ISOLATION OF HOMOLOGOUS NUCLEAR DNAs FROM SEA-URCHIN EMBRYOS

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SUMMARY

Studies are presented on the ability of low-molecular-weight nuclear DNA fractions to hybridize to higher-molecular-weight nuclear DNAs taken from different stages of early developing sea-urchin embryos, Strongylocentrotus purpuratus. Using preparative DNA-DNA hybridization, a fraction of 60-s mid-blastula DNA was isolated for its ability to anneal to 10-s morula DNA. Approximately 80 regions on each 60-s molecule were found to be homologous to 10-s DNA. High-molecular-weight (> 240-s) nuclear DNA from mesenchyme blastula stage and later stages (compared with DNA from pre-blastula nuclei) shows an increase in the number of regions homologous to fractions of 10- and 60-s DNAs.

INTRODUCTION

In previous experiments (Baker, 1971) replicating DNA was labelled in vivo for various periods of time and then isolated under mild conditions by detergent-enzyme treatment (without phenol extraction) from nuclei of sea-urchin embryos at various stages of development. A portion of the DNA labelled with [3H]thymidine for a period of less than about 2 min was found to be single-stranded, having a sedimentation constant corresponding to 1.5 to 1.9 x 10^3 nucleotides per strand. (This single-stranded DNA is perhaps analogous to the pieces seen in bacteria (Okazaki et al. 1968; Oishi, 1968) and in eukaryotic cells (Painter & Schaefer, 1969; Schandl & Taylor, 1969; Kidwell & Mueller, 1969).) Most of the radioactive DNA labelled for periods longer than 2 min was found to be double-stranded and to sediment, for the most part, in 2 heterogeneous portions: at greater than 240 s, and at less than 70 s on alkaline sucrose gradients. The sedimentation pattern of the latter portion of the nuclear DNA was found to change during the course of early development: prior to blastulation this lower-molecular-weight portion of labelled DNA sedimented predominantly at 10–15 s; after the onset of blastulation a spectrum of size classes of heavier DNA (up to approximately 70 s) was found. Fractionation of isolated labelled native DNA by sedimentation on neutral sucrose gradients followed by hydroxyapatite chromatography of the gradient fractions has shown that the 10–15 s DNA of the various stages is composed of molecules which are a mixture of double-stranded DNA and single-stranded DNA. DNA heavier than 15 s was found to be entirely double-stranded DNA.

The occurrence of the changing size spectrum of double-stranded nuclear DNA with the course of development suggests the possibility that some of the lower-molecular-weight double-stranded DNA made early in development may become part of
higher-molecular-weight DNA found later in development. A further and testable possibility is that nucleotide sequences similar to those sequences represented in low-molecular-weight DNA molecules appearing early in development may be found as new redundant regions on higher-molecular-weight DNA occurring later in development. This latter possibility has been examined here by testing for the presence of multiple copies of nucleotide sequences homologous to early stage 105-s DNA on: (1) later stage 60-s DNA; and (2) high-molecular-weight (> 240-s) chromosomal DNA from gametes and nuclei of different stages of early development of the sea urchin.

MATERIALS AND METHODS

Development of embryos

Strongylocentrotus purpuratus eggs were obtained from gravid females (Pacific Biomarine Supply, Venice, California) by injection of 0.5-1 ml of 0.53 M KCl and inversion of the animal over a beaker containing cold seawater. Eggs were filtered through cheesecloth, washed 4 times by centrifugation through cold Millipore (0.45 μm) filtered seawater, resuspended at about 3 x 10⁶ eggs per ml and fertilized at 15 °C with a minimum quantity of sperm (obtained by KCl injection of mature males) which allowed greater than 96% fertilization. Embryogenesis was allowed to proceed in 200-ml volumes in 2-l Erlenmeyer flasks with gentle gyratory agitation at 15 °C. Penicillin G to 100 μg/ml and streptomycin sulphate to 50 μg/ml were added to the seawater in all experiments at 10 min after fertilization. At a prescribed stage, development was terminated by the addition of sodium azide to a concentration of 0.02 M. The embryos were immediately chilled, pelleted, and washed twice with cold Ca²⁺-Mg²⁺-free seawater (Cavanaugh, 1956), followed by 2 washes with cold 1 M glucose.

The possibility of bacterial contamination of nuclei preparations has been minimized by the conditions used in washing the eggs, the treatment of the embryos, the growth conditions, and the method of harvesting embryos and obtaining nuclei. Labelled or unlabelled bacterial ribosomal RNA in preparations of sea-urchin RNA (Glisen & Glisen, 1964) derived from nuclei has not been detected even when the development period was as much as 24 h with continuous label.

Isolation of nuclei

At morula stage there are about 100-200 cells per embryo and at blastula stage 400-600 cells per embryo (Hinegardner, 1967). Embryos from which the fertilization membranes had been removed (constituting a total of up to 10⁸ cells and washed as indicated above) were resuspended in 10 volumes of ice-cold 0.05 M EDTA, 0.15 M NaCl, 0.015 M Na citrate, 0.05 M Tris, pH 8.4 measured at 4 °C (Tris—EDTA—SSC buffer). These embryos were immediately forced slowly through a number 20-gauge hypodermic needle (Hinegardner, 1962) into 2 vol. of cold 2.0 M sucrose (Wilt, 1967). After hand centrifugation followed by centrifugation of the supernatant at 10000 g for 15 min, the pellet was resuspended in Tris—EDTA—SSC buffer containing 1.0 M sucrose and 2 ml were layered on to a sucrose step gradient (Hinegardner, 1962) made up in Tris—EDTA—SSC buffer. After centrifugation in a Spinco SW 25:1 rotor at 20000 rev/min for 45 min, the nuclei were collected from the lowest sedimenting band (except for pluteus stage nuclei which did not separate well from slower sedimenting material). The fraction containing the nuclei was then quickly frozen and stored at −25 °C. Nuclei were later pooled with other similar stage frozen nuclei which had been prepared over a period of up to 6 months. For preparation of high-molecular-weight nuclear DNA, nuclei which had not been frozen were used.

