Identification of Human RNA Transcripts among Heterogeneous Nuclear RNA from Man–Mouse Somatic Cell Hybrids

(human X chromosome/repetitive DNA sequences/DNA–RNA hybridization)

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ABSTRACT In a man–mouse hybrid line from our cell library, the only cytologically detectable portion of the human genome is the X chromosome, and the only genetic markers regularly expressed are coded by genes known to be X-linked. A component of the heterogeneous nuclear RNA of these cells was found to be complementary to repetitive human DNA sequences by means of RNA–DNA hybridization on nitrocellulose filters. The same procedure also permitted the identification of hybrid cell DNA sequences that are complementary to human heterogeneous nuclear RNA. This experimental approach, coupled with hybridization studies in situ, is expected to yield critical data on the distribution and the specificity of the repetitive DNA sequences present in the human genome and to provide a new tool for cytological mapping of human chromosomes.

Highly unstable somatic cell hybrids offer a unique opportunity for basic experimentation of molecular biology at the level of single human chromosomes. The Sendai virus-mediated fusion between somatic cells of human and rodent origin usually yields interspecific somatic cell hybrids which undergo the progressive loss of the human genome (1–5). When the rodent parental cell line is deficient in a metabolic function that is essential for survival in a given selective medium, these types of somatic cell hybrids evolve rapidly towards a reduced karyotype where the only component of the human genome retained is the chromosome (or chromosome portion) carrying the human gene that codes for the essential metabolic function.

The present report describes the rationale and the methodologies devised for the identification of human RNA transcripts among the heterogeneous nuclear RNA (hnRNA) isolated from a man–mouse somatic cell hybrid line (A9/HRBC2) that has lost almost entirely the parental human genome with the exception of the human X chromosome.

The selective system that has permitted the isolation of this hybrid cell line was devised by Szybalski and Szybalska (6) to select against mammalian cells deficient in hypoxanthine phosphoribosyltransferase (EC 2.4.2.8; IMP:pyrophosphate phosphoribosyltransferase). This is achieved by carrying out cell growth in the so-called selective medium, containing a potent inhibitor of purine and pyrimidine synthesis (A for aminopterin) together with preformed nitrogen bases (H for hypoxanthine and T for thymidine). Cell survival in this selective system depends upon the ability of the cell to use the preformed bases as a salvage pathway for nucleic acid synthesis. This ability is provided by normal activity of the phosphoribosyltransferase, whose structural gene is known to be X-linked in man (7). Consequently, when phosphoribosyltransferase-negative mouse cells are fused with phosphoribosyltransferase-positive human cells, the resulting hybrid clones grown in HAT medium may lose every part of the human genome but the X chromosome, or at least the portion of it bearing the locus for the human enzyme (8, 9).

The identification of human RNA transcripts in man–mouse somatic cell hybrids of the type described above is based on RNA–DNA hybridization. Molecular hybrids formed by annealing RNA from A9/HRBC2 cells with human DNA are expected to allow detection for human RNA synthesized in A9/HRBC2 cells. Likewise, molecular hybrids obtained by annealing RNA from human cells with DNA from A9/HRBC2 should detect the human DNA carried by this hybrid cell line. To be meaningful, the levels of molecular hybridization obtained in such experiments should be constantly higher than those between the RNA of the parental mouse cells with human DNA and vice versa, since these measure the amount of interspecific crosshybridization. Soeiro and Darnell (10) have demonstrated that hybridization between L-cell hnRNA and HeLa cell DNA and vice versa is negligible. For this reason, hnRNA was chosen as experimental material. While this work was in progress, Coon et al. (11) found that complementary RNA of human mitochondrial or nuclear DNA does not significantly hybridize with rodent DNA and vice versa.

MATERIALS AND METHODS

The A9/HRBC2 hybrid line was derived from the man–mouse hybrid line established by Miller et al. (8) through the fusion of hypoxanthine phosphoribosyltransferase-positive human male diploid cells (HRBC2) with mouse A9-cells that lack this enzyme (12) as well as adenine phosphoribosyltransferase (EC 2.4.2.7; AMP:pyrophosphate phosphoribosyltransferase) (13). The original hybrid line had been propagated in the HAT-selective medium for about 1 year, before being stored in liquid nitrogen. During that period it had lost almost the entire human genome, with the exception of the human X chromosome and all or some of its markers (8, 9). The present clone derivative originated from the few cells that recovered in HAT medium after 4 years of storage.

The HeLa-S3 cell line (14) was used as a source of human hnRNA. This line is known to have from 43 to 69 chromosomes with two or more doses of each human chromosome (15) and exhibited normal activity for all X-linked and autosomal enzyme markers mentioned in Table 1.

