Biochemical and Cytogenetic Characterization of Rat Hepatoma Cell Lines in Vitro

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SUMMARY

Comparative studies on H4-II-E-C3 cells, originating from a rat hepatoma, and two lines derived therefrom are described. On the basis of morphology, kinetics of growth, tumorigenicity, and karyotype, the three lines segregated into two distinct groups. All three cell lines were, however, related as evidenced by the presence of certain marker chromosomes. Isozyme measurements could be used to separate these cells into three distinct groups. Quantitative biochemical differences were also discerned among all three lines. These were based on the inducibility of tyrosine aminotransferase with steroid and the activity profile of pyridine-adenine dinucleotide transhydrogenase during the growth cycle.

These studies illustrate the necessity for use of multiple parameters to characterize evolving cell lines in vitro. Furthermore, they suggest the feasibility of using a combination of cytogenetic and biochemical measurements, in conjunction with model systems, for the study of mammalian cell genetics and the question of tumorigenesis.

INTRODUCTION

The in vitro culture technique allows direct exposure of mammalian cells to a wide variety of agents and conditions, which may lead to the evolution of new strains. Montagnier et al. (11) reported the isolation of an epithelioid variant of the BHK21 hamster fibroblastic line. Dawe et al. (3) described the emergence of a fibroblast-like variant from an epithelioid cell line derived from a chemically induced hepatocarcinoma in the rhesus monkey. The ease with which changes can occur in vitro poses a problem when genetic stability is desirable. The control of such changes, on the other hand, can be most useful as in the production of complementary strains of cells for genetic studies. In addition to the obvious morphological changes described above, more subtle differences also have been reported. Bottomley et al. (1), for example, have shown widely varying biochemical and cytogenetic differences in a random collection of HeLa strains. Higgins et al. (7) studied variability of several traits of the KB cell line. This communication describes the use of biochemical and cytogenetic parameters for the characterization of 2 cell lines derived from an established rat hepatoma line cultured in vitro.

MATERIALS AND METHODS

Cell Cultures. The H4-II-E-C3 line was originally established (15) from the H35 minimal-deviation hepatoma (18) in the rat. This line is the parent of the 2 derived lines to be described and will be designated the H4 line. The H4 cells used in these studies originated from the 2nd of 3 cultures obtained from Dr. V. R. Potter at the McArdle Laboratory. These cells are epithelioid and form discrete colonies when grown on glass or plastic surfaces; they reach their stationary phase in 4 to 5 days at 80 to 90% confluency. When inoculated s.c. or i.m. into rats of the ACI strain (Microbiological Associates, Walkersville, Md.), H4 cells invariably form tumors that kill their hosts in about 6 weeks (C. DeLuca and J. A. Mason, in preparation).

Line F/rh was observed as a morphologically different cell in the 1st sample of H4 cells following a brief period in a variety of test growth media. This cell type was easily purified since it outgrew the parent cells on selected media (C. DeLuca and B. DeWald, unpublished observations). F/rh cells appear spindle shaped in low-density cultures; they reach confluency in 3 to 4 days and appear epithelioid in dense culture. Unlike the parent line, F/rh cells fail to form tumors when inoculated into ACI rats.

Line 9Q was isolated from a tumor formed by s.c. inoculation of H4 cells into a female ACI rat. This line is epithelioid in vitro and exhibits a growth rate and pattern similar to those of the parent line. When inoculated in vivo, 9Q cells form tumors that are histologically indistinguishable from those formed with H4 cells.

All 3 cell lines described were cultured as reported previously for H4 cells (6). They were maintained by subculture every 5 to 7 days into Medium S20 (Associated Biomedic Systems, Inc., Buffalo, N. Y.) supplemented with 20% (v/v) heat-inactivated fetal calf serum. The studies presented here were performed simultaneously on all 3 lines within 7 or 8 weeks.
Cytogenetic Studies. Chromosome preparations were obtained following a slight modification of the method of Moorhead et al. (12). At 60 to 70% confluency, cells of all 3 lines were treated with Colcemid (0.05 μg/ml culture medium) for the last 6 hr of culture and harvested by gentle scraping with a rubber policeman. Following centrifugation, the supernatant was discarded, and the cells were resuspended in 1% sodium citrate and incubated at 37° for 10 to 15 min. Fixation was achieved by 3 changes (10 min each) of 3:1 absolute methanol:glacial acetic acid. Four drops of cell suspension were placed on a microscope slide, prewet in 70% methanol, and passed through a flame for blaze-drying. Staining was achieved with 2% aceto-orcein; the chromosomes were examined by phase-contrast microscopy.