Isolation of DNA from nuclei or sperm

Sperm for preparation of DNA was collected from mature male sea urchins by injection of 0.53 M KCl and inversion of the animal over beakers containing 2 ml of ice-cold Tris—EDTA—
Homologous nuclear DNAs from embryos

SSC buffer. Concentrated ('dry') sperm, as obtained directly from the animal, has a concentration of about $2 \times 10^{10}$ spermatozoa per ml (Harvey, 1956).

Nuclei, in Tris-EDTA-SSC buffered sucrose, were used as obtained from gradients as described above.

To a suspension of either sperm or nuclei (each adjusted to approximately $5 \times 10^9$ per ml), pronase (Calbiochem) was added to 1 mg per ml followed by the addition of sodium lauryl sulphate to 0.25% (Thomas, Berns & Kelly, 1966). After incubation at 37°C for 10-12 h, RNase (Worthington; stock solution at 2 mg per ml in 0.15 M NaCl, heated to 85°C for 20 min to destroy DNase) was added to 20 μg per ml and α-amylase (Worthington, 650 units per mg) to 30 μg per ml, and an additional incubation was allowed at 37°C for 1 h.

In order to obtain DNA of the various natural size classes shown in Table 1, the DNA was fractionated on sucrose gradients prior to phenol extraction. (It was found that direct phenol extraction of DNA caused a reduction in the molecular weight of the different size classes of DNA examined.) Sucrose gradient fractionation was carried out by layering 1.5 ml of the enzyme-treated DNA solution on to 30 ml, 5-20% (w/v) linear sucrose gradients, neutral or alkaline as indicated below, and centrifuging at 18°C in a Spinco SW 25-1 rotor for the appropriate period of time required to allow separation of a particular weight class of DNA. For collection of the highest-molecular-weight chromosomal DNA, sedimentation was for 10 h at 10000 rev/min, through alkaline sucrose gradients made up in 0.1 M NaOH, 0.9 M NaCl, 0.001 M EDTA. This period of centrifugation allowed the collection of denatured DNA of greater than 240-s from the lower 50% of the gradients. In order to preserve double-strandedness, lower-molecular-weight DNA was obtained by fractionation on neutral gradients (made up in 0.1 M NaCl, 0.02 M Tris, pH 7.3, 0.001 M EDTA) centrifuged at 24000 rev/min. Centrifugation for 22 h allowed 50-s-60-s pieces (used to obtain blastula type C DNA) to sediment to near the centre of the gradient. DNA directly from gradient fractions, while indistinguishable from clean DNA by sedimentation, showed some variable results in hybridization reactions. This variability was eliminated by extracting the appropriate gradient fractions 3 times at room temperature (with gentle swirling for 5 min each time) with an equal volume of neutralized water-saturated phenol in the presence (Thomas et al. 1966) of 27% (w/v) sucrose. The DNA was then dialysed against 3 or 4 changes of 1000 volumes of the appropriate solution as indicated.

Isolation and enrichment for homologous DNA

The general method used for isolation of low-molecular-weight early stage DNA homologous to higher-molecular-weight later stage DNA is shown in Fig. 1 (p. 158). The characteristics of the designated types of DNA are shown in Table 1 (p. 159) and defined in the Results. The yield of the different DNAs isolated is shown in Table 2 (p. 159).

Isolation of type A' DNA from morula stage nuclear DNA. The starting DNA was unlabelled low-molecular-weight (10- to 13-s when double-stranded on a neutral sucrose gradient) nuclear DNA from morula stage embryos obtained as described above. After dialysis against 0.05 M phosphate buffer, pH 6.9, the DNA solution was applied to a 1 x 7 cm hydroxyapatite column equilibrated with the same buffer. Naturally occurring single-stranded DNA was eliminated by washing the column with 0.16 M phosphate buffer, pH 6.9, and then double-stranded DNA was eluted with 0.25 M phosphate buffer, pH 6.9. Normally, this DNA (designated type A DNA) was then subjected to radioactive labelling in vitro as described below. After dialysis against 3 changes of 0.1× SSC (SSC is 0.15 M NaCl, 0.015 M Na citrate), the DNA was denatured at 0.4°C by the addition of one-tenth volume of 1 M NaOH. After 5 min the solution was neutralized quickly by the addition of 1 M HCl. Morula type A DNA was then hybridized as described below to DNA (designated type C) which was isolated from middle blastula stage embryos by the same method used to obtain type A DNA. Blastula type C DNA was of higher molecular weight (50-60-s when double-stranded on a neutral sucrose gradient) than morula type A DNA. For this preparative hybridization step, type C DNA was denatured as described above and immobilized (Gillespie & Spiegelman, 1965) at 100 μg per filter on 25-mm nitrocellulose filters. As many as 25 filters were utilized in each hybridization vial containing a volume of hybridization medium just sufficient to cover the filters. The excess amount of input DNA added to the
medium was at least 5–10 times the total amount of DNA immobilized on the filters. After annealing, the filters were washed as described below. The post-hybridization input DNA was normally used 5 more times in the same hybridization reaction with fresh type C DNA filters. The hybridized DNA was then eluted by incubating the filters for 5 min at room temperature in a minimal volume of 0.05 M NaOH, 0.01 M EDTA. This eluted DNA was then pooled and 0.2 ml layered on to 4.8 ml alkaline sucrose gradients made up as before except without EDTA and centrifuged for 6 h at 50,000 rev/min at 18 °C in a Spinco SW 50-1 rotor. This step was necessary to eliminate small amounts of contaminating higher-molecular-weight, previously immobilized, DNA which also eluted from the filter papers. The low-molecular-weight DNA, sedimenting approximately half-way from the top of the gradient at 10-5-8, was obtained and designated type A' DNA.

