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NEOPLASTIC TRANSFORMATION OF MOUSE C3H 10T1/2 AND SYRIAN HAMSTER EMBRYO CELLS BY HEAVY IONS

L. Hieber,* G. Ponsel,** K. Trutschler,* S. Fenn* and A. M. Kellerer*

**Institut für Medizinische Strahlenkunde der Universität Würzburg, Versbacher Strasse 5, D-8700 Würzburg, F.R.G.*
***Institut für Biotechnologie der Universität Würzburg, Röntgenring 11, D-8700 Würzburg, F.R.G.*

ABSTRACT

C3H 10T1/2 mouse-embryo fibroblasts were used for transformation experiments to study the effectiveness of various heavy ions with energies up to 20 MeV/u and LET values from 170 to 16.000 keV/μm. The transformation frequency per unit absorbed dose decreased with increasing ionization density; at the highest values of LET we found a decrease even of the transformation efficiency per unit fluence. Uranium ions at energies of 5, 9, and 16.3 MeV/u did not induce any transformation.

In additional studies primary Syrian hamster embryo cells (SHE) were exposed to heavy ions in order to characterize cytological and molecular changes which may be correlated with neoplastic transformation. Growth behaviour, chromosomal status, tumorigenicity in nude mice, and expression of oncogenes of transformed cell lines were examined.

INTRODUCTION

Radiation carcinogenesis is seen as the dominant risk of ionizing radiations at low doses. In view of a possible, more general introduction of heavy ion radiotherapy and also in view of any future manned space activities, it is of especial interest to assess the cancer risk due to heavy ion exposure. The lack of epidemiological data and the paucity of relevant animal studies make it necessary to base risk considerations also on transformation studies in vitro.

The transformation efficiencies of different ionizing radiations have been studied in different cell systems, i.e. C3H 10T1/2, Balb/c 3T3 and Syrian hamster embryo cells (SHE). Transformation studies with γ- and x-rays have been performed by Borek and Hall /1/, Terzaghi and Little /2/, Miller et al. /3/, Han et al. /4/, Miller and Hall /5/, and others. Results for neutrons have been given by Borek et al. /6/, Barendsen and Gaiser /7/, Han and Elkind /8/, Hill et al. /9/, and Balcer-Kubiczek et al. /10/. There have been also a number of transformation studies with densely ionizing α-particles by Robertson et al. /11/, Lloyd et al. /12/, Hall and Hei /13/, and Hieber et al. /14/. Data for heavy ions of intermediate LET results were reported by Yang et al. /15/. The aim of the present study with C3H 10T1/2 mouse embryo fibroblasts was to extend the informations about the inactivation and transformation effectiveness for different heavy ions in the LET range from 170 keV/μm up to 16,000 keV/μm.

Beyond the direct interest in the transformation effectiveness of different ionizing radiations, it is desirable to obtain information about the mechanisms underlying the transformation process(es) induced by ionizing radiation. Therefore, it is important to investigate parameters which may correlate with the transformed phenotype of a cell. The growth behaviour, i.e. plating efficiency, population doubling time, and growth in semi-solid medium, the induction of tumors in athymic nude mice, and the chromosomal status of Syrian hamster embryo (SHE) cell lines transformed by carbon ions were investigated. In addition, recent results are given on the expression of the H-ras-oncogene in cells transformed after exposure to densely ionizing heavy ions (carbon ions) and in the related tumor-cell lines.

METHODOLOGY

Cell culture and irradiation procedures

The transformation study was performed with C3H 10T1/2 mouse-embryo fibroblasts developed by Reznikoff et al. /16/. The cells were maintained in Eagle's basal medium (Gibco) supplemented with 10 per cent heat-inactivated fetal bovine serum (Boehringer) 50u/ml penicillin, and

50μg/ml streptomycin (BRL). Cells of passage 12 were cultured in 75cm^2 flasks (Greiner) and incubated in a humidified gas atmosphere (95 per cent air and 5 per cent CO_2) at 37°C. The plating efficiency of control cultures was between 20 and 30 per cent. Twenty-four hours before irradiation the cells were plated in petri dishes of 3.5cm diameter at a final density (at the time of irradiation) of 10^4/cm^2 in order to achieve exponential growing cell populations during exposure.

Irradiations with different heavy ions were performed at the linear accelerator UNILAC of the Gesellschaft für Schwerionenforschung (GSI) in Darmstadt. Just before the irradiation the petri dishes were transferred into a medium-filled teflon magazine and were irradiated vertically in an exposure facility with automatic sample changer constructed by the GSI /17/. The heavy ion beams were defocussed and the samples were wobbled to achieve homogenous irradiations. An integrated part of the exposure facility is a secondary electron emission chamber that monitors the ion beam current. This monitor was calibrated by comparison with the particle fluence determined from track counts on etched glass or on CR39-samples.