All cells were grown in monolayer cultures in 1585 cm² roller bottles. HeLa and A9 cells were constantly propagated in minimum essential medium, and A9/HRBC2 cells in HAT medium, hypoxanthine/aminopterin/thymidine medium.

Abbreviations: hnRNA, heterogeneous nuclear RNA; HAT medium, hypoxanthine/aminopterin/thymidine medium.
medium (16), both supplemented with the standard additions of sera and antibiotics reported in full elsewhere.† All cell stocks were checked for mycoplasma with Levine's method (17) and frozen. Each experiment was conducted with a freshly thawed vial of mycoplasma-free frozen cells. Confluent monolayers of cells were labeled for 1 hr with 2 μCi of [3H]adenine (27 Ci/mmol) in 25 ml of medium. The cells were chilled to 4°C and harvested, and their nuclei were isolated (18, 19). Nuclear RNA was extracted (10, 19) and hnRNA, with a sedimentation constant greater than 45 S, was isolated (10). DNA was extracted (20) from isolated nuclei of cultured cells (18, 19) and of human placenta (21). Minor modifications of these classical nucleic acid isolation procedures were applied and are described elsewhere.† RNA-DNA hybridization on nitrocellulose filters (22) was carried out as described by Soeiro and Darnell (10). Background binding to blank filters under these experimental conditions ranges from 1 to 3% of the radioactive RNA hybridized to DNA-bearing filters.

Multistep hybridization experiments were conducted as described by Bävre and Szybalski (23) with the following modifications. The first step or preparative hybridization was conducted as described by Soeiro and Darnell (10). After radioactivity determination, the filter was removed from the scintillation fluid, air-dried, and treated with iodoacetate (23). Then 0.75 ml of 0.3 M NaCl/0.030 M sodium citrate (pH 7) containing 0.1% sodium dodecyl sulfate was added to the vial containing the filter, and the solution with the filter was heated in a boiling-water bath for 10 min. The solution was immediately chilled to 4°C, and the filter was carefully removed and dried for radioactivity measurements to determine the efficiency of elution. The eluted RNA was then cross-hybridized to a new set of filters under the same conditions used in the preparative hybridization.

Electrophoretic characterization of enzyme markers was performed by the techniques reported by Shin et al. (24), Meera Khan (25), Migeon et al. (26), and Grzeschik (27). The screening for human adenine phosphoribosyltransferase was performed at the single cell level by autoradiography after growth of the hybrid cells in HAT medium containing 10 μCi/ml of [3H]adenine; since the parental mouse cells lack the enzyme, incorporation of labeled adenine is considered evidence of the retention of the human gene coding for this enzyme. Standard techniques for centromeric (C-) banding (28), Giemsa (G-) banding (29), and quinacrine (Q-) banding (30) were applied for chromosome studies, sometimes with slight modifications as described in the legends to the figures.

RESULTS AND DISCUSSION

Characterization of Hybrid Line A9/HRBC2: Enzyme and Chromosome Studies. Celloidin electrophoresis (25) of cell lysates and chromosome banding techniques were used to establish the amount of human genetic information residual in the A9/HRBC2 hybrid line. Such studies were performed whenever a large batch of A9/HRBC2 cells was propagated for isolation of nucleic acids. A small sample of the cell population was grown in parallel in two Falcon flasks. When these were nearly confluent, cells in metaphase were removed by shaking for chromosomal analysis, while the remaining attached cells were harvested and lysed for enzyme electrophoresis. This procedure ensures that conclusions drawn from enzyme and chromosomal studies can be confidently considered pertinent to the same population of A9/HRBC2 cells used in the experiments of molecular hybridization, despite possible variations of the hybrid genome known to occur during prolonged growth in HAT selective medium (8). Cell lysates of A9/HRBC2 were always studied in parallel with lysates of A9 (mouse parental) and of normal human cells. All lysates were screened for the presence of 28 human genetic markers which identify the human X chromosome and 14 of the 22 autosomes (Table 1). These genetic markers include only constitutive enzymes whose electrophoretic mobility differs in the parental species and whose regular expression is well documented in man–mouse somatic cell hybrids before they undergo the loss of the specific human chromosomes bearing the corresponding structural loci (31).

Table 1 shows that hybrid line A9/HRBC2 has regularly retained only the three human X-linked markers: hypoxanthine phosphoribosyltransferase (HPRT), glucose-6-phosphate dehydrogenase (G6PD), and phosphoglycerate kinase (PGK). All human autosomal markers are absent, with the exception of those of chromosome no. 2: NAD-dependent malate dehydrogenase (NAD-MDH) and cytoplasmic isocitrate dehydrogenase (IDH-1) (32). The latter ones must, however, be present only in a small proportion of cells, as is suggested by the finding that the human bands as well as the heteropolymeric mouse–human components of these enzymes have always been found to be much fainter than the mouse bands in all lysate preparations of A9/HRBC2 cells.