Isolation of type B' DNA from blastula stage nuclear DNA. The procedure was the same as that used for the isolation of type A' DNA above except that the 10-13-S double-stranded low-molecular-weight starting DNA was from early blastula stage embryos. The immobilized DNA was mid-blastula type C DNA as before. The 10-5-S denatured DNA, obtained after sucrose gradient sedimentation, was designated type B' DNA.

Isolation of type C' DNA from blastula stage nuclear DNA. The procedure was the same as that used for the isolation of type A' DNA except that the starting DNA was 50-60-S from middle blastula stage embryos (type C DNA above) and the DNA immobilized on the filters was 10-13-S from morula stage embryos (type A DNA above). Post-hybridization sedimentation as before on alkaline sucrose gradients in a Spinco SW 50-1 rotor for 2-2.5 h at 30,000 rev/min was used to obtain the eluted C' DNA which sedimented at 60 S (near the centre of the centrifuged gradient).

Isolation of selected fragments of blastula type C' DNA which possess morula A' homology. The procedure was the same as that used for the isolation of type A' DNA except for the following modifications: the starting DNA was 50-60-S middle blastula stage type C DNA. After the hydroxyapatite chromatography and dialysis steps, the double-stranded DNA in 0.1 X SSC was sheared by 1-3 passages through a number 28-gauge hypodermic needle or by sonication for up to 1 min with a Branson Biosonic III sonicator. The sheared DNA was then fractionated into size classes by sedimentation (4 h at 50,000 rev/min, Spinco SW 50-1, 18 °C) through alkaline sucrose gradients (made up as above except without EDTA). Pieces of DNA ranging from 6-3 S up to 28 S were isolated in different fractions obtained from the gradients. After neutralization with HCl and making the solution 2 X SSC and 30 % formamide, /v/v, the DNA in these fractions was hybridized to type A DNA immobilized on filters. Elution of hybridized DNA and resedimentation through alkaline sucrose gradients was used to obtain the various size classes of fragmented C' DNA. It should be noted that this procedure results in eliminating pieces of C' DNA which do not contain a certain minimum sequence homology with A' DNA.

Isolation of unselected fragments of blastula type C' DNA. Renatured C' DNA was sheared and fractionated into size classes as in the last paragraph, except that the final selection for A' homology was omitted.

Radioactive labelling of DNA

DNA was labelled in vitro by 'H-methylation using the following modification of the method of Smith, Armstrong & McCarthy (1967): one part by weight of DNA was mixed with 1 part by weight of 'H-dimethyl sulphate (International Chemical and Nuclear Corp.) at a specific activity of 100 or 120 mCi/mmol in 0.25 M phosphate buffer, pH 7.2, containing 10 %, v/v, water-saturated diethyl ether. The mixture was incubated at 15-16 °C for 12 h with gentle agitation. After incubation the mixture was chilled and the DNA precipitated by the addition of 2 vol. of ethanol at —20 °C. After redissolving the DNA in 0.1 X SSC, precipitation was repeated 2 additional times.

Preparative and analytical DNA–DNA hybridization

In the immobilization of DNA on filters (Gillespie & Spiegelman, 1963), the amount of DNA passing through a filter under suction varied with the batch of filters and with the amount and type of DNA presented to the filter. Also, a variable low percentage (less than 5 %) of the immobilized DNA was released during hybridization and washing of the filters. Accordingly, the
Homologous nuclear DNAs from embryos

amount of DNA actually immobilized and the amount of immobilized DNA retained after hybridization procedures were determined by control experiments. DNA of the same type and amount used in the analytical studies was labelled in vitro and immobilized on filters. Control hybridization experiments in which the input DNA was unlabelled and immobilized DNA was radioactive allowed for the assessment of the amount of immobilized DNA retained by the filter after exposure to hybridization conditions.

Although preincubation at 65 °C by the method of Denhardt (1966) was used to reduce background binding during hybridization (Smith et al. 1967; Searcy, 1968), annealing was carried out for 12 h at 25 °C in 2 x SSC with 30 %, v/v, formamide (Bonner, Kung & Bekhor, 1967). Control experiments have shown that this period of hybridization was sufficient to obtain maximal hybridization in the analytical experiments. In the preparative hybridization steps preferential hybridization of the specific redundant DNAs was enhanced by these conditions. Longer hybridization time (and higher temperatures) in the preparative steps increases the amount of unwanted non-redundant DNA extraneous to the selected A', B' or C' types of DNA. This relatively low-temperature hybridization prevented, as suggested by Smith et al. (1967), a reduction during hybridization in the specific activity of input DNA which had been labelled by alkylation. (Analytical hybridization under conditions of higher salt and temperature (Gillespie & Spiegelman, 1965) in control assays on experiments shown in Results gave the same qualitative results and, after correction for loss of 3H specific activity, essentially the same quantitative results. This gave some confidence that the hybridization conditions used here allow sufficiently accurate pairing of strands during annealing. See also Discussion.) After hybridization the filters were washed 5 times in 2 x SSC solution (10 ml of 2 x SSC solution added to the aspirated hybridization vial containing the filters, then vortexing 15 s and aspirating). In the saturation hybridization experiments, the amount of DNA which hybridized was determined by counting the heat-dried filter and dividing by the specific activity of the input DNA. The justification for this method of determining the amount of hybridized DNA depends upon: (1) labelling to the same degree all DNA molecules which hybridize; (2) the specific activity of the hybridized DNA being the same as the input DNA. Both of these conditions are met by labelling in vitro. Control hybridization experiments carried out under both saturation and subsaturation conditions indicate that the percentage of hybridization is not dependent upon the specific activity of the input DNA when the latter is varied over a 3-fold range. This indicates that preferential labelling of a fraction of the hybridizing DNA is probably not taking place and that the degree of methylation is not affecting the efficiency of hybridization under the conditions used. It was found that the in vitro labelled homologous input DNAs (designated A', B' and C' above) each had specific activities within 4% of that found in the respective hybridized DNA eluted from filters and separated on alkaline sucrose gradients from higher-molecular-weight DNA which was originally immobilized. In all hybridization assays the amount of radioactivity binding to background filters having an equivalent amount of immobilized φ80 phage DNA was subtracted from the amount binding to sea-urchin DNA filters. The maximum background radioactivity binding to φ80 DNA in any saturation experiment or at zero competing DNA in competition experiments was less than 6% of the radioactivity annealing to the corresponding sea-urchin DNA filter.

