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Identification of Chick Chromosomes in Cell Hybrids Formed Between Chick Erythrocytes and Adenine-Requiring Mutants of Chinese Hamster Cells*

(heterokaryon/chick gene mapping/prematurely condensed chromosomes/auxotrophic mutants)

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ABSTRACT **When chick erythrocytes were fused with adenine-requiring mutants of Chinese hamster cells of two different complementation classes and grown in adenine-free medium for 1 week or longer, mitotic cells were observed containing both chick and Chinese hamster metaphase chromosomes within a single cell. Stable hybrid clones were subsequently isolated that possessed, in addition to a complete Chinese hamster genome, a single specific chick chromosome. Hybrids resulting from fusions involving different adenine mutants retained different, identifiable chick chromosomes. Thus, the two chick genes for the endogenous synthesis of adenine were assigned to the respective chick chromosomes.**

Extensive studies on reactivation of chick erythrocytes in heterokaryons formed with mammalian cells have demonstrated the initiation of DNA and RNA synthesis in chick nuclei and, after formation of chick nucleoli, the appearance of chick-specific proteins or enzymes (1). In these experiments, the mammalian cells were usually irradiated with gamma radiation to prevent them from entering mitosis during reactivation of the erythrocyte nuclei (2). Thus, in such fusions, the appearance of mitotic cells containing metaphase chromosomes of both species within a single cell and the subsequent isolation of hybrids capable of continuous proliferation were rarely observed.

If one uses conditional lethal mutants of mammalian cells in these fusions, however, it becomes possible to arrest the growth of the mammalian cell and to allow completion of erythrocyte reactivation in the heterokaryons. Schwartz *et al.* (3) used mouse A9 cells, which lack hypoxanthine phosphoribosyl transferase (EC 2.4.2.8.) activity and do not grow in HAT medium (which contains hypoxanthine, aminopterin, and thymidine), a medium in which normal cells possessing the transferase do grow (4). When the A9 cells were fused with chick erythrocytes and grown in HAT medium, clones that survived were isolated and apparently possessed the chick-specific transferase; however, no chick chromosomes could be identified in these clones.

In our laboratory, various auxotrophic mutants of Chinese hamster cells have been isolated by use of 5-bromodeoxyuridine plus near-visible light (5). These mutants have been shown by complementation analysis to involve mutations in 15 different genes (6). They exhibit all-or-none growth characteristics with respect to availability in the medium of nutrilites such as glycine, proline, adenine, inositol, thymidine, etc. All of the mutants studied are highly stable, with a spontaneous reversion rate of 10^{-7} or less. Several of these auxotrophs have been hybridized with human cells and are useful in human linkage analysis (7–10). The human chromosomes are rapidly eliminated from such hybrids, except for one or a few that are retained in order to provide necessary genes for survival of the hybrids in the selective medium.

When such auxotrophic mutants were inoculated into the respective deficient, selective media, they began to attach and stretch on the surface of the dishes. After incubation at 37° overnight, some of the mutants, e.g., those requiring glycine, became rounded and then shrank and were released into the medium; these cells died at a rapid rate. However, two of the adenine-requiring (ade^-) mutants, adeA and adeB, could remain attached for 2 weeks or longer during starvation for adenine. Thus, it seemed possible that if these mutants were fused with chick erythrocytes and the resulting heterokaryons were grown in adenine-free medium, the mutant, while unable to divide, would be able to survive while waiting for reactivation of erythrocyte nuclei and development of chick nucleoli. As soon as the chick adenine-synthesizing enzymes are produced in the heterokaryon, the two nuclei may become synchronized and enter mitosis together, since no lethal irradiation was used to inactivate the hamster nuclei. If this should be the case, then mitotic cells with metaphase chromosomes of both parents confined in a single cell should result. Furthermore, in the subsequent divisions after the first synchronous mitosis, the resulting hybrids may lose unnecessary chick chromosomes as the hybrids do in the Chinese hamster–human crosses (7, 10). However, one critical chick chromosome carrying the necessary adenine gene should always be retained as the hybrids grow in the selective medium. Hybrid clones could then be isolated that may carry an identifiable chick chromosome to which the chick adenine gene may be assigned.

This report describes observations on the events that occurred after fusion between chick erythrocytes and ade^- mutants of Chinese hamster cells and isolation of hybrid clones that no longer required adenine and that possess single, specific, and readily identifiable chick chromosomes.