Survival and transformation assay

Immediately after irradiation the cells were trypsinized, and held on ice till plating. If more than about 10^5 cells were necessary for the transformation and survival assay at a specified dose, two or more dishes were irradiated successively and the samples were pooled.

For the survival assay the numbers of plated cells were suitably chosen to attain about 80 viable cells per 25cm^2 flask. The flasks were incubated for 10 days, then fixed and stained with 10 per cent Giemsa, colonies with more than 50 cells were counted as survivors.

For the transformation assay the cells were plated in 25cm^2 flasks with about 300 viable cells per flask. The cells were incubated for 6 weeks with a weekly medium change. After fixation and staining the numbers of transformed foci were determined; only foci of type 2 and 3, as described by Reznikoff et al. /16/, were scored as transformants. The transformation frequency per surviving cell were determined as described earlier /14/.

Transformation of Syrian hamster embryo cells

Primary Syrian hamster embryo cells isolated from 12 days old embryos were frozen in liquid nitrogen as stock cultures. For the experiments cells were thawed and maintained in IBR medium (Gibco) supplemented with 20 per cent fetal bovine serum and antibiotics. The cells were incubated in a humidified gas atmosphere of 90 per cent air and 10 per cent CO_2 at 37°C. Cells of passage 2 to 3 were used for the experiments. The irradiation procedure for SHE cells was the same as described above. After irradiation the cells were trypsinized and $5x10^5$ cells were plated in 75cm^2 flasks. Before reaching confluency the cells were subcultured repeatedly until the primary cells aged and immortal or transformed cells overgrew the aged, dying cells.

Tumorigenicity in athymic nude mice

The transformed cell lines were grown in 75cm^2 flasks, trypsinized and resuspended in glucose-containing phoshate buffered saline (10^7 cells per ml). $2x10^6$ cells were subcutanously injected into athymic nude Balb/c mice. The animals were weekly controlled for tumor induction. Tumors growing under the skin of mice were isolated from killed animals one week after appearance. The tumors were minced, transferred to petri dishes, fed with medium containing 10 per cent fetal bovine serum, and incubated at 37°C. One day later the remaining fragments of the tumor were removed during a medium change. Cells attached to the dishes were grown for a few days and were then frozen in liquid nitrogen, to serve as tumor-cell lines for further experiments.

Growth behaviour assays

Plating efficiency. The plating efficiency of the transformed cell lines was determined by the usual colony formation assay, as described above for the survival assay.

Population doubling time. The population doubling time was estimated from the exponential part of the growth curves. $5x10^4$ cells of each cell line were plated in 6 cm dishes and every 24 hours one sample was trypsinized and the cell number was counted using a Coulter Counter.

Anchorage independent growth. The competence for anchorage independent growth was tested in semi-solid medium. A solution of 1.2 per cent Bactoagar (Difco) and 0.4 per cent Bactopeptone (Difco) mixed with an equal volume of double-concentrated medium was used as base layer and was pipetted into 6cm petri dishes and cooled at 4°C. Between 10^4 and 10^6 cells suspended in normal medium mixed with the double volume of the base-layer solution were plated on the base layer. The cells were incubated for four weeks; after half the incubation time the cells were fed with fresh medium. One day before scoring colonies, 1 ml of a 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium-chloride solution (1 mg/ml, Aldrich Chemie) was added to the cultures according to the procedure of Schaeffer and Friend /18/. The plating efficiency in semi-solid medium was estimated from the number of colonies divided by the number of cells initially plated.

Determination of chromosome numbers

Cells were grown in 75cm^2 flasks for 24 hours, 0.05µg/ml Colcemide was added for 3 hours, and mitotic cells were harvested by shaking the culture flasks. The mitotic cells were incubated in 75mM potassium chloride, fixed with Carnoy solution, spred on microscopic slides, air dried, and stained with 10 per cent Giemsa. Chromosome number per cell were counted from at least 50 metaphase cells.

Expression of H-ras oncogene

Total RNA of transformed and tumor-cell lines was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction according to the method of Chomczynski and Sacchi /19/. H-ras-expression was determined by Northern-blotting. Equal amounts of total RNA were separated by electrophoresis on agarose gels (1 per cent) and were blotted onto Nylon filters (Amersham). The RNA was fixed on the filters by UV-irradiation and baking the filters for 2 hours at 80°C. The filters were hybridized with a 32-P-nick-translated human H-ras DNA-probe (Amersham). The relative amount of ras-mRNA was estimated from the autoradiograms (Kodak XAR or S x-ray film) of the filters by comparing with signals of hybridizations of the same filters with an α-actin probe.