Radioactive counting

Radioactivity was determined by liquid scintillation spectrometry. In all cases the samples were counted while immobilized on 25-mm type HA Millipore filters in toluene containing diphenyloxazole and dimethyl-1,4-bis-(2-phenyloxazolyl)-benzene.

Sedimentation constants and the size of DNA molecules

Marker DNA from φ80 phage, 32 x 10^6 Daltons (Yamagashi, Nakamura & Ozeiki, 1965), sedimenting at 35 s_m as double-stranded DNA (Studier, 1965) or DNA from P1 KC phage, 60 x 10^8 Daltons (Tomizawa & Anraku, 1965), sedimenting at 56-1 s_m as double-stranded DNA (Abelson & Thomas, 1966) on parallel gradients and also calculation using the equation of Abelson & Thomas (1966) were used to determine and verify sedimentation constants on the preparative gradients. Molecular weights of DNA fractions were estimated from sedimentation constants by the equations of Studier (1965).
Isolate DNA from morula stage nuclei

Fractionate on neutral sucrose gradient

Obtain 10-13-s DNA

Phenol extract

Obtain double-stranded type A DNA from hydroxyapatite chromatography

Denature

Input DNA

Preparative hybridization

Wash filters

Elute hybridized DNA

Fractionate on alkaline sucrose gradient

Obtain single-stranded morula type A' DNA

--- Label in vitro

Isolate DNA from blastula stage nuclei

Fractionate on neutral sucrose gradient

Obtain 50-60-s DNA

Phenol extract

Obtain double-stranded type C DNA from hydroxyapatite chromatography

Denature

Immobilized DNA

Fig. 1. Isolation and enrichment scheme for DNAs having mutual homology. The method of isolation of A' DNA from morula stage embryos is shown here. The method of isolation of B' and C' DNA from blastula stage embryos is similar and is described in Materials and Methods.

RESULTS

Isolation of low-molecular-weight morula stage DNA having a high degree of homology with higher-molecular-weight blastula stage DNA

In order to test the possibility that some low-molecular-weight double-stranded DNA sequences made early in development are homologous to multiple regions on intermediate-molecular-weight DNA appearing later in development, 2 size classes of nuclear DNA, each selected to have a high degree of homology for the other, were taken from different stages of development as detailed in Materials and Methods.
Table 1. Characteristics of the different nuclear DNA fractions

<table>
<thead>
<tr>
<th>Type of DNA</th>
<th>Stage of embryo used as source</th>
<th>Sedimentation coefficient in alkaline sucrose, s</th>
<th>Approximate molecular weight (single-stranded), $X 10^8$</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Morula</td>
<td>10.5</td>
<td>0.55</td>
<td>Separated from native nuclear DNA by fractionation on alkaline sucrose gradient</td>
</tr>
<tr>
<td>B</td>
<td>Blastula</td>
<td>10.5</td>
<td>0.55</td>
<td>The portion of type A DNA which has homology with type C DNA</td>
</tr>
<tr>
<td>C</td>
<td>Blastula</td>
<td>~ 60</td>
<td>44</td>
<td>The portion of type B DNA which has homology with type C DNA</td>
</tr>
<tr>
<td>A'</td>
<td>Morula</td>
<td>10.5</td>
<td>0.55</td>
<td>The portion of type C DNA which has homology with type A DNA</td>
</tr>
<tr>
<td>B'</td>
<td>Blastula</td>
<td>10.5</td>
<td>0.55</td>
<td>Not isolated: defined as the portion of B' DNA which is not homologous with A' DNA</td>
</tr>
</tbody>
</table>

Table 2. Yield of prepared nuclear DNAs per $6 \times 10^8$ embryos

<table>
<thead>
<tr>
<th>Stage of embryo used as source of nuclei</th>
<th>Total amount of bulk nuclear DNA isolated, mg</th>
<th>Amount of derived type of DNA, mg</th>
<th>A</th>
<th>A'</th>
<th>B</th>
<th>B'</th>
<th>C</th>
<th>C'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morula</td>
<td>153</td>
<td>---</td>
<td>3.44</td>
<td>0.97</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Early blastula</td>
<td>479</td>
<td>---</td>
<td>---</td>
<td>14.81</td>
<td>0.98</td>
<td>1.21</td>
<td>0.24</td>
<td>---</td>
</tr>
<tr>
<td>Mid-blastula</td>
<td>594</td>
<td>---</td>
<td>---</td>
<td>7.05</td>
<td>2.14</td>
<td>5.06</td>
<td>1.36</td>
<td>---</td>
</tr>
<tr>
<td>Late blastula</td>
<td>731</td>
<td>---</td>
<td>---</td>
<td>1.13</td>
<td>0.08</td>
<td>0.01</td>
<td>0.28</td>
<td>---</td>
</tr>
<tr>
<td>Gastrula</td>
<td>858</td>
<td>---</td>
<td>---</td>
<td>0.08</td>
<td>0.01</td>
<td>1.01</td>
<td>0.28</td>
<td>---</td>
</tr>
<tr>
<td>Pluteus</td>
<td>1022</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

The amounts shown are the average yields of 2 or more preparations of a particular DNA.