MATERIALS AND METHODS

The two adenine-requiring mutants, adeA and adeB, used in these fusions are clonal populations isolated just before these experiments. They contain a highly uniform and stable

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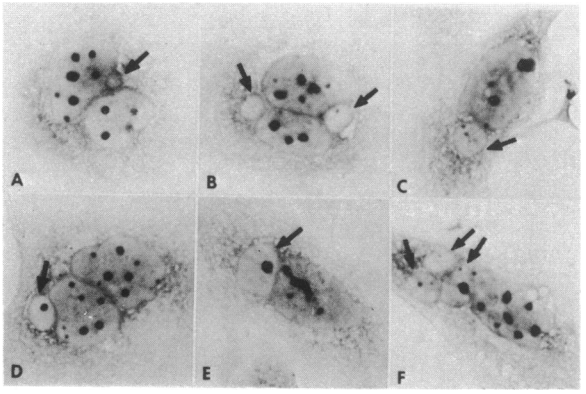

FIG. 1. Enlargement of chick erythrocyte nuclei and development of chick nucleoli in the heterokaryons. The cells were sampled between 1 and 5 days after fusion. *Arrows* indicate chick nuclei. (*A*) A slightly enlarged chick nucleus with no visible nucleoli; (*B* and *C*) further enlargement of chick nuclei with discernible nucleoli; (*D* and *E*) well-developed nucleoli in the maximally enlarged chick nuclei; (*F*) three chick nuclei with various degrees of nucleolar development.

karyotype, of which the adeA mutant has 21 while the adeB mutant has 20 chromosomes. They have enzyme deficiencies in the early steps of *de novo* purine biosynthesis, possibly between 5-phosphoribosyl pyrophosphate and 5-aminoimidazole-4-carboxamide ribonucleotide (11). Their growth requirement can be fulfilled by supplementing minimal medium with adenine, hypoxanthine, inosinic acid, their ribonucleoside or ribonucleotide, or 5-aminoimidazole-4-carboxamide (11). These mutants were induced by treatment of the parental CHO-Kl cells with ICR-191, an acridine mustard (12).

The cells were routinely cultivated in F12 medium (13) plus 10% fetal-calf serum. Methods of cell cultivation and handling of hybrid clones have been described (5, 7). When selective medium is needed for specific experiments, a minimal medium, F12D, supplemented with the macomolecular fraction of fetal-calf serum is used (5, 6). This deficient medium

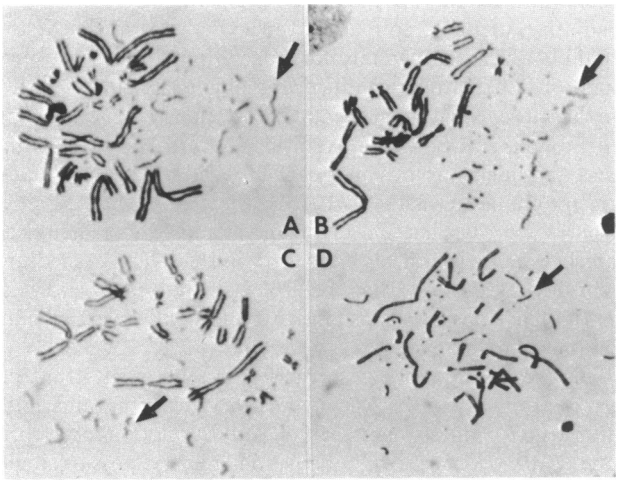

FIG. 2. Cells showing G_1 prematurely condensed chromosomes of the chick genome (*arrows*). The 21 large, double-stranded metaphase chromosomes are of adeA. The chick genome has many small, dot-like chromosomes, some of which can not be seen in these photographs.

