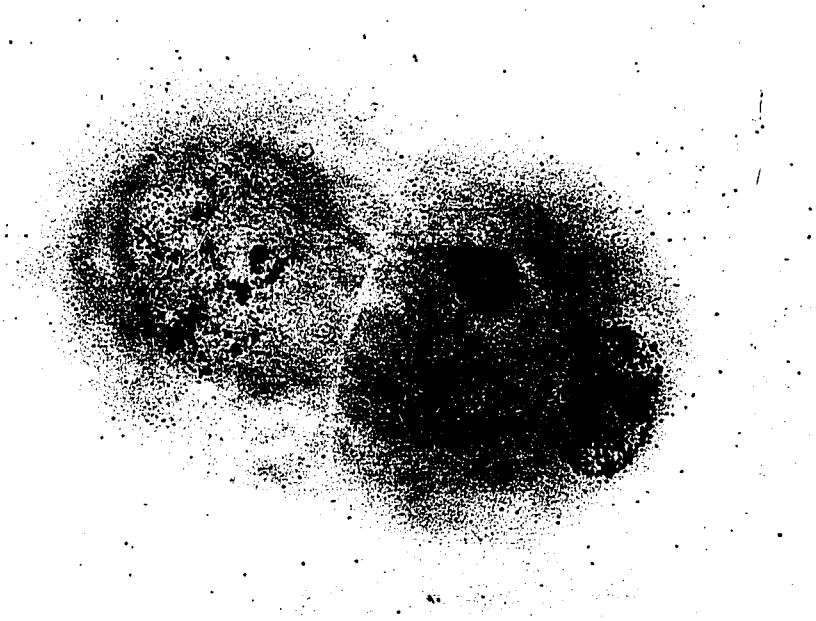
PLATE 37

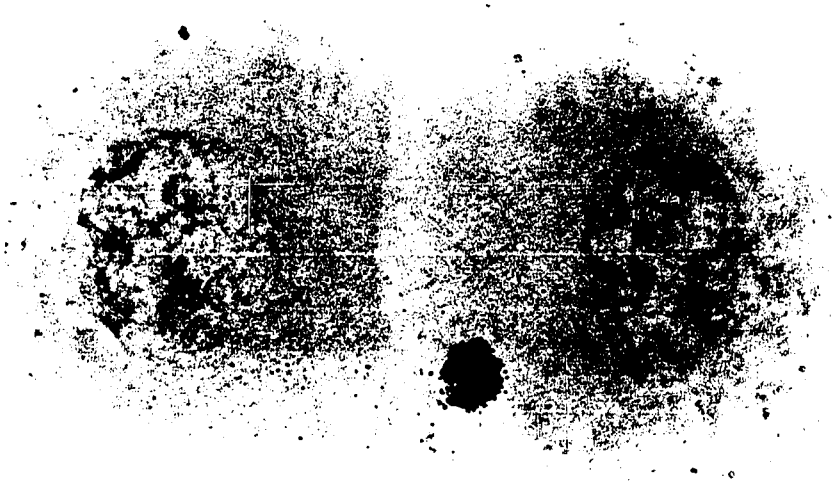
a. Autoradiogram of 2-cell/A31 heterokaryon labelled with H3-thymidine. The A31 nucleus was derived from the same culture as the cells in plate 36. The heterokaryon was labelled in the same way as in plate 36. DNA synthesis has begun in the A31 nucleus in the cytoplasm of a blastomere in the S period.

a. 400X.

b. Autoradiogram of a heterokaryon obtained in the same way as "a". DNA synthesis has commenced in the A31 nucleus in the cytoplasm of a blastomere in the G2 period.

b. 640X.





BIBLIOGRAPHY

ARONSON, A. I., and WILT, F. H. (1969). Properties of nuclear RNA in sea urchin embryos. Proc. Nat. Acad. Sci. 62: 186-193.

BACHVAROVA, R., DAVIDSON, E. H., ALLFREY, V. G., and MIRSKY, A. E. (1966). Activation of RNA synthesis associated with gastrulation. Proc. Nat. Acad. Sci. U.S. 55: 358-365.

BARANSKA, W., and KOPROWSKI, H. (1970). Fusion of unfertilized mouse eggs with somatic cells. J. Exp. Zool. 174: 1-4.

BRAMWELL, M. E., and HANDMAKER, S. D. (1971). Ribosomal RNA synthesis in human-mouse hybrid cells. Biochim. Biophys. Acta 232: 580-583.