The 2 size classes of DNA selected were: 10.5-8, which was the lowest-molecular-weight duplex DNA found at the different stages of early development; and 60-s, from middle to late blastula embryos, which was the highest-molecular-weight DNA found (except for the very high-molecular-weight (> 240-s) chromosomal DNA) (Baker, 1971). A subfraction of each of these size classes was selected by preparative DNA-DNA hybridization such that the subfraction of one size class carried a high degree of homology for the subfraction of the other size class. The methods used for isolation and enrichment toward mutual homology of the various subfractions of DNA are shown schematically in Fig. 1, the derivation and characteristics of the different DNAs in Table 1 and their yields in Table 2. The subfraction of low-molecular-weight
Fig. 2. Saturation hybridization of immobilized blastula C' DNA (0.1 µg per filter) with increasing amounts of morula ³H-A' (8712 cpm per µg) or blastula ³H-B' (8429 cpm per µg) input DNA. — O —, ³H-A' DNA; — • —, ³H-B' DNA.

10⁻⁵-S DNA having a high degree of homology for 60-s blastula stage DNA was designated type A' if from morula stage or type B' if from blastula stage. The subfraction of the 60-s blastula stage DNA which bore a high amount of homology for A' and B' DNA was designated type C' DNA.

**Homology between A' or B' DNA and C' DNA**

The relative amount of homology between 60-s blastula C' DNA and 10⁻⁵-S morula A' or 10⁻⁵-S blastula B' DNA was assessed by DNA-DNA hybridization under saturation conditions and also by competition hybridization. In saturation studies the amount of C' DNA immobilized on nitrocellulose filters was held constant with increasing amounts of in vitro labelled A' or B' input DNA. The results shown in Fig. 2 indicate that at saturation 0.044 µg of A' DNA or 0.089 µg of B' DNA anneal to 0.1 µg of C' DNA. Thus, by this criterion, 44% of the average C' molecule would be homologous to sequences carried by the A' molecules and 89% of C' would be homologous to B' sequences if the entire length of each input DNA molecule were homologous to a region of a C' molecule and if the input molecules did not overlap one another on the hybrid. However, in either case, if only a section of a hybridized input molecule was hydrogen bonded to a C' molecule, the same amount of radioactivity would be present on the reaction filter as in the case in which the entire length of the hybridized input molecule was homologous and bonded to a C' region. Consequently, the degree of sequence homology of A' or B' molecules with C' molecules was tested by competition hybridization. Type A' or B' molecules were competed with short pieces (< 0.3 x 10⁶ Daltons, single-stranded) of labelled C' molecules for sites on immobilized full-length C' molecules. The results are shown in Fig. 3. A subsaturating amount of the input DNA was used in this experiment. (Another experiment, not shown here, using a saturating amount of the input DNA followed by competition with A' or B' DNA.
Homologous nuclear DNAs from embryos

161

Fig. 3. Competition of fragments (∈ $10^{-3}$ Daltons, single-stranded) of blastula 
$^3$H-C' DNA with unlabelled morula A' or blastula B' DNA for hybridization sites on 
immobilized full-length blastula C' DNA (0.1 μg per filter). The input DNA consisted 
of a subsaturating amount (6-2 μg) of fragmented $^3$H-C' DNA (8247 cpm per μg) 
mixed with increasing quantities of competing A' or B' DNA. (Saturation occurred at a 
ratio of input to immobilized DNA of approximately 200:1 when competing DNA was 
not present.) The amount of $^3$H-C' DNA hybridizing (574 cpm) when competing 
unlabelled A' or B' DNA was not present, was set at 100%. — ○ —, A' DNA; 
—— ● —, B' DNA. 
gave substantially the same results.) Type A' molecules can compete for approxi-
mately 42% of the sites (100–58%) on full-length C' molecules. Type B' molecules 
can compete for approximately 89% of the C' sites (100–11%). The results and agree-
ment of the competition and saturation experiments indicate that approximately one-
half of the C' DNA contains sequence homology for A' molecules and almost all of 
the C' DNA has B' homology.

Homology between morula 10^{-5} A' DNA and blastula 10^{-5} B' DNA

The DNA selection procedure required that all C' molecules contain some A' 
homology. Since there is approximately twice as much B' homology as there is A' 
homology per C' molecule (Figs. 2, 3), it seems likely that the B' set of molecules is 
made up of both the A' set of molecules and some other set of molecules nearly all of 
which are homologous to C' DNA. This possibility has been tested and the results are 
shown in Fig. 4. Hybridization analysis indicates that 43% of the B' DNA can be 
saturated with A' DNA. Results of competition of A' and B' molecules for sites on 
immobilized C' molecules is shown in Fig. 5. Approximately 51% of C' sites which 
hybridize B' molecules can be competed by A' molecules. Almost all (90%) of C' sites 
which hybridize A' molecules can be competed by B' DNA. Since A' and B' mole-
cules have approximately the same molecular weight, the saturation and competition 
hybridization data together indicate that the set of molecules designated B' is made up, 
qualitatively, of about one-half A' molecules and one-half other molecules which will be
Fig. 4. Saturation hybridization of immobilized blastula B' DNA (0.1 μg per filter) with increasing amounts of morula 3H-A' (8712 cpm per μg) input DNA.