Biochemical Studies. Vertical electrophoresis (Buchler Instruments, Fort Lee, N. J.) was accomplished at 4° with 14% starch (Electro-Starch Company, Madison, Wis.) gels in the Tris:borate:EDTA buffer system of Boyer et al. (2). After 4 days of growth, cells were treated with trypsin, harvested, and washed once with cold 0.9% NaCl solution. They were resuspended in 5 mM phosphate buffer, pH 7.2 (approximately 8 X 10^6 cells/ml), homogenized on ice, and stored at −15°. The homogenates were thawed at the time of electrophoresis, and aliquots were taken for the determination of protein concentration by the method of Lowry et al. (9). For comparative runs on the 3 cell lines, homogenates were adjusted to the same total protein concentration with phosphate buffer. Forty-μl aliquots (approximately 100 μg protein) of 600 X g supernatant were placed in the preformed sample slots of the starch gel, and electrophoresis was accomplished with a constant voltage gradient of 12 V/cm for 17.5 to 22.0 hr. Following this, the gels were sliced horizontally and stained for enzyme activity with the use of specific assay mixtures. Preparation of the starch gel and staining procedures have been described in detail previously (10, 19).

Studies on the induction of tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.8) were performed essentially according to the method of Thompson et al. (20). The medium of replicate cultures was decanted, and cell monolayers were washed 3 times with basal medium not containing serum. Washed cultures were randomized and split into 2 groups. Fresh basal medium was added to each group; that applied to the experimental group contained 1 μM hydrocortisone acetate.

RESULTS
The distribution of chromosome numbers for the parent and the 2 derived cell lines is presented in Table 1. The H4 and 9Q lines exhibited a sharp modal number of 52 chromosomes; a considerable number of cells of each line showed 53 chromosomes. Both lines exhibited an identical karyotype (Fig. 1, lower), consisting of 31 acrocentric chromosomes and 17 metacentrics with 3 and sometimes 4 large submetacentric marker chromosomes. With the exception of these markers, all elements resembled normal ACI rat chromosomes. This may best be seen by direct comparison to the karyotype published by Nowell et al. (14) for the normal ACI rat. This is shown in Fig. 1 (upper). It appears from this comparison that duplications may have occurred at chromosome 12 and also in the 14 to 20 region.

No sharp mode could be established for the F/rh line (Table 1). The chromosome number in most cells ranged between 69 and 74. Furthermore, the karyotype of this line was quite different from that of the other 2 lines (Fig. 2). The largest acrocentric chromosomes characteristic for the rat were missing. However, 6 to 8 new pairs of acrocentrics were present. These were similar to those of the 4 to 10 group. In addition, 9 new pairs of metacentrics were present that did not resemble those normally present in the ACI rat. Marker chromosomes like those observed in the H4 and 9Q lines were also present in F/rh cells.

Isozymic distribution for certain specific enzymes was examined concurrently with the cytogenetic analyses. Lactate dehydrogenase (L-lactate:NAD oxido-reductase, EC 1.1.1.27)
Tyrosine aminotransferase activity can be induced in H4 cells was quite distinct. Two other enzymes, phosphoglucomutase (6-phospho-D-gluconate:NADP oxidoreductase, EC 1.1.1.44) and pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) showed the inverse to be true. Phosphoglucomutase activity in the 9Q line differed from that in the H4 line in the migration of the fastest of the anodal bands (Fig. 4). Despite the distinct karyotypic differences between the F/rh and H4 lines, the isozymic expression for this enzyme was identical for these 2 cell lines. The same comparative relationships held for pyruvate kinase activity, as shown in Fig. 5. Here, however, the differences exhibited by the H4 and 9Q lines were more striking. Furthermore, although the H4 and F/rh lines displayed qualitatively similar patterns, the intensity of staining indicated quantitative differences.

Differential expression among all 3 cell lines was exhibited by both 6-phosphogluconate dehydrogenase (6-phospho-D-glucuronate:NADP oxidoreductase, EC 1.1.1.44) and phosphoglucose isomerase (D-glucose 6-phosphate ketolisisomerase, EC 5.3.1.9). This is illustrated in Figs. 6 and 7. Quantitative differences in enzymic activity also were observed between cells of the parent line and the derived lines. Tyrosine aminotransferase activity can be induced in H4 cells in vitro in the presence of hydroxycortisone (15, 17). Comparative studies have demonstrated that the enzyme of the H4 line exhibited a 3- to 4-fold greater induction, in the presence of the steroid, than that of the derived 9Q line (Chart 1). Constitutive or preinduced levels of tyrosine aminotransferase were essentially the same for both these cell lines. These were determined on cells grown and harvested simultaneously and under identical conditions to avoid any influence of the physiological state of the cultures (5).

