Identification of key regulated events early in the life of hybrid animal cells constructed by nuclear transplantation

(cytoskeleton/DNA synthesis/gene expression/two-dimensional gel electrophoresis)

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ABSTRACT Reconstituted cells were constructed by fusion of cytoplasts from the human diploid fibroblast cell strain Detroit 532 and karyoplasts from the mouse fibroblast cell line A9. Several cellular properties were examined during the first 48 hr after nuclear transplantation. (i) The overall morphology of the cells originally resembled that of the cytoplasmic donor, Detroit 532, but rapidly changed to approximate that of the nuclear donor, A9. However, definitive changes in the microfilament structure of the reconstituted cells were not seen until 24–48 hr after fusion. These observations support the idea that the presence or absence of an ordered array of microfilament bundles is not the sole determinant of cell shape. (ii) Although cytoplasts and karyoplasts were prepared from cultures of randomly growing cells, the first division of reconstituted cells occurred in a synchronous manner. However, the initiation of DNA synthesis was not synchronized. It thus appeared that, in their first cell cycle, the cells had a G2 period of variable length. The results further suggest that the cytoplasm of interphase fibroblasts contains the material necessary to initiate or support DNA synthesis in a transplanted nucleus and entry into mitosis. (iii) A two-dimensional gel electrophoretic analysis of polypeptide synthesis in reconstituted cell cultures showed that synthesis directed by transplanted mouse nuclei could be detected as early as 3–6 hr after fusion. Some of the mouse polypeptides detected at the earliest time points studied were not among the major polypeptides synthesized by the parental A9 cells. By about 48 hr after fusion, the pattern of polypeptides produced by reconstituted cells was almost indistinguishable from that of the nuclear donor parent cells.

Cytoplasts and karyoplasts prepared from animal cells by cytochalasin-induced enucleation (1, 2) can be fused together to reconstitute whole viable cells (3–5). If the fragments are from two different cell types, cells containing single nuclei within foreign cytoplasm can be constructed (6, 7). By judicious choice of the parental cell types and the use of a variety of staining techniques, such reconstituted cells can be produced in large numbers and definitively identified immediately after fusion (8–11). These cells, having a minimal contribution of cytoplasm from the nuclear donor cell type (12–15), would appear to be excellent systems for investigating the interaction of cytoplasm and nucleus in determining a variety of cellular properties and events, including cell shape and cytoarchitecture and genome replication and expression. However, analyses of both reconstituted and hybrid cells performed so far have indicated that, except for a few instances (16–21), exposure to a foreign cytoplasm has little long-term effect on a transplanted nucleus. In this report it is shown that, in most respects, cells constructed by the method of nuclear transplantation are indistinguishable from the nuclear donor parental cell type as early as 48 hr after fusion. However, results of an examination of such cells during this initial 2-day period suggest that they will be useful in the identification and characterization of a variety of cellular regulatory mechanisms.

MATERIALS AND METHODS

Detroit 532, a low-passage human fibroblast strain (ATCC-CCL54), was maintained in Eagle’s minimal essential medium supplemented with 10% fetal calf serum. Cells used in experiments described here were derived from the 18th–25th generations. Cells of the A9 line, a mutant of mouse L929 cells that lacks the enzyme hypoxanthine phosphoribosyltransferase (22), were grown in Dulbecco’s modification of Eagle’s medium supplemented with 10% calf serum. Cells were demonstrated to be free of mycoplasma contamination (23).

As described in detail previously (8–10), cytoplasts and karyoplasts were prepared and used to construct mouse–human hybrid cells. They could be definitively identified by using a variety of morphological, immunological, and genetic criteria, including nuclear pattern of staining with the fluorochrome Hoechst 33258, appearance of the actin-myosin-containing cytoskeleton, presence of fibronectin, and sensitivity to both aza-guanine and diphertheria toxin. The reconstituted cells gradually became resistant to both agents, as they lost hypoxanthine phosphoribosyltransferase molecules from their cytoplasm and toxin receptors from their surface (8, 10). Staining of cell nuclei with the fluorochrome Hoechst 33258 and indirect immunofluorescence using rabbit antiserum to myosin were performed as previously (8). Observation of fluorescent staining, combined with autoradiography, was accomplished by the method devised by Lockwood (24). For autoradiography, cells were labeled for 6-hr periods with [3H]thymidine (8 Ci/mmol, 1 Ci = 3.7 × 10¹² Bq) at 5 μCi/ml in normal growth medium supplemented with 5% diazoyed fetal calf serum. Preparation of [³⁵S]methionine-labeled cell extracts and two-dimensional gel electrophoresis in the nonequilibrium pH gradient electrophoresis (NEPHGE) system were performed as described by Bruno et al. (25).