Fig. 5. Competition of unlabelled morula A' DNA with blastula 3H-B' DNA (--- ○ ---) and unlabelled blastula B' DNA with morula 3H-A' DNA (--- ● ---) for hybridization sites on immobilized blastula C' DNA (0.1 μg per filter). The input DNA consisted of a subsaturating amount (see Fig. 3) of 3H-A' DNA (5 μg, 9163 cpm per μg) or 3H-B' DNA (5 μg, 8931 cpm per μg) mixed with increasing amounts of the opposite type of unlabelled A' or B' DNA. The amount of 3H-DNA hybridizing (316 cpm for 3H-A' DNA and 543 cpm for 3H-B' DNA) when competing unlabelled A' or B' DNA was not present, was set at 100%.
Designated type B* molecules. By definition then, B* molecules are those molecules of the B' type which show homology to C' molecules but not to A' molecules. It is consistent with the data of Figs. 2-5 that C' molecules are composed almost entirely of regions homologous to A' and B* molecules and that A' and B* regions occur with approximately equal frequency on the collective C' molecules.

Evidence that the majority of individual C' molecules contain both A' and B* homology

The results just described do not indicate whether: (1) there is an exclusive population of C' molecules which possesses mostly A' homology while a different population has mostly B* homology; (2) both A' and B* homology resides on the same C' molecules; or (3) the population of C' molecules is a mixture of the first 2 alternative types. In order to decide between these alternatives, the following experimental approach was used. Fragments of C' DNA which possessed A' homology were prepared as described in Materials and Methods. The C' fragments utilized were of 2 size classes separated by sedimentation on alkaline sucrose gradients: (a) 16-8 (corresponding to $1.7 \times 10^6$ Daltons) which is about 3 times larger than A' or B' pieces; and (b) 21.5-8 (corresponding to $3.3 \times 10^6$ Daltons) which is about 6 times larger than A' or B' pieces. The fragments of either size class were immobilized on filters and tested by saturation hybridization for the presence and amount of B' and A' homology. The results are shown in Fig. 6. Both size classes of C' fragments show approximately 90% homology.
for B' DNA [(0.00/0.1) x 100] and 44-45% homology for A' DNA [(~ 0.045/0.1) x 100] by this criterion. Comparison of the levels of saturation of non-fragmented C molecules by $^3$H-A' or $^3$H-B' DNA (Fig. 2) with the corresponding levels of saturation of C' fragments selected to have A' homology (Fig. 6) indicates that the selection procedure did not raise the level of A' saturation or lower the level of B' saturation. (This latter possibility should have occurred if, for instance, the family of unselected C' molecules contained a significant proportion of species having more A' homology than B' homology.) Therefore, it appears that the majority of natural blastula C' molecules (not fragmented and selected in this special way) have about one-half A' and one-half B' sequences on each molecule.

**Hybridization of A', B' and C' DNA to high-molecular-weight nuclear DNA from various stages of development**

Hybridization of increasing amounts of A', B' or C' homologous DNA with a fixed amount (1 or 2 filters per hybridization reaction at 100 $\mu$g per filter) of immobilized high-molecular-weight nuclear DNA (> 240-s on alkaline sucrose gradients; see Materials and Methods) from various stages of early development was carried out, and the results are shown in Figs. 7-9. The saturation plateau is about 0.0014% for $10.5$-s morula A' DNA [(0.0028/200) x 100] hybridized to high-molecular-weight chromosomal DNA from either sperm, egg, or morula stage (Fig. 7). Increasing the ratio of input DNA to immobilized DNA to as high as 0.5:1:0 did not significantly increase the saturation level in these studies. Using the value of 1.8 x 10^{-12} g of DNA per diploid nucleus of *S. purpuratus* (Hinegardner, Rao & Feldman, 1964), the number of A' regions (1.10 x 10^6 Daltons per region) per haploid genome corresponding to this saturation level is calculated as being approximately 7. At the early blastula stage the amount of the genome saturated increases to 0.0335% and further increases at the late blastula stage to 0.0535%. High-molecular-weight DNA from gastrula or pluteus shows approximately the same degree of saturation as that from late blastula stage (0.057-0.060%), reflecting an increase of the order of 40-fold in the available hybridization sites on the genome at these stages compared with gamete and early cleavage stage embryos. If the A' region(s) of the high-molecular-weight genome in each of the cells of all embryos were equally amplified, then the number of A' regions per haploid genome, calculated as before, is thus about 280 for these later stages of development compared with 7 for pre-blastula stage embryos. If A' DNA were composed of only one species of molecule, then this increase in hybridization plateau with development would reflect a 40-fold increase in gene redundancy in the chromosomes for the A' genetic regions. However, neither the proportion of genomes undergoing this possible A' amplification nor the number of species in the hybridization input A' DNA is known. Therefore, this type of experiment reflects a minimum increase at the blastula stage in A' regions on high-molecular-weight nuclear DNA. Saturation hybridization of the various high-molecular-weight chromosomal DNAs with 10.5-s blastula B' DNA is shown in Fig. 8. The saturation plateaus for the respective stages of development are in general about twice those obtained by saturation with A' DNA as shown in Fig. 7. This is consistent with the previously suggested probability that B'
Fig. 7. Saturation hybridization of morula

DNA is composed of A' and B" molecules and contains twice as many different kinds of sequences as does A' DNA. Since the calculated number of B' sites saturated on high-molecular-weight DNA increases about 40-fold (560/14) at blastula stage, the possibility can be entertained that an increase in gene redundancy on high-molecular-weight nuclear DNA was effected at this stage of development for both the A' family of sequences (40-fold increase) and the B" family of sequences (40-fold increase).