BRAY, G. A., (1960). A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Bioch. 1:279-85.

BRINSTER, R. L. (1963). A method for in vitro cultivation of mouse ova from 2-cell to blastocyst. Exp. Cell Res. 32: 205-208.

BROWN, D. D. (1964). RNA synthesis during amphibian development. J. Exp. Zool. 157: 101-114.

BROWN, D. D., and GURDON, J. B. (1964). Absence of ribosomal RNA synthesis in the enucleolate mutant of Xenopus laevis. Proc. Nat. Acad. Sci. U.S. 51: 139-146.

BROWN, D. D., and LITTNA, E. (1964). RNA synthesis during the development of Xenopus laevis, the South African clawed toad. J. Mol. Biol. 8: 669-687.

BROWN, D. D., and LITTNA, E. (1966). Synthesis and accumulation of DNA-like RNA during embryogenesis of Xenopus laevis. J. Mol. Biol. 20: 81-84.

COMINGS, D. E. (1966). Incorporation of 3H-5-uridine into DNA. Exp. Cell Res. 41: 677-681.

COON, H. G., and WEISS, M. C. (1969). A quantitative comparison of formation of spontaneous and virus-produced viable hybrids. Proc. Nat. Acad. Sci. U.S. 62: 852-859.

CRIPPA, M., and GROSS, P. R. (1969). Maternal and embryonic contributions to the functional messenger RNA of early development. Proc. Nat. Acad. Sci. U.S. 62: 120-127.

DAENTL, D. L., and EPSTEIN, C. J. (1971). Developmental interrelationships of uridine uptake, nucleotide formation and incorporation into RNA by early mammalian embryos. Develop. Biol. 74: 428-442.

DARNELL, J. E. (1968). Ribonucleic acid from animal cells. Bacteriol. Rev. 32: 262-290.

DAVIDSON, E. H. (1968). Gene activity in early development. Academic Press, New York.

deVINCENTIIS, M., and LANCIERI II, M. (1970). Observations on the development of the sea urchin embryos in the presence of actinomycin. Exp. Cell Res. 59: 479-481.

DiBERARDINO, M. A., and KING, T. J. (1965). Transplantation of nuclei from the frog renal adenocarcinoma. II. Chromosomal and histologic analysis of tumor nuclear-transplant embryos. Develop. Biol. 11: 217-242.

ELICEIRI, G. L., and GREEN, H. (1969). Ribosomal RNA synthesis in human-mouse hybrid cells. J. Mol. Biol. 41: 253-260.

ELLEM, K.A.O., and GWATKIN, R.B.L. (1968). Patterns of nucleic acid synthesis in the early mouse embryo. Develop. Biol. 18: 311-330.

EMERSON Jr., C. P., and Humphreys, T. (1970). Regulation of DNA-like RNA and the apparent activation of ribosomal RNA synthesis in sea urchin embryos: quantitative measurements of newly synthesized RNA. Develop. Biol. 23: 86-112.

FRACCARO, M., HANSSON, K., HULTEN, M., LINDSTEN, L., and TIEPOLO, L. (1969). Heterochromatin in pre-implantation mouse embryos. Exp. Cell Res. 55: 427-430.

GAMOW, E. I., and PRESCOTT, D. M. (1970). The cell life cycle during early embryogenesis of the mouse. Exp. Cell Res. 59: 117-123.

GRAHAM, C. F. (1969). Fusion of cells with one- and 2-cell mouse embryos. Wistar Inst. Symp. Monogr. 9: 19-35.

GRAHAM, C. F., ARMS, K., and GURDON, J. B. (1966). The induction of DNA synthesis by frog egg cytoplasm. Develop. Biol. 14: 349-381.

GRIMES, W. J. (1970). Sialic acid transferases and sialic acid levels in normal and transformed cells. Biochem. 9: 5083-5092.

GROSS, P. R., and COUSINEAU, G. H. (1963). Effects of actinomycin D on macromolecule synthesis and early development in sea urchin eggs. Biochem. Biophys. Res. Comm. 10: 521-526.

GUGGENHEIN, M. A., TYRRELL, S., and RABSON, A. S. (1968). Studies on Sendai virus cell fusion factor. Proc. Soc. Exp. Biol. Med. 129: 854-857.

GUIDICE, G., and VINCENZO, M. (1969). Synthesis of ribosomal RNA during sea urchin development. II. Electrophoretic analysis of nuclear and cytoplasmic RNA's. Biochim. Biophys. Acta 197: 341-347.