RESULTS AND DISCUSSION

Inactivation and transformation of C3H 10T1/2 cells by heavy ions

C3H 10T1/2 cells were exposed to various heavy ions of energies between 4.6 and 18.6 MeV/u, e.g. carbon, oxygen, argon, iron, krypton, lead, and uranium ions. Beam parameters, such as energy and linear energy transfer (LET), are listed in table 1. Irradiations with all heavy ions led to exponential survival curves. The inactivation cross section, σ, for the different heavy ions, was derived from the slope of the survival curves:

$$-\ln S(\Phi) = \sigma \Phi \qquad (1)$$

where S is the surviving fraction and Φ the particle fluence. The heavier ions with their larger LET-values were associated with the highest inactivation cross sections up to 89µm^2. These maximal inactivation cross sections for 10T1/2 cells are similar to those found for V79 Chinese hamster cells /20/; however, the geometrical cross section of 10T1/2 cells is about twice that of V79 cells.

TABLE 1 Energy, Linear Energy Transfer (LET), and Inactivation Cross Sections, σ, for C3H 10T1/2 Cells of Different Heavy Ions and 241-Americium α-Particles

Particle	Energy (MeV/u)	LET (keV/µm)	σ (µm^2)
α-particle	0.67	147	40
Carbon	5.5	220	57
	8.0	170	58
Oxygen	9.0	275	40
Argon	4.6	1800	51
	6.5	1500	39
	14.0	1050	32
Iron	17.3	1500	52
Krypton	8.5	4300	65
	16.7	3200	67
Lead	7.8	13900	89
Uranium	6.3	15700	83
	9.0	15300	77
	16.0	12800	74

The increase of the cross section is less than proportional to LET and, accordingly, the relative biological effectiveness for inactivation decreases with increasing LET of the heavy ions, as shown in figure 1. At about 900 to 1000 keV/μm the RBE vs. γ-rays becomes smaller than unity; it decreases to about 0.1 for uranium ions with LET values in excess of 10,000 keV/μm.

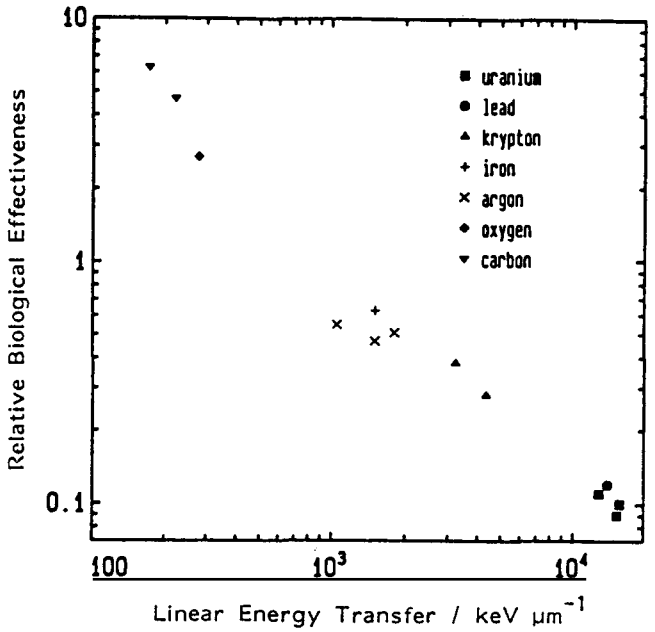

Fig. 1. The relative biological effectiveness of different heavy ions for the inactivation of C3H 10T1/2 cells versus the linear energy transfer (LET). The data for heavy ions were compared with that of γ-rays.

Transformation frequencies per surviving cells for different heavy ions at various fluences, Φ, are listed in table 2. The data are based on single experiments only, and they are, therefore, subject to substantial statistical uncertainties. However, there is a consistent decrease of the transformation frequencies with increasing mass of the ions at similar velocities. Transformation efficiencies appear to increase with increasing energy, i.e. decreasing LET, as shown for argon and krypton ions. The LET dependencies are in substantial agreement with the results of Yang et al. /15/ for ions with LET values in excess of about 200 keV/μm. The data support the general statement in ICRU 40 /21/ that the efficiency of densely ionizing radiations decreases at LET in excess of a few hundred keV/μm.

TABLE 2 Transformation Frequencies per Surviving Cell Exposed to Heavy Ions at Various Fluences, Φ.