The 35-40 regions of both A' and B" DNA sequences on the C' molecule (see Discussion) and the size of the large C' DNA molecule being about 80 times that of the A' or B' molecules (Table 1), suggest the possibility that C' DNA contains the same sequence homology to high-molecular-weight nuclear DNA as does B' DNA. This possibility is consistent with the results shown in Fig. 9 in which increasing quantities of sonicated C' DNA (pieces having a molecular weight of < 3.5 x 10⁶ Daltons, single-stranded, and derived from 60-s blastula C' DNA) are hybridized to high-molecular-weight chromosomal DNAs from gametes and nuclei of various stages of development. The results are similar to those shown in Fig. 8, strengthening the possibility that C' DNA contains much the same genetic homology for the high-molecular-weight DNA as does B' DNA. Also, the C' DNA plateau heights for each stage of development
Fig. 8. Saturation hybridization of blastula \(^{3}H\)-B' input DNA (specific activity 8931 cpm per \(\mu\)g) to sonicated immobilized chromosomal DNA from various stages of development. Increasing the ratio of input DNA to immobilized DNA, for the curves shown in the inset graph, to as high as 0.5:1 did not significantly alter the saturation plateau levels. Other details are as in Fig. 7. — x —, sperm; — Δ —, egg; — ▲ —, morula; — ○ —, mid-blastula; — ⋄ —, late blastula; — ● —, gastrula; — ● —, pluteus.

The possibility that the isolated high-molecular-weight nuclear DNAs from blastula, gastrula and pluteus stages contained trapped (but not covalently bound) lower-molecular-weight copies of A', B' or C' DNA (and thereby falsely indicated chromosomal gene redundancy for the lower-molecular-weight homologous DNAs) was minimized by control experiments in which isolated excess unlabelled A', B' and C' DNA was mixed with sperm or morula stage high-molecular-weight chromosomal DNA prior to sedimentation on gradients and collection of DNA sedimenting at > 240-5. These high-molecular-weight DNAs had the same percentage of saturation per unit weight of DNA for saturation hybridization with either A', B' or C' \(^{3}H\)-DNA as did the corresponding high-molecular-weight DNA which had not been treated in this manner.

**DISCUSSION**

In these studies a naturally occurring low-molecular-weight size class of duplex DNA was isolated separately from both preblastula and blastula stage nuclei. A subfraction of each of these 2 DNA preparations was subsequently isolated, by means of preparative DNA-DNA hybridization, as having a high degree of homology for
another naturally occurring intermediate-molecular-weight class of DNA obtained from middle to late blastula stage nuclei. The properties of the derived types of DNA are summarized in Table 1. The selected subfractions of the low-molecular-weight DNAs, designated A' if from preblastula stage nuclei and B' if from blastula stage nuclei, each consisted of pieces sedimenting at 10-5-s on alkaline sucrose gradients, corresponding (Studier, 1965) to DNA having approximately 1·7 x 10^3 nucleotides per single strand of originally duplex DNA. A subfraction, designated C' DNA, of the intermediate-molecular-weight blastula stage nuclear DNA was also selected by preparative hybridization as having homology for preblastula A' and blastula B' DNA. Blastula type C' DNA consisted of pieces sedimenting at approximately 60-s (~ 1·4 x 10^4 nucleotides per strand if considered to be linear molecules (Studier, 1965)). Competition and saturation hybridization experiments indicate that A' DNA has homology for 40-45 %, and B' DNA for 90-94 %, of the collective C' DNA sequence. By calculation, the average full-length C' strand has about 37 A' regions (0·45 x 1·4 x 10^6/1·7 x 10^3) and 77 B' regions (0·94 x 1·4 x 10^6/1·7 x 10^3) of homology. As previously defined, those regions on the C' molecules which are not homologous to A' regions are designated as B* regions. The number of B' regions per full-length C' molecule is equal to about 40 regions (77-37).
Other experiments now in progress, utilizing variously sized pieces of C' DNA selected to possess A' homology, suggest that A' and B* regions probably alternate with one another in making up C' molecules. It also has been found that nicking of type C DNA by mild treatment with DNase II results in a shift in the sedimentation constant of a portion of this DNA from approximately 60 s to predominantly 55 s as measured on alkaline sucrose gradients. This suggests that type C DNA as first isolated may contain some circular forms. A 55-s type C' DNA was subsequently isolated from 55-s type C DNA. The hybridization properties (in annealing with $^3$H-A' DNA or $^3$H-B' DNA) of 55-s type C' DNA are the same as 60-s type C' DNA (Figs. 2, 5). Calculation (Studier, 1965) of the molecular weight of linear 55-s type C' DNA (i.e. $3.5 \times 10^7$ Daltons) indicates that there are 32 A' and 32 B' size regions per C' molecule.

At the mesenchyme blastula stage of development, comparative saturation hybridization has shown that, on the average, each high-molecular-weight (> 240-s) nuclear DNA genome equivalent apparently acquires an approximately 40-fold increase in homology for both A' and B* regions relative to earlier stage and gamete high-molecular-weight DNA (Figs. 7-9). Since there are approximately 35-40 A' and B' regions per full-length 60-s C' molecule, one possible explanation for this result is that an average of about one C' sequence is acquired at the blastula stage by each high-molecular-weight genome of every cell in the embryo for each A' or B' region originally present on the pre-blastula genome. (Another possibility which cannot be rigorously excluded by these data is that in the isolation of post-blastulation high-molecular-weight DNA there is some unknown selection toward enriching for DNA carrying A' and B* sequences.) The actual number of cells per embryo which may undergo a possible increase in specific nucleotide sequences cannot be determined from the experiments shown here.