Chromosome studies were regularly carried out on each batch of thawed cells propagated for isolation of nucleic acids. Under the experimental conditions used, the mouse chromosomes exhibited a distinct centromeric band while human ones did not. Giemsa and quinacrine banding were used for the identification of individual human chromosomes. The A9 parental mouse line has a heteroploid genome with an average

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number of 51 chromosomes, 28 of which are telocentric and 23 biarmed believed to be the result of centromeric fusion (33). All these chromosomes showed a distinct centromeric band except one or two telocentric chromosomes per metaphase analyzed. The A9/HRBC2 hybrid line used in this study has a karyotype which would be indistinguishable from that of the A9 parental cell line, except for the regular presence of one biarmed chromosome without centromeric banding. From G- and C- banding, this chromosome was identified as the human X in 26 out of 27 karyotyped metaphases (Fig. 1). However, this conclusion could not be confirmed in 19 additional metaphases which were studied for C- and Q- banding. In these metaphases the non-C-banded human X-like chromosome failed to show the characteristic Q-banding pattern of the human-X, while the mouse chromosomes were banded as expected. We have no explanation for this inconsistency. We are presently investigating whether this is a phenomenon of general occurrence in man-mouse hybrids that have retained very few human chromosomes. The C- and G-banding studies revealed also the presence of an extra biarmed non-C-banded chromosome with an atypical G-banding pattern in six out of the 27 above-mentioned metaphases karyotyped. In view of the enzyme data reported above, it is conceivable that this chromosome may be human autosome no. 2, probably modified in its banding pattern as result of de novo chromosomal rearrangements occurring in culture.

In conclusion, both the enzymatic and the cytological studies suggest that the only human chromosome regularly present in the A9/HRBC2 hybrid cells is the X chromosome. However, the occasional presence of additional human genetic information in a small proportion of these hybrid cells cannot be excluded.

Identification of Human hnRNA and of Human DNA in A9/HRBC2 Hybrid Cells. [3H]Uridine-labeled hnRNA from hybrid cells, parental A9 mouse cells, and human HeLa cells were separately exposed to nitrocellulose filters bearing A9/HRBC2, A9, or human placental DNA. Throughout these hybridization experiments, the filters bearing A9 or A9/HRBC2 DNA were exposed simultaneously to each RNA preparation, while filters bearing human placental DNA were exposed individually. The results of these experiments can be summarized as follows (Fig. 2).

(i) Under the given experimental conditions the percent of hybridization between human DNA and human DNA (as is the A9 DNA for the A9/HRBC2 RNA and vice versa) than they do to human placental DNA (Fig. 2A, B, D, and E). Correspondingly, human HeLa hnRNA binds to human DNA much more efficiently than to A9 or to A9/HRBC2 DNA (Fig. 2C and F).

(ii) The A9/HRBC2 hnRNA anneals better to human DNA than does the A9 hnRNA. Namely, 0.18% (0.33 − 0.15 = 0.18%) more of hybrid hnRNA binds to filters bearing human DNA than does mouse hnRNA (Fig. 2D and E). Correspondingly, human HeLa hnRNA binds to A9/HRBC2 DNA better than to A9 DNA. Namely, 0.22% (0.28 × 2 − 0.17 × 2 = 0.22%) more of human hnRNA anneals to hybrid DNA than it does to mouse DNA (Fig. 2F).

(iii) Both A9 and A9/HRBC2 hnRNA were found to bind slightly better to A9 DNA than to A9/HRBC2 DNA when each of the RNA preparations was exposed simultaneously to both types of DNA (Fig. 2A and B). Since the chromosomal content of these two cell lines is almost identical, their hnRNA is expected to anneal to A9/HRBC2 DNA and A9 DNA identically. However, because the differences in hybridization are small, it is difficult to ascribe significance to them, especially in view of the finding that differences of the same magnitude have been found also when two sets of filters bearing the same DNA (A9/HRBC2) were exposed to the homologous RNA preparation. At any rate, whatever its ex-