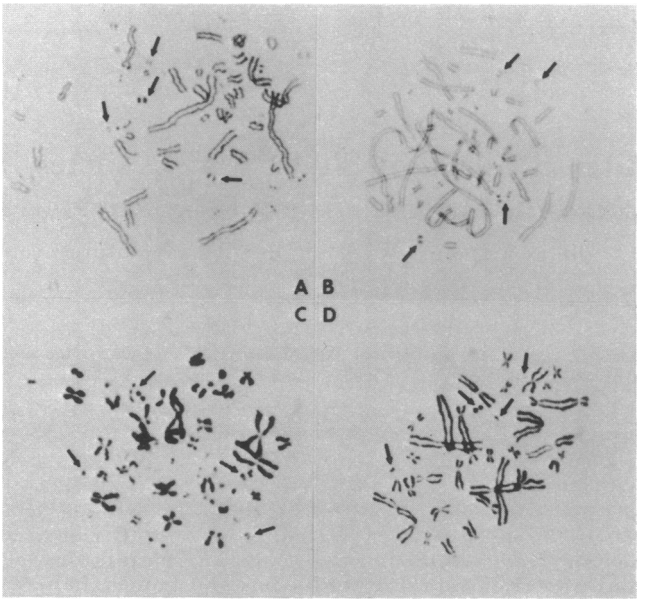

FIG. 3. Mitotic cells containing metaphase chromosomes of both the chick and the Chinese hamster genomes. *Arrows* indicate examples of the small dot-like chick chromosomes, which can be distinguished from the large, double-stranded adeA chromosomes. The cells in (*A*) and (*B*) were sampled 10 days after fusion; those in (*C*) and (*D*) were sampled 14 days after fusion. About 38 chromosomes can be counted in (*D*), of which 21 may be of adeA and 17 of chick. Although the exact identification of these chromosomes is difficult, the small dot-like chromosomes (*arrows*) can be unambiguously assigned to the chick genome.

contains no nutrilites that can support growth of the ade$^-$ mutants.

Erythrocytes were obtained from 12-day-old chick embryos and were purified of leukocytes. Cell fusion was done by mixing 10^7 erythrocytes and 10^6 ade$^-$ cells and suspending them in serum-free F12D; ultraviolet-inactivated Sendai virus of 500 hemagglutinating units was then added, and the fusion mixture was kept at 4° for 15 min and incubated at 37° for another 20 min. The mixture was then diluted with F12D and inoculated into a series of petri dishes containing coverslips

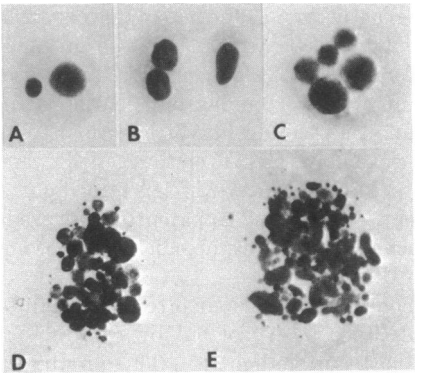

FIG. 4. Cells observed 2 weeks after fusion. (*A*) Heterokaryon containing a highly enlarged chick nucleus (*left*); (*B*) homokaryon of two ade$^-$ nuclei (*left*) and an unfused ade$^-$ cell (*right*); (*C*) heterokaryon containing three enlarged chick and two ade$^-$ nuclei; (*D* and *E*) multiple fusions resulted in heterokaryons containing numerous ade$^-$ and chick nuclei, the latter exhibiting various degrees of enlargement.

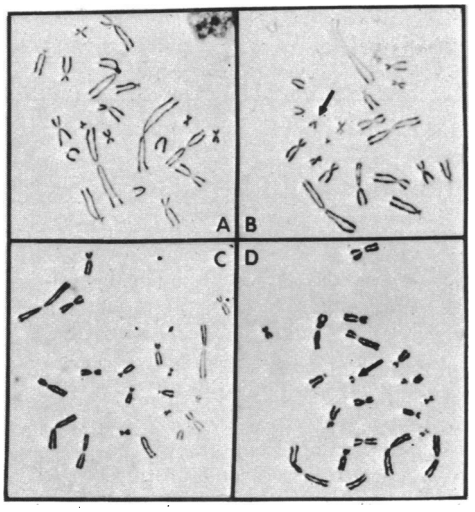

FIG. 5. Metaphase cells of adeA (*A*) and adeB (*C*) and hybrid clones from adeA–chick (*B*) and adeB–chick (*D*) fusions. *Arrows* in (*B*) and (*D*) indicate the foreign chromosomes present in hybrid, but not in parental, clones.