DISCUSSION

Comparative studies between wild-type and complementary mutant strains of microorganisms have led to the basic tenets of modern molecular genetics. Before this could be accomplished, at least 3 things were required: (a) the provision or production of stable mutants at single loci; (b) the recognition of such mutants by the use of sensitive biochemical or pharmacological parameters; and (c) the selection and preservation of these mutants. Currently, an attempt is being made to develop and adapt appropriate models for the study of mammalian cell genetics by the methods of molecular biology (16). The results described in this paper provide an example of the feasibility of applying a variety of techniques to characterize mammalian cells in order to uncover subtle differences among them. Variations in specific isoymic patterns in the H4 and 9Q cells, in the face of karyotypic similarity, suggest that small genetic differences may exist between these 2 cell lines. These might provide a complementary combination of possible use in the genetic analysis of mammalian cells.

The mechanism of host-induced variation or selection in vivo, apparently responsible for the derivation of the 9Q line, is not understood. The recent work of Muragishi et al. (13) illustrated a strong selective effect at the karyotype level when cells were grown in the cheek pouch of the hamster. Alternate and sequential culture in vivo and in vitro was reported to cause a shift in derived populations with a progressive sharpening of the modal number of chromosomes. The quantitative difference in the inducibility of tyrosine aminotransferase observed between the H4 and 9Q lines is noteworthy. Levisohn and Thompson (8) have isolated stable derivatives from the HTC line that induce only to the extent of one-twentieth that of the parent. Earlier, Higgins et al. (7) reported the isolation of clones from heterogeneous KB cultures which differed in the inducibility of alkaline phosphatase.

The origin of the F/rh line remains an unresolved question that demands further study. Since the H4 line was not a clone in the strictest sense, the F/rh line may represent a latent cell type carried in the original expant population. The presence of chromosomes normally found in the ACI rat precludes the possibility that this was merely a heterologous contaminant. The presence of other chromosomes may be indicative of adaptation to the environment. Because this line appeared some time after passage of H4 cells into different media, it could have survived by virtue of its ability to respond to pressures exerted by nutritional factors. Alternatively, it may have been selected by the environment because of its preexisting aberrant chromosome constitution. On the other hand, the possibility also exists that this cell arose by fusion of
the parent H4 cells with an unrelated or heterologous contaminant. This latter possibility is presently under investigation.

The question of the effect of extra chromosomes on gene expression and cell survival and tumorigenicity is one of fundamental importance. Weiss and Chaplain (21) clearly showed the effect of extra chromosomes on tyrosine aminotransferase induction. Hybrids of H4 cells with diploid epithelioid cells from rat liver completely lost the ability to respond to steroid hormone. An isolated clone, in which the component cells contained only 60 to 70% of the original chromosomes, and all its derivatives showed increased activity in tyrosine aminotransferase in the presence of dexamethasone. The striking difference in tumorigenicity between the H4 and F/rh cells is of interest to the field of cancer research. The possibility of using combinations like these cell lines and their derivatives as experimental tools to approach the question of tumorigenicity needs to be evaluated.

As stated earlier (7) the development and characterization of a wide variety of mutants is necessary for continued progress in the genetic analysis of mammalian cells. The results presented here emphasize the necessity of using parameters encompassing widely different disciplines comprehensively to characterize and identify complementary mammalian cells in vitro. The judicious use of carefully selected lines with specific properties underlies the approach to an understanding of molecular biological mechanisms relating genetic potential and phenotypic expression.

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REFERENCES

Figs. 1 and 2. Representative karyotypes of the parent, H4, and derived cell lines.

Fig. 1. Upper, normal karyotype of the ACI rat [Nowell et al. (14)]; lower, karyotype of both the H4 (155th to 162nd passage) and 9Q cell lines (18th to 26th passage).

Fig. 2. Karyotype of the F/rh cell line (190th to 198th passage).
Figs. 3 to 7. Isozyme patterns for various enzymes in the parent and 2 derived cell lines. In all cases, the origin was at the sample wells shown, the anode was toward the top, and the cathode was toward the bottom of each photograph. Aliquots were placed in the order 9Q, H4, and F/rh, reading from left to right, in every case.

Fig. 3. Lactate dehydrogenase. Arrow, position of a weakly staining band for F/rh cells. Fig. 4. Phosphoglucomutase. Fig. 5. Pyruvate kinase. Fig. 6. 6-Phosphogluconate dehydrogenase. Fig. 7. Phosphoglucone isomerase.
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