GURDON, J. B. (1966). The cytoplasmic control of gene activity. Endeavour 75: 95-99.

GURDON, J. B. (1967). On the origin and persistance of a cytoplasmic state inducing nuclear DNA synthesis in frogs' eggs. Proc. Nat. Acad. Sci. U.S. 58: 545-552.

GURDON, J. B. (1968). Changes in somatic cell nuclei inserted into growing and maturing amphibian oocytes. J. Embryol. Exp. Morph. 20: 401-414.

GURDON, J. B., BIRNSTIEL, M. L., and SPEIGHT, V. A. (1969). The replication of purified DNA introduced into living egg cytoplasm. Biochim. Biophys. Acta 174: 614-628.

GURDON, J. B., and BROWN, D. D. (1965). Cytoplasmic regulation of RNA synthesis and nucleolus formation in developing embryos of Xenopus laevis. J. Mol. Biol. 12: 2'-35.

GURDON, J. B., and FORD, P. E. (1967). Attachment of rapidly labelled RNA to polysomes in the absence of ribosomal RNA synthesis during normal cell differentiation. Nature (London) 216: 666-668.

GURDON, J. B., and LASKEY, R. A. (1970). The transplantation of nuclei from single cultured cells into enucleat frogs eggs. J. Embryol. Exp. Morph. 24: 227-248.

GURDON, J. B., and WOODLAND, H. R. (1969). The influence of the cytoplasm on the nucleus during cell differentiation, with special reference to RNA synthesis during amphibian cleavage. Proc. Roy. Soc. 173: 99-111.

HARRIS, H. (1965a). Hybrid cells derived from mouse and man: artificial heterokaryons of mammalian cells from different species. Nature (London) 205: 640-646.

HARRIS, H. (1965b). Behavior of differentiated nuclei in heterokaryons of animal cells from different species. Nature 206: 583-588.

HARRIS, H. (1967). The reactivation of the red cell nucleus. J. Cell Sci. 2: 23-32.

HARRIS, H., MILLER, O. J., KLEIN, G., WORST, P., and TACHIBONA, T. (1969). Suppression of malignancy by cell fusion. Nature (London) 223: 363-368.

HARTMANN, J. F., and COMB, D. G. (1969). Transcription of nuclear and cytoplasmic genes during early development of sea urchin embryos. J. Mol. Biol. 41: 155-158.

HENNEN, S. (1963). Chromosomal and embryological analyses of nuclear changes in embryos derived from transfers of nuclei between Rana pipiens and Rana sylvatica. Develop. Biol. 6: 133-183.

HILLMAN, N., and TASCA, R. J. (1964). Ultrastructural and autoradiographic studies of mouse cleavage stages. Amer. J. Anat. 126: 151-174.

IVES, D. H., MORSE, P. A., and POTTER, V. R. (1963). Feedback inhibition of thymidine kinase by thymidine triphosphate. J. Biol. Chem. 238: 1467-1474.

KEDES, L. H., and GROSS, P. R. (1969a). Identification in cleaving embryos of three RNA species serving as templates for the synthesis of nuclear proteins. Nature (London) 223: 1335-1339.

KEDES, L. H., and GROSS, P. R., (1969b). Synthesis and function of messenger RNA during early embryonic development. J. Mol. Biol. 42: 559-575.

KIEFER, B. I., ENTELIS, C. F., and INFANTE, A. A. (1969). Mitotic abnormalities in sea urchin embryos exposed to dactinomycin. Proc. Nat. Acad. Sci. U.S. 64: 657-862.

KING, T. J., and DiBERARDINO, M. A. (1965). Transplantation of nuclei from the frog renal adenocarcinoma. I. Development of tumor nuclear-transplant embryos. Ann. N.Y. Acad. Sci. 126: 115-126.

LANDESMAN, R., and GROSS, P. R. (1968). Patterns of macromolecule synthesis during development of Xenopus laevis. I. Incorporation of radioactive precursors into dissociated embryos. Develop. Biol. 18: 571-589.

LANDESMAN, R., and GROSS, P. R. (1969). Patterns of macromolecule synthesis during development of Xenopus laevis. II. Identification of the 40s precursor to ribosomal RNA. Develop. Biol. 19: 244-260.

LASKEY, R. A., and GURDON, J. B. (1970). Genetic content of adult somatic cells tested by nuclear transplantation from cultured cells. Nature (London) 228: 1332-1334.

LEWIS, W. H., and WRIGHT, E. S. (1935). On the early development of the mouse egg. Contributions to embryology. Carnegie institute of Washington 25: 115-144.