RESULTS

Both the overall morphology and the actin- and myosin-containing cytoskeleton (as visualized by indirect immunofluorescent staining) of the two cell types used in these studies appeared quite different. The human Detroit 532 fibroblasts (Fig. 1a) were long, thin and flat, and generally bipolar, and they deployed a highly ordered array of stained microfilament bundles. The mouse A9 cells (Fig. 1b) were less well spread upon the substrate, were often tripolar or quadrupolar, and exhibited

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only a diffuse staining of the cytoplasm when examined by the immunofluorescence technique. Reconstituted cells were prepared by fusion of Detroit 532 cytoplasts and A9 karyoplasts. As described previously (8-10), cultures of such reconstituted cells, with nonenucleated Detroit 532 cytoplasts present, have no detectable contamination by the A9 nuclear donor cell type and no more than 1% contamination by nonenucleated Detroit 532 cells. Thus, in an experiment in which 20% of the cytoplasts were reenuclated with mouse nuclei—the average experimental result with these cell types—at least 95% of the observed nucleus-containing cells would be true reconstituted cells. They possessed mouse nuclei—identified by their staining pattern with the fluorochrome Hoechst 33258—within bodies having the overall morphology and cytoskeletal structure of the human cytoplasmic donor cell type (8). One reconstituted cell is shown in Fig. 2 a and b. Examination of numerous cells showed that they began to change shape within a few hours after transplantation was completed. By 16-18 hr, they clearly resembled the mouse nuclear donor; by about 48 hr, they were generally indistinguishable from A9 cells (8). Occurrence of these events was confirmed by time-lapse cinematography.

In order to determine if this changing morphology could be correlated with alterations in the cytoskeletal structure of the cells, the pattern of immunofluorescent staining, detected by using antiserum directed against myosin, was examined at various times after nuclear transplantation. In these experiments, the cells were also stained with the fluorochrome Hoechst 33258, so that mouse and human nuclei could be identified. For the first 24 hr, all reconstituted cells (and also nonenucleated cytoplasts) possessed easily identifiable myosin-containing cables running throughout their cytoplasm (see Fig. 2 a and c). The only feature that occasionally distinguished the cytoarchitecture of reconstituted cells from that of Detroit 532 cells was the appearance of the star-like structures shown in Fig. 2c. Sometime after 24 hr, the ordered cable structure disappeared from the cells; by 48 hr after preparation, none of the cells having mouse nuclei possessed the ordered arrays characteristic of the human cytoplasm donor (see Fig. 2 e and f). This cytoarchitectural reordering (or disordering) apparently occurred under the direction of the new transplanted nuclei. As shown in Fig. 2 e and f, nonenucleated Detroit 532 cytoplasts in the same culture dishes still contained microfilament networks identical to those of Detroit 532 whole cells.

During determinations of the viability of both reconstituted L929 cells (5) and Detroit 532–A9 reconstituted cells (8), it was noted that there was a lag period before the cells began to divide. This observation was confirmed and quantitated by monitoring both the increase in number of nuclei and the frequency of appearance of mitotic cells in cultures of reconstituted cells. As shown in Fig. 3, few cells divided in the first 15-20 hr after preparation; then a fairly synchronous round of division occurred between 20 and 25 hr. This round of division involved essentially all reconstituted cells: the cell number approximately doubled. This was also confirmed directly in experiments in which the fate of reconstituted cells was carefully followed. Only a small portion of the cells—no more than 8-10%—became highly vacuolated and died within the first 2 days after reconstitution. The burst of mitotic activity was seen in numerous experiments (the results presented in Fig. 3 are typical of four experiments in which extensive data were collected), even in those in which great care was taken to ensure that both the karyoplast and cytoplasm donor populations were in an asynchronous growth phase before enucleation. The observation was also confirmed by viewing time-lapse films of reconstituted cell cultures. Furthermore, observation of the growth of both A9 and Detroit 532 whole cells that had been treated with cytochalasin but not enucleated indicated that the synchrony of division seen with reconstituted cells was not induced simply by exposure to the drug.