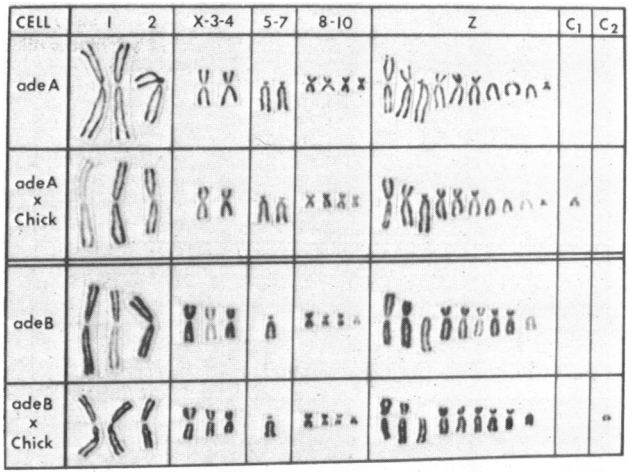

FIG. 6. Karyotype of adeA and adeB parents and hybrids.

plus F12D supplemented with the standard macromolecular serum component. Incubation was resumed, and samples were removed daily for the following 2 weeks. The medium was changed every 3 days. The coverslips were fixed in Carnoy's solution (absolute ethanol–glacial acetic acid 3:1) and stained with Giemsa. In some samples colcemid (0.01 μg/ml) was added to the dishes 5 hr before fixation.

For treatment by the trypsin–Giemsa banding procedure, 75 mM KCl was added to harvested cells for 15 min at room temperature. The cells were fixed in freshly prepared solutions of methanol—glacial acetic acid 3:1. Air-dried slides were stored for 1 day before trypsin treatment. Slides were immersed in freshly prepared 0.01% tryspin (Nutritional Biochemicals, Corp., 3× crystallized) in phosphate-buffered saline (pH 7.4) for 10–15 sec in an ice bath. Further trypsin action was stopped by transferring the slides quickly to phosphate-buffered saline containing 1% fetal-calf serum. The slides were then rinsed well in phosphate-buffered saline, stained with Giemsa, and mounted.

RESULTS

Reactivation of Chick Erythrocyte Nuclei. Enlargement of erythrocyte nuclei began 1 day after fusion. The extent of enlargement and the number of erythrocytes undergoing enlargement continued to increase in the following several days, accompanied by development of nucleoli in the enlarged chick nuclei (Fig. 1). All these observations are in agreement with those described by Harris and his colleagues (2).

Appearance of Prematurely Condensed Chick Chromosomes. During the first and second day after fusion, mitotic cells were observed which contained, in addition to a complete set of Chinese hamster metaphase chromosomes, a set of fine thread-like chromosomes (Fig. 2, *arrows*) that were similar in morphology to the prematurely condensed chromosomes of the G_1 type described by Johnson and Rao (14). When they fused mitotic HeLa cells with G_1 (pre-DNA synthetic period) HeLa cells, horse lymphocytes, or chick erythrocytes, prematurely condensed chromosomes exclusively of the G_1 type, exhibiting single-stranded chromatid appearance, were observed (14, 15).

Because asynchronous Chinese hamster cells were used in the present experiments and because the fraction of mitotic cells in an asynchronous cell population is small, about 3–5% (16), the chance for the mitotic cells to fuse with the erythrocytes and to induce G_1 prematurely condensed chromosomes would be extremely small. This was shown from the fact that no cells with G_1 prematurely condensed chromosomes were ever observed in samples taken within 2 hr after fusion. However, after 1 day of incubation, cells with G_1 prematurely condensed chromosomes became evident, although they were reduced in number in 2-day and 3-day samples and were no longer observed in the following days. In samples that had been treated with colcemid for 5 hr before fixation, more cells showing G_1 prematurely condensed chromosomes accumulated.

These observations thus suggest that those ade^- cells that were in late G_2 phase (post-DNA synthetic period) at the time of fusion had moved through the cell cycle inside the heterokaryon at a reduced rate under starvation conditions, and finally entered mitosis and elicited G_1 prematurely condensed chromosomes in the chick erythrocytes (a cell type known to be arrested in G_1). These mitotic heterokaryons may enter interphase again, but their fate was not followed in this study.

Appearance of Metaphase Chromosomes of Both Chick and Ade^- Genomes in the Same Cell. During the first week after fusion, reactivation of chick erythrocytes and development of nucleoli progressed; but no single cells with metaphase chromosomes of both species were found except those containing G_1 prematurely condensed chromosomes, which appeared and then disappeared during the first 3 days after fusion. On the seventh day after fusion, however, the first mitotic cell with complete sets of both chick and Chinese hamster metaphase chromosomes emerged. The number of such cells increased in the following days. Samples of some of these mitotic cells are shown in Fig. 3.