§ When two filters bearing the same amount of identical or quasihomologous DNA are exposed simultaneously to the same RNA preparation, the true percent of input RNA hybridized is to be considered twice as much as the one observed per filter.
**Fig. 2.** RNA-DNA filter hybridization experiments carried out with [3H]uridine-labeled hnRNA of various kinds. The percents of RNA hybridized at each DNA concentration have been computed from the ratios between cpm bound per filter (average of two determinations) and total cpm of RNA preparation. (A) Mouse (A9) or hybrid cell (A9/HRBC2) DNA exposed to $4.7 \times 10^4$ cpm of A9/HRBC2 hnRNA (1.3 $\times 10^4$ cpm/µg). (B) Same DNA filters as in A, exposed to $4 \times 10^4$ cpm of A9 hnRNA (1.6 $\times 10^4$ cpm/µg). (C) Human placental DNA exposed to 2.2 $\times 10^4$ cpm of HeLa hnRNA (9.84 $\times 10^4$ cpm/µg). (D) Same DNA filters as in C, exposed to 3.8 $\times 10^4$ cpm of A9/HRBC2 hnRNA (1.3 $\times 10^4$ cpm/µg). (E) Same DNA filters as in C and D, exposed to $4 \times 10^4$ cpm of A9 hnRNA (1.6 $\times 10^4$ cpm/µg). (F) Same DNA filters as in A and B, exposed to $8.4 \times 10^4$ cpm HeLa hnRNA (9.9 $\times 10^4$ cpm/µg). All experiments were performed in duplicate. The percent of A9/HRBC2 RNA that is specifically hybridizable to human DNA can be obtained by subtracting the single values reported in E from the corresponding ones reported in D. For 100 µg of DNA per filter, this amounts to 0.18% (i.e., 0.33 - 0.15). Correspondingly, the percent of HeLa RNA that is specifically hybridizable to cell hybrid DNA can be obtained from the difference of the two sets of values reported in F (interrupted line). For 100 µg of DNA per filter, this amounts to 0.22% (i.e., 0.28 $\times 2 - 0.17 \times 2$).

In conclusion, it is clear that this finding strengthens the significance of the above-mentioned higher affinity of the human RNA for filters bearing A9/HRBC2 DNA when the former is exposed simultaneously to filters bearing the hybrid cell or the mouse DNA (Fig. 2F).

**Multistep Hybridization.** The nature of the cell hybrid hnRNA that hybridizes to human DNA and of HeLa hnRNA that hybridizes to cell hybrid DNA, was further analyzed by multistep hybridization experiments, which confirm the observations reported in the preceding paragraph. Labeled RNA was eluted from filters each bearing a given combination of RNA-DNA molecular hybrid (Table 2A) and reannealed to a second set of filters bearing 100 µg of human, mouse, or hybrid cell DNA (Table 2B). The percent of eluted RNA ranged from 5% to 91. In Table 2, the radioactivity bound to each filter in the series of cross-hybridizations has been corrected for background by subtracting the radioactivity bound to filters bearing *Escherichia coli* DNA. The data indicate that A9/HRBC2 hnRNA eluted from an A9/HRBC2 human DNA molecular hybrid reanneals 1.9 times more to human than to A9 DNA (Table 2, Exp. 2). In contrast, RNA isolated from an A9/human-DNA molecular hybrid exhibits a slight preference for A9 DNA (Table 2, Exp. 4). Correspondingly, HeLa hnRNA eluted from a HeLa DNA-A9/HRBC2 RNA molecular hybrid anneals 4.9 times more to A9/HRBC2 DNA than to A9 DNA, while HeLa RNA isolated from a HeLa hnRNA-A9 DNA molecular hybrid binds only 2.3 times more (Table 2, Exp. 5). In the control experiments, RNA isolated from molecular hybrids of various combinations of A9/HRBC2 and A9 nucleic acids anneals preponderantly to A9 DNA (Table 2, Exps. 1 and 3). Correspondingly, HeLa RNA eluted from HeLa hnRNA–human DNA molecular hybrids and re-exposed simultaneously to various combinations of human, mouse (A9), and cell hybrid (A9/HRBC2) DNA, prefer—as expected—human to either mouse or cell hybrid DNA (Table 2, Exps. 7–9) and cell hybrid to mouse DNA (Table 2, Exps. 6 and 7).