This observed synchronization of cell division suggested the possibility that DNA synthesis might likewise be synchronized in reconstituted cells. Therefore, cells were constructed and labeled with [3H]thymidine for 6-hr periods at various times after preparation. DNA synthesis was monitored by autoradiography. The identity of nuclei was again determined by using Hoechst 33258. In these experiments, the Detroit 532 cells were enucleated at suboptimal conditions to increase somewhat the number of contaminating human cells. These cells were likewise scored for DNA synthesis and thus served as an internal control to ensure that no part of the experimental procedure (except nuclear transplantation) induced synchrony in the cells. Autoradiography combined with fluorescence microscopy was performed. Only cells containing nuclei that could be definitively identified as mouse or human nuclei were scored. As shown in Table 1, during any 6-hr period, approximately 30% of the "contaminating" Detroit 532 whole cells were actively engaged in the synthesis of DNA, thus verifying that growth of the cytoplast donor population was indeed asynchronous throughout the experiment. The same result was seen with reconstituted cells: approximately 30% of the cells containing mouse nuclei.
were labeled during any 6-hr period. Separate A9 cultures treated with cytochalasin B likewise showed no marked degree of synchronization of DNA synthesis. These results suggest that (i) the observed synchrony in cell division was not caused by a regulation of DNA replication and (ii) during their first cell cycle, reconstituted cells apparently had a G2 period of variable length.

To complete this initial analysis of events that might be regulated during the early life of reconstituted cells, polypeptide synthesis was examined by using high resolution two-dimensional gel electrophoresis. Such a study was possible because of the high efficiencies of enucleation and nuclear transplantation achieved with this system and because the ability of enucleated Detroit 532 cells to synthesize protein decreases rapidly after their preparation. In the experiment described here, more than 40% of the bodies in a freshly prepared culture were reconstituted cells. At various times after preparation, samples were labeled for 3 hr with [35S]methionine. A9 and Detroit 532 whole cells were similarly labeled. Fig. 4 shows the polypeptide patterns obtained with samples prepared from parental cells and reconstituted cultures labeled at 3, 18, and 48 hr after nuclear transplantation. The 3-hr pattern appeared very similar to that produced by the cytoplasm donor cell type, Detroit 532, and the 48-hr pattern appeared quite similar to that produced by the nuclear donor cell type, A9. The 18-hr pattern resembled both parental cell fluorograms. Several mouse- and human-specific polypeptides whose behavior was observed throughout the experiment are marked with arrows. These few selected examples illustrate that (i) the rate of synthesis of some Detroit 532 polypeptides was already greatly reduced by 3 hr after fusion, whereas synthesis of others was detectable as late as 48 hr; and (ii) synthesis of A9 polypeptides was detected as early as 3 hr after transplantation was completed. Note also that some of the mouse polypeptides detected at 3 hr were not among the major polypeptides made by the parental mouse A9 cells. Some implications of these observations are discussed below.

### Table 1. DNA synthesis in reconstituted cells

<table>
<thead>
<tr>
<th>Labeling period, hr</th>
<th>A9 nuclei (in reconstituted cells)</th>
<th>Detroit 532 nuclei (in contaminating parental cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA−</td>
<td>DNA+</td>
</tr>
<tr>
<td>0.5−6</td>
<td>213</td>
<td>84</td>
</tr>
<tr>
<td>6−12</td>
<td>236</td>
<td>103</td>
</tr>
<tr>
<td>12−18</td>
<td>266</td>
<td>100</td>
</tr>
<tr>
<td>18−24</td>
<td>172</td>
<td>84</td>
</tr>
</tbody>
</table>

Cells were formed from A9 nuclei and Detroit 532 cytoplasts. Cultures also contained nonenucleated Detroit 532 cells, which served as an internal control to ensure that synchrony was not induced by the experimental protocol. Cells were labeled with [3H]thymidine for 8-hr periods beginning 30 min after transplantation. Cells that incorporated label into DNA were identified by autoradiography and mouse and human nuclei were distinguished by staining with Hoechst 33258. Unlabeled (DNA−) and labeled (DNA+) nuclei in a unit area were counted for each time point and the percentages of nuclei labeled were calculated.
DISCUSSION

A combination of techniques for preparing nearly pure populations of cytoplasts and karyoplasts, fusing these bodies with high efficiency, and definitively identifying and enumerating reconstituted cells permitted an analysis of some events occurring soon after a nucleus was placed into a foreign environment. These initial studies yielded the following noteworthy results.