It thus appears that, after erythrocyte nuclei had been reactivated, chick nucleoli had developed, and chick adenine enzymes had been produced, starved Chinese hamster nuclei in the heterokaryons revived and achieved synchrony with the chick nuclei; the two then entered mitosis hand in hand, a situation analogous to that described by Rao and Johnson

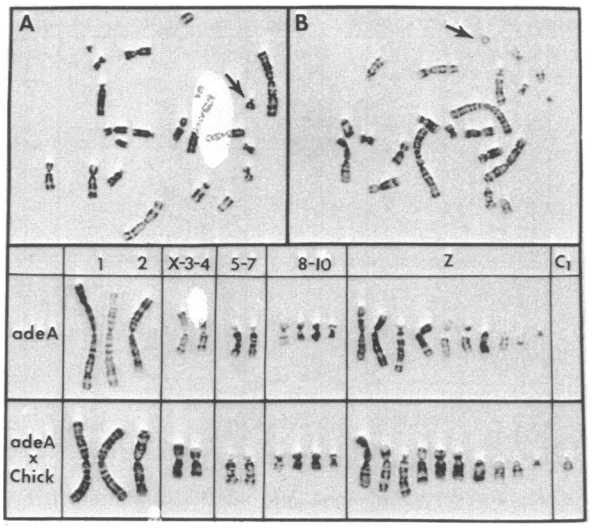

FIG. 7. (*A* and *B*) Trypsin–Giemsa banding patterns of two representative hybrid clones from adeA x chick. *Arrows* indicate the foreign chromosome C_1. The hybrid karyotype shown in the bottom row was prepared from the cell in (*B*).

(17). These workers observed that, in fusions of cells from different phases of the cell cycle, synchrony could be achieved in the heterokaryons at the entry of either the S phase (DNA-synthetic period) or mitosis, depending on the stages of the cell cycle in which each of the fusion partners resided before fusion.

In the latter part of the second week after fusion, some of the mitotic cells that were observed contained a complete adeA genome but reduced numbers of the chick chromosomes (e.g., Fig. 3*D*). These cells possibly resulted from loss of chick chromosomes during subsequent divisions of the hybrid cells after the first synchronous mitosis.

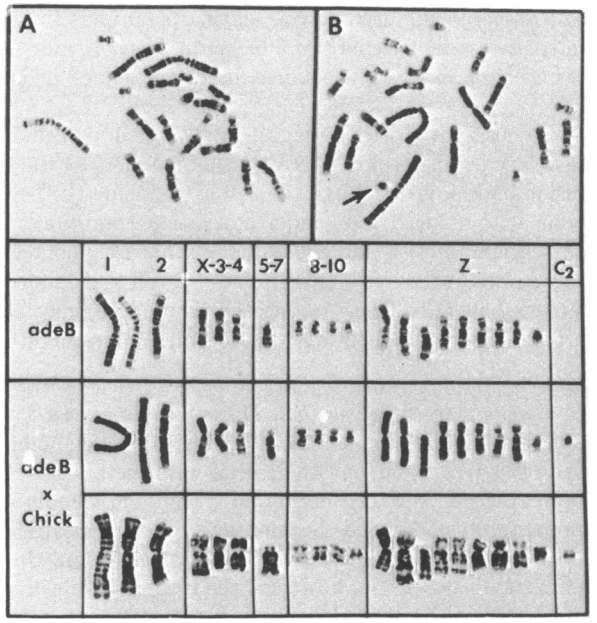

FIG. 8. Trypsin–Giemsa banding patterns of adeB (*A*) and a hybrid clone from adeB x chick (*B*). *Arrow* in (*B*) points to the C_2 chromosome. The hybrid karyotype in the bottom row was prepared from a hybrid cell that had undergone endoreduplication; the identical pairs of each chromosome make the bands especially distinct. In the hybrid karyotype shown in the middle row, one of the Z-group chromosomes (second one from right) apparently had some extra chromosomal material added to the distal end of the long arm, which was resolved by the banding procedure. This chromosome appeared normal in other spreads.