Multiple copies of a particular type of low-molecular-weight DNA (A' or B* DNA) could possibly be generated by (1) multiple replication of a short section of high-molecular-weight chromosomal DNA, and/or (2) autonomous replication of low-molecular-weight DNA where the initial copy may or may not have been replicated from the high-molecular-weight genome. The first alternative would seem unlikely if the high-molecular-weight chromosomes lose the A' or B* initial genetic templates during the period of synthesis of these DNAs. (It is conceivable that this could happen as a result of circularization of a short region on the preblastula genome followed by its excision to produce a parent A' or B* DNA template.) However, the results of experiments shown in Figs. 7 and 8, in which the level of saturation of the DNA genome of different stages of preblastula development by the low-molecular-weight A' or B' DNA is found to be at least as high as egg or sperm genome, argues that the high-molecular-weight DNA does not temporarily lose these genetic regions during the synthesis of multiple copies of the low-molecular-weight DNAs.

The use of preparative hybridization in eliminating non-homologous DNA species from both immobilized and input hybridization A', B' and C' DNA may increase the degree of comparative accuracy of the subsequent analytical hybridization assays of these DNAs. In saturation hybridization studies, Fig. 2 shows that, on the average, about one-half of the collective C' molecules can be saturated by A' molecules and that
Homologous nuclear DNAs from embryos

Each C' molecule is largely saturated by B' molecules. This suggests that, if the hybridization assays are quantitative in a comparative way, then the level of saturation of immobilized B' molecules by A' molecules should approach the same level as A' molecules saturating immobilized C' molecules. This condition is fulfilled in comparing the results shown in Fig. 4 with the data shown in Fig. 2. This equivalence of saturation levels may not necessarily be a result of improved matching of homologous nucleotide strands during annealing but rather may result from a high efficiency of saturation of the (redundant) immobilized DNA. (See McCarthy & Church (1970) for a critique of molecular hybridization as an analytical method.) However, regardless of whether saturation hybridization as used here gives an exact quantitative measure of the degree of homology that an immobilized DNA has in common with input DNA, comparison of saturation plateaus produced by 2 different input DNAs on the same type and amount of immobilized DNA (Fig. 2) or the same type of input DNA with different immobilized DNAs (Figs. 7–9) increases the reliability of assessments of relative homology.

Although not important to those results (Figs. 7–9) suggesting an increase in specific nucleotide sequences present on post-blastula high-molecular-weight DNA, several features of the methods and results argue that the majority of the homologous pieces of DNA (A', B' or C') are not the result of mechanical shear or degradation of higher-molecular-weight DNA during the isolation of the DNA: (1) a discrete size class of DNA molecules (10-5-s on alkaline sucrose gradient) was isolated at morula stage to have A' nucleotide sequences and the same size class at blastula stage to have B' sequences; (2) since A' DNA does not carry B' homology, it seems probable that neither A' nor B' DNA is a product of sheared C' DNA or high-molecular-weight DNA carrying redundant A' and B' regions; (3) at the morula stage, before higher-molecular-weight nuclear DNA (C' or > 240-s DNA) begins to show increased quantities of B' regions, 10-5-s A' DNA pieces can be isolated. At the gastrula stage, after an apparent increase in A' and B' regions on the high-molecular-weight genome has taken place, little B' DNA can be found in the nuclei. This argues that DNA having a molecular weight higher than that of A' and B' pieces does not shear, under the isolation methods used, in some special way to produce A' and B' pieces.

Evidence has been obtained which suggests that neither A', B' nor C' DNA contains genes coding for ribosomal or transfer RNA (R. F. Baker & L. C. Fitzmaurice, in preparation): (1) banding of labelled A', B' or fragmented C' DNA in CsCl has shown that all 3 DNAs have the same buoyant density as the major nuclear DNA band of S. purpuratus (1.695 g/ml, relative to a value of 1.710 g/ml for E. coli DNA (Piko, Blair, Tyler & Vinograd, 1968)); (2) compared with bulk nuclear DNA, A', B' or C' DNA is unable to hybridize labelled sea-urchin ribosomal or transfer RNA; (3) these DNAs will hybridize (to a high preference compared with bulk nuclear DNA) messenger-like RNA from gastrula stage embryos labelled just prior to isolation.

The possibility that the isolated homologous DNA pieces are of viral origin seems remote considering that the homologous DNAs hybridize to high-molecular-weight sperm and egg as well as embryo DNA (Figs. 7–9) (and also to messenger-like RNA made at the gastrula stage as mentioned above). Perhaps the most cogent reason for
believing the homologous DNA pieces to be of non-viral origin is that the A’ and B" molecules (each with a molecular weight affording only about one average gene) appear to hybridize almost equally well and in multiple copies to separate regions of the higher-molecular-weight C’ DNA molecule (Fig. 6).

Conceivably, many different species of types A’ and B" molecules may be synthesized and then link together to make a variety of C’ molecules. A minimum variety of C’ molecules would result if the set of A’ molecules making up a particular C’ molecule consisted of the same species (and the companion B" molecules were all of the same species). A maximum variety of C’ molecules would result if different species of A’ and B" molecules were totally interchangeable in linking together to make individual C’ molecules.

I thank Drs C. Brunk, P. Denny and D. Nierlich for their helpful comments during preparation of the manuscript. This work was supported by a grant (HD 04015) from the National Institutes of Health, U.S. Public Health Service and a Biomedical Sciences Support grant (RR 07012) from the same agency.

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Homologous nuclear DNAs from embryos


(Received 4 January 1972)