(i) Changes in the morphology of reconstituted cells occurred before observable alterations in cytoskeletal structure. Reconstituted cells appeared to possess both the shape and microfilament architecture of the cytoplast donor cell type, Detroit 532. They rapidly began to assume the morphology of the nuclear donor cell type, A9 (see also ref. 8). However, when the cells had an appearance intermediate between the two cell types, they possessed an ordered array of microfilament bundles like that seen in Detroit 532 cells. It seemed, then, that the mere presence or absence of these cables was not the sole determinant of cell shape. A massive change in cytoarchitecture was seen only after 24 hr—that is, when the first, synchronous burst of mitotic activity had begun. This leads to the speculation that the ordered array of bundles found in the cytoplasm of young hybrid cells cannot reform after the disruption of structure associated with mitosis—a testable hypothesis.

(ii) The first round of division of reconstituted cells occurred in a synchronous manner, but DNA synthesis did not. Two facts originally suggested that DNA synthesis in reconstituted cells formed from cytoplasts and karyoplasts prepared from nonsynchronized parental cells might be initiated in a synchronous manner: mitosis occurred as a fairly synchronous event (see Fig. 3) and, in general, the G1, rather than G2, phase is the most variable phase of the animal cell cycle (see refs. 26 and 27). The finding that no measurable degree of synchronization of DNA synthesis occurred may indicate that cytoplasts are capable of supporting—and perhaps initiating—DNA synthesis in a transplanted nucleus (see also ref. 28). The results presented here suggest further that the cytoplasm of interphase cells cannot provide the information necessary for entry into mitosis. Presumably, a portion of the cytoplasm and karyoplast donor cells were in the G2 phase of the cell cycle at the time of enucleation, and yet there was a substantial lag before any cell division was observed. It is also instructive to compare these results with observations on the regeneration of karyoplasts to reform whole viable cells (14, 15, 29). Karyoplasts, even when prepared from synchronized cells poised at the G1/S interface, do not begin extensive replication of their genome until they have regenerated an entire complement of cytoplasm—that is, at about 72 hr after preparation (unpublished data). These facts may in-
dicate, then, that a foreign cytoplasm can supply to a body essentially incapable of genome replication—the young karyoplast—the material necessary for DNA synthesis; however, this cytoplasm cannot support mitosis, suggesting perhaps that structural components of the cell must be reformed under the direction of the new nucleus. Further experimentation with reconstituted cells and regenerating karyoplasts should provide valuable insight into the relationships of these various cellular events.

(iii) The patterns of polypeptides synthesized in reconstituted cells suggested a complex interaction of cytoplasm and nucleus in regulation of gene expression. When new nuclei were placed into cytoplasts, synthesis of various polypeptides encoded in mRNAs contributed by the cytoplasm donor decreased with various rates: some human-specific polypeptides were no longer made as early as 3 hr after transplantation, whereas others were still made as late as 48 hr. In contrast, analyses of protein synthesis in enucleated cells showed that although the overall rate of synthesis decreased rapidly with time, the two-dimensional gel patterns obtained from cytoplasts radiolabeled for short periods at various times—up to 10–12 hr after enucleation—remained almost identical (1, 30–32). Also of interest was the observation that some mouse-specific polypeptides could be detected as early as 3 hr after transplantation. Some of these molecules were not among the major polypeptides synthesized in the mouse nuclear-donor cell line. These findings are consistent with several interpretations, including the possibility that some mouse genes were actively repressed during the early life of reconstituted cells. Results of analyses of the synthesis of some specific gene products in cells constructed by hybridization or nuclear transplantation suggested that cytoplasm contains elements that can inhibit or repress specific gene expression (20, 33). Direct measurement of the rates of transcription of specific genes in cybrid and reconstituted cells is now clearly essential for further evaluation of this hypothesis. The possibility that genes might be activated by exposure to foreign cytoplasm was also considered. Evidence for the activation of liver-specific enzymes in cells formed by fusion of hepatoma cytoplasts to either erythroleukemia cells (18) or fibroblast karyoplasts (19) has been reported. No evidence for such activation (the appearance of polypeptides present in neither parental cell pattern) was obtained here. However, further experiments, in which extracts from purified populations of reconstituted cells are evaluated by electrophoresis techniques that better resolve subsets of the polypeptides seen in the gel patterns illustrated in Fig. 4 are warranted before the possibility of gene activation occurring in the cell type described here is eliminated.

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