TABLE 1. *Summary of the events that occurred after fusion between chick erythrocytes and ade⁻ mutants of Chinese hamster cells*

Stage	No. of days after fusion	Characteristics	Reference figs.
I	1–7	Enlargement of erythrocyte nuclei and development of chick nucleoli	Fig. 1
	1–3	Appearance of cells with a complete set of ade⁻ metaphase chromosomes plus G_1 prematurely condensed chromosomes of the chick genome	Fig. 2
II	7–14	Appearance of mitotic cells with metaphase chromosomes of both parental genomes in the same cell	Fig. 3
III	14–21	Isolation of hybrid clones containing complete ade⁻ karyotype plus a single chick chromosome	Figs. 5–8

Toward the end of the second week after fusion, most of the heterokaryons containing reactivated erythrocyte nuclei showed no sign of growth; some of them remained attached to the surface of the coverslips while others were detached and died. Because the ade⁻ mutants suffer reduced viability after starvation for adenine (11), many ade⁻ nuclei in the heterokaryons could have been irreversibly impaired by the time the erythrocytes became reactivated and the chick adenine enzymes were synthesized. In such cases, the heterokaryons would not move to mitosis even though the erythrocytes had been reactivated (Fig. 4*A* and *C*). In addition, heterokaryons that resulted from a high degree of multiple fusions apparently had difficulty in all of their nuclei achieving synchronous entry into motisis (Fig. 4*D* and *E*).

Isolation of Hybrid Clones from Selective Medium. During the third and the following weeks after fusion, discrete colonies began to appear in plates containing selective F12D medium. Six clones were picked from adeA–chick and five from adeB–chick fusions. These clones grew actively in F12D medium at a rate somewhat slower than in complete F12 medium. Hybrid clones from the adeA–chick cross grew faster than those from the adeB–chick cross in either medium.

Karyotypic Analysis of Hybrid Clones. As soon as enough cells were obtained in each of the hybrid clones, the standard karyotypic analysis was performed. Fig. 5 shows the metaphase cells of the parent and the typical hybrid clones. Fig. 6 presents the karyotype of these cells. All of the adeA–chick hybrid clones had 22 chromosomes of which 21 were identical to those of the adeA parent; the extra chromosome, designated C_1, was clearly not one of the adeA chromosomes. The hybrid clones resulting from adeB–chick fusions all had 21 chromosomes, one more than in the adeB parent. Karyotypic analysis revealed that this foreign chromosome, designated C_2, differed in morphology not only from the 20 adeB chromosomes but also from the C_1 chromosome.

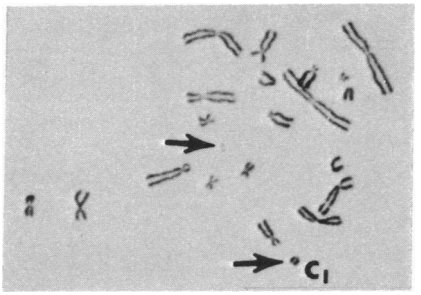

FIG. 9. A hybrid cell observed in the uncloned population of adeA x chick. *Arrows* indicate the chick chromosomes C_1 and a pair of dot-like chromosomes.

The trypsin–Giemsa banding analysis was performed on these cells (Figs. 7 and 8). Based on the specific bands resolved in each chromosome, it became clear that all of the 21 chromosomes in the adeA–chick hybrids, except C_1, could be matched with an identical counterpart of the 21 adeA chromosomes (Fig. 7). This finding suggests that no chromosomal changes had occurred in the hybrids and the foreign chromosome C_1 was probably from chick. Similarly, no counterpart of the C_2 chromosome could be found in the adeB karyotype (Fig. 8); thus, this foreign chromosome probably was also of chick origin.

The uncloned hybrid-cell population derived from the adeA–chick fusion was grown in selective medium for 1 month, after which chromosome analysis was performed. Although the majority of the hybrid cells had the karyotype identical to that shown in Fig. 6, a few cells possessed, in addition to the C_1 chromosome, a pair of dot-like chromosomes characteristic of the small chick chromosomes (Fig. 9).