LITTLEFIELD, J. W. (1966). The use of drug resistant markers to study the hybridization of mouse fibroblasts. Exp. Cell Res. 41: 190-196.

MERRIAM, R. W. (1969). Movement of cytoplasmic proteins into nuclei induced to enlarge and initiate DNA or RNA synthesis. J. Cell Sci. 5: 333-349.

MINTZ, B. (1964a). Synthetic processes and early development in the mammalian egg. J. Exp. Zool. 157: 85-100.

MINTZ, B. (1964b). Formation of genetically mosaic mouse embryos, and early development of "lethal" (t12/t12)-normal mosaics. J. Exp. Zool. 157: 273-292.

MINTZ, B. (1967). Mammalian embryo culture. In "Methods in Developmental Biology. Ed. Wilt, F., and Wessels, N. Thomas Y. Crowell Company, New York. pp. 379-400.

MONESI, V., MOLINERO, M., SPALLETTE, E., and DAVOLI, C. (1970). Effects of metabolic inhibitors on macromolecular synthesis and early development in the mouse embryo. Exp. Cell Res. 59: 197-206.

MONESI, V., and SALFI, V. (1967). Macromolecular syntheses during early development in the mouse embryo. Exp. Cell Res. 46: 632-635.

PIKO, L. (1970). Synthesis of macromolecules in early mouse embryos cultured in vitro: RNA, DNA and a polysaccharide component. Develop. Biol. 21: 257-279.

ROVERA, G., BERMAN, S., and BASERGA, R. (1970). Pulse labelling of RNA of mammalian cells. Proc. Nat. Acad. Sci. U.S. 65: 876-8

SCONZO, G., PIRRONE, A. M., MUTOLO, V., and GUIDICE, G. (1970). Synthesis of ribosomal RNA during sea urchin development. III. Evidence for an activation of transcription. Biochim. Biophys. Acta 199: 435-440.

SHIOKAWA, K., and YAMANA, Y. (1967). Inhibitor of ribosomal RNA synthesis in Xenopus laevis embryos. Develop. Biol. 16: 389-406.

SHIOKAWA, K., and YAMANA, Y. (1969). Inhibitor of ribosomal RNA synthesis in Xenopus laevis embryos. II. Effects on ribosomal RNA synthesis in isolated cells from Rana japonica embryos. Exp. Cell Res. 55: 155-160.

SILAGI, S. (1963). Some aspects of the relationship of RNA metabolism to development in normal and mutant mouse embryos cultured in vitro. Exp. Cell Res. 32: 149-152.

SKALKO, R. G., and MORSE, J.M.D. (1969). The differential response of the early mouse embryo to actinomycin D treatment in vitro. Teratology 2: 47-54.

SLATER, D. W., and SPIEGELMAN, S. (1970). Transcriptive expression during sea urchin embryogenesis. Biochim. Biophys. Acta 213: 194-207.

STAVY, L., and GROSS, P. R. (1969). Availability of mRNA for translation during normal and transcription-blocked development. Biochim. Biophys. Acta 182: 203-213.

TASCA, R. J., and HILLMAN, N. (1970). Effects of actinomycin D and cyclohexamide on RNA and protein synthesis in cleavage stage mouse embryos. Nature (London) 225: 1022-1025.

TAYLOR, J. H. (1960). Asynchronous duplication of chromosomes in cultured cells of Chinese hamster. J. Biophys. Biochem. Cytol. 7: 455-463.

TERMAN, S. A. (1970). Relative effect of transcription-level and translation-level control of protein synthesis during early development of the sea urchin. Proc. Nat. Acad. Sci. U.S. 65: 985-992.

THOMSON, J. L., and BIGGERS, J. D. (1966). Effects of inhibitors of protein synthesis on the development of preimplantation mouse embryos. Exp. Cell Res. 41: 411-427.

WARNER, T., SOEIRO, R., BIRNBOIM, H. C., and DARNELL, J. E. (1966). Rapidly labelled HeLa cell nuclear RNA. I. Identification by zone sedimentation of a heterogeneous fraction separate from ribosomal precursor RNA. J. Mol. Biol. 14: 349-361.

WILT, F. H. (1970). The acceleration of ribonucleic acid synthesis in cleaving sea urchin embryos. Develop. Biol. 23: 444-455.

WOODLAND, H. R., and GRAHAM, C. F. (1969). RNA synthesis during early development of the mouse. Nature (London) 221: 327-332.