The data presented provide preliminary evidence that it is possible to identify human RNA and DNA in human–mouse somatic cell hybrids with a greatly reduced human chromosomal content. Additional studies, to be reported elsewhere, provide further evidence that molecular hybrids between A9/HRBC2 and human nucleic acids, detected by nitrocellulose filter assay, are more specific than molecular hybrids between A9 and human nucleic acids. These studies also provide data identifying the presence of human RNA in A9/HRBC2 cells that is complementary to "few gene copy" human DNA, as well as data identifying the presence of human RNA in HeLa cells that is complementary to "few gene copy" A9/HRBC2 DNA.†

Since the enzyme and chromosomal data suggest that the only human chromosome regularly present in A9/HRBC2 cells is the human X chromosome, it can be concluded that the hnRNA identified by the experiments described in Fig. 2D and in Table 2, Exp. 2, must evidently be transcribed from repetitive DNA sequences of the human X chromosome. However, despite the evidence favoring this conclusion, we cannot as yet exclude the possibility that additional human genetic information, besides the one identified through enzymatic and chromosomal studies, may be present in A9/HRBC2 hybrid cells in the form of undetectable chromosomal fragments translocated to mouse chromosome. We intend to test this possibility with DNA preparations from human strains with multiples of the X-chromosome. If the human-X is essentially the only human chromosome in A9/HRBC2 cells, the human component of A9/HRBC2 hnRNA should hybridize proportionately to human DNA derived from XY, XXX, or XXXXY human cells. Should this prove to be the case, then the in situ hybridization of this human component of A9/HRBC2 cells to human metaphase chromosomes would pro-
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Table 2. Multistep hybridization

<table>
<thead>
<tr>
<th>hnRNA (cpm)</th>
<th>DNA</th>
<th>cpm bound and %</th>
<th>% of eluted RNA</th>
<th>Hybridization of eluted RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A9/HRBC2 (5 x 10⁴) Mouse (A9)</td>
<td>Cell hybrid (A9/HRBC2)</td>
<td>(1.10)</td>
<td>88</td>
<td>0.03</td>
</tr>
<tr>
<td>Human (4 x 10⁶) (placenta)</td>
<td>1515</td>
<td>86</td>
<td>1.90</td>
<td></td>
</tr>
<tr>
<td>A9 (4 x 10⁴) Mouse (A9)</td>
<td>Cell hybrid (A9/HRBC2)</td>
<td>(1.20)</td>
<td>720</td>
<td>88</td>
</tr>
<tr>
<td>Human (4 x 10⁴) (placenta)</td>
<td>624</td>
<td>86</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>A9 Human (4 x 10⁴) (placenta)</td>
<td>(0.17)</td>
<td>675</td>
<td>91</td>
<td>0.70</td>
</tr>
<tr>
<td>HeLa Mouse (A9) (8.4 x 10⁴)</td>
<td>Cell hybrid (A9/HRBC2)</td>
<td>(0.17)</td>
<td>1428</td>
<td>91</td>
</tr>
<tr>
<td>Human (8.4 x 10⁴) (placenta)</td>
<td>(2.20)</td>
<td>1848</td>
<td>88</td>
<td>3.6</td>
</tr>
<tr>
<td>Human (8.4 x 10⁴) (placenta)</td>
<td>(2.10)</td>
<td>1763</td>
<td>85</td>
<td>27.0</td>
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<td>HeLa Human (8.4 x 10⁴) (placenta)</td>
<td>(2.00)</td>
<td>1626</td>
<td>89</td>
<td>2.7</td>
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<tr>
<td>HeLa Human (8.4 x 10⁴) (placenta)</td>
<td>(2.30)</td>
<td>1931</td>
<td>90</td>
<td>26.0</td>
</tr>
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</table>

(A) Preparative hybridization performed with various preparations of [H]uridine-labeled RNA (1-hr pulse) and filters bearing 100 µg of a given type of DNA. Types and amount of input RNA, type of DNA on filters, cpm bound, and percent of eluted RNA are specified. Exps. 1, 3, and 5 were conducted with two DNA filters simultaneously exposed to the same RNA preparation. Specific activities of the input RNA preparations are the same as those reported in the legend to Fig. 2. (B) Cross-hybridization of the eluted RNA to filters bearing 100 µg of human (H), mouse (M), or hybrid cell (hy) DNA. The ratios H/M, H/hy, and hy/M express the relative cpm of eluted RNA bound to various filters in the second step hybridization experiments.

vide critical information about the distribution and/or the chromosomal specificity of repetitive DNA sequences of the human genome.

If a regional chromosomal specificity of the human repetitive DNA is demonstrated, the experimental approach described may be used to gather indirect information about the precise chromosomal location of given human structural loci with particular reference to those for human hypoxanthine phosphoribosyltransferase, glucose-6-phosphate dehydrogenase, and phosphoglycerate kinase, whose cytological mapping is hitherto still a matter of dispute (9). This could be achieved by annealing in situ to normal and abnormal human metaphases (notably those with X-autosomal translocations) different preparations of hnRNA derived from man-mouse hybrid cell lines that are known to have retained different segments of the X-chromosome and its genetic markers as the only residual of the human genome (8, 9).