Karyotype of the Chick Genome. Embryonic chick cells were established in culture and the karyotype was prepared in the early cell passages (Fig. 10). Because of the small, dot-like chromosomes in the chick genome, the exact number of chromosomes is difficult to determine. By counting a large number of spreads, the numbers usually ranged between 70 and 78. The largest eight pairs of autosomes and the two sex chromosomes were karyotyped and presented in the lower part of Fig. 10. The karyotype is in agreement with that described by others (18, 19). The numbering system adopted here differs slightly from that proposed by Owens (18) in that the sex chromosomes are not numbered in sequence with the autosomes.

After we examined a large number of spreads, it seemed fairly certain that the C_1 chromosome was likely to be chick chromosome no. 6, and C_2 to be no. 7. Thus, the genes responsible for the enzyme deficiencies in the adeA and adeB mutants can be assigned to chick chromosomes no. 6 and no. 7, respectively.

Stability of the Chick Chromosomes in the Hybrids. The hybrid clones that were isolated had been grown in F12D medium for over 100 generations without exogenous supplement of adenine. Another series of the same hybrid clones had been grown in complete F12 medium for the same period. Karyotype analysis of these clones was done every week. The C_1 and C_2 chromosomes were present in the respective hybrids even when the cells were grown in complete F12 medium, a condition where the presence of these chick chromosomes in the hybrids was not essential.

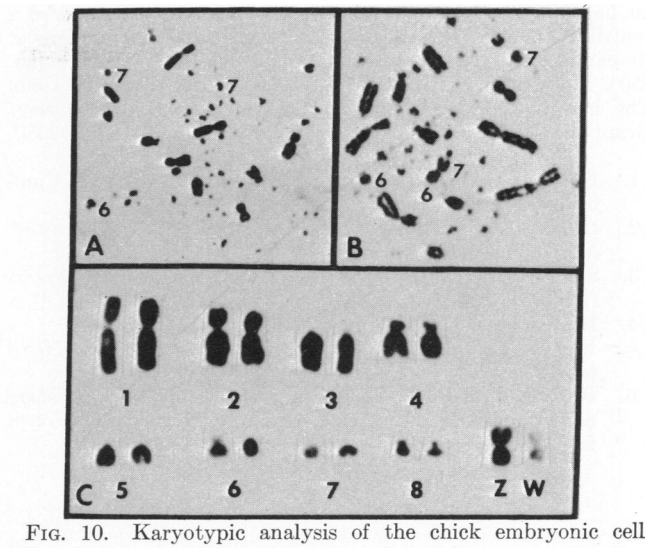

FIG. 10. Karyotypic analysis of the chick embryonic cell culture. The chromosomes shown in (*B*) had been treated by the trypsin–Giemsa banding procedure. The karyotype presented in the lower part of the figure was prepared from the cell in (*A*), which possesses the female sex chromosomes ZW. One of the no. 6 chromosomes (*right*) in the karyotype appeared distorted in this preparation; but the pair of no. 6 chromosomes appeared normal in other karyotypes analyzed. In (*B*) the no. 6 chromosome on the right (near center) was located very close to the no. 7 chromosome.

DISCUSSION

The sequence of events that occur after fusion of chick erythrocytes and Chinese hamster ade$^-$ mutants is summarized in Table 1. The following implications emerge from the findings presented in this study: (*1*) The entire chick genome in erythrocytes can be reactivated to undergo mitosis after fusion with mammalian cells. While it was previously concluded that the blocked macromolecular synthesis of chick erythrocytes could be relieved by appropriate fusions (1), there was no evidence that a similar situation could be achieved with respect to the mitotic process. It has now been shown that all of the steps in the resumption of cell multiplication occur, since synchronous entry into mitosis of heterokaryons formed between chick erythrocytes and mammalian cells is achieved. Long-term cultivation of hybrid cells that include the chromosomes of both species has been demonstrated. (*3*) Single, specific chromosomes of the chick genome have been identified in the stable chick–Chinese hamster hybrid cell. This is the first demonstration of the chromosomal localization of chick genes by use of somatic cell hybridization.

These observations show that essentially the same operations that have been so useful for genetic analysis in mammalian somatic cells can now be done even for cells of different phylogenetic classes. The use of somatic cells *in vitro* for genetic study has represented a new development in biology. It has made possible various genetic studies, including mutational analysis and genetic exchange, without necessitating meiosis and the mating of individuals. Moreover, whereas conventional mating procedures for genetic analysis are limited to crosses between members of the same species, somatic cell techniques can now be used even for members of different classes in the animal kingdom.

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