Ion	Energy (MeV/u)	LET (keV/μm)	Transformants per 10^4 survivors at fluences /cm^2 2.5x10^5	5x10^5	1x10^6	1.5x10^6	2x10^6	3x10^6	4x10^6	6x10^6
C	8.0	170		<0.4	<0.5	1.3±1.3	<0.6	<0.8	1.3±1.3	
	5.5	220		<0.4	<0.5	<0.4	1.5±0.9	0.5±0.5	2.3±1.2	
O	9.0	275				2.2±1.1		2.0±0.9		1.6±0.8
Ar	18.6	780				5.0±3.5		2.6±1.2		1.7±1.0
	4.6	1800	1.1±0.8	0.7±0.7	1.5±0.9		2.7±1.2		1.1±0.8	
Fe	17.3	1500	<0.6	0.7±0.7	1.3±0.9		1.4±1.0		2.4±1,1	
Kr	16.7	3200				1.7±1.2		2.3±1.4		<0.4
	8.5	4300				0.8±0.8		0.6±0.6		<0.5
Pb	7.8	13900	0.6±0.6	0.6±0.6	0.7±0.7		<0.6		<0.6	
U	16.3	12800				<0.5		<0.4		<0.4
	9.0	15300				<0.9		<0.4		<0.4
	6.3	15700	<0.5	<0.4	<0.5	<0.6	<0.5	<0.5		

Tumorigenicity of SHE cells transformed by carbon ions

Syrian hamster embryo cells were exposed to the fluences $5x10^5/cm^2$, $2x10^6/cm^2$, and $4x10^6/cm^2$ of 8 MeV/u carbon ions; the resulting absorbed doses were 0.14, 0.56, and 1.12 Gy. At each dose we succeeded to isolate two independent transformed cell lines. After injection of $2x10^6$ cells at passage 5 of the six cell lines into athymic mice each animal developed a subcutaneous tumor, which was classified as fibrosarcoma. Thus all isolated cell lines were neoplastically transformed and tumorigenic. The time periods between injection and appearance of tumors were 5 to 6 weeks.

Growth parameters of cells transformed by carbon ions and of the resulting tumor-cell lines

Growth parameters, e.g. plating efficiency, population doubling time, and the cloning efficiency in semi-solid medium, have been determined for the transformed cell lines and for the tumor-cell lines from the nude mice and have been compared with untransformed primary SHE cells 82-9 (see table 3). The plating efficiencies of transformed and tumor cells was about 0.5 to 0.6 without feeder cells. With or without feeder layer primary cells had plating efficiencies of 0.2 and 0.3, respectively.

TABLE 3 Growth Parameters of Cell Lines Transformed by 8 MeV/u Carbon Ions and of Tumor-Cell Lines Derived from the Transformed Cell Lines

Cell Line	Particle	Dose (Gy)	Passage Number	PE*	DT** (hrs)	CE in soft agar# ($x10^3$)
82-9			4	0.2-0.3	20	<0.001
A40I-1	Carbon	0.14	8	n.d.	n.d.	0.04
T 2802			3	n.d.	n.d.	9.0
A40I-2	Carbon	0.14	7	0.6	14.1	<0.01
T 2792			4	0.31	20.4	35.8
A40II-1	Carbon	0.56	8	n.d.	n.d.	0.96
T 2800			3	n.d.	n.d.	10.5
A40II-2	Carbon	0.56	7	0.63	13.6	<0.01
T 2794			4	0.53	14.4	4.6
A40III-2	Carbon	1.12	7	0.60	13.3	0.44
T 2793			4	0.55	12.6	3.9

*PE, plating efficiency. **DT, population doubling time derived from growth curves. #CE in soft agar, cloning efficiency in semi-solid medium.

The population doubling time estimated from the exponential part of growth curves was significantly shorter (about 13 hours) than that of the 82-9 cells (about 20 hours). The transformed cells, and the tumor cells have, thus, a pronounced growth advantage; they overgrow untransformed normal cells rapidly.

The cloning efficiency in semi-solid medium of primary cells was less than 10^{-6}. In contrast, between 1 out of 10^5 and 1 out of 10^3 cells of the transformed lines were able to grow in soft agar. The tumor-cell lines showed even higher cloning efficiencies, between roughly 0.004 and 0.04. The tumor-cell line T2792 is a seeming exception, with its low plating efficiency of 0.31 and its long doubling time of 20.4 hours but with the highest cloning efficiency in soft agar. However, the low plating efficiency and the low growth rate may be experimental artefacts; T2792 cells had a tendency to grow unattached and only the attached cells and clones were counted. The ability to grow unattached is in line with the high cloning efficiency in semi-solid medium. Since a part of the T2792 cells grow in suspension they were not included in the data to establish growth curves.

Chromosome number per cell of transformed and tumor-cell lines

The diploid set of chromosomes of normal Syrian hamster cells consists of 44 chromosomes (see figure 2 a). We determined the number of chromosomes per cell of 3 transformed cell lines, 3 soft agar subclones of one transformant, and one tumor-cell line (T2800) with, additionally, 3 subclones grown in semi-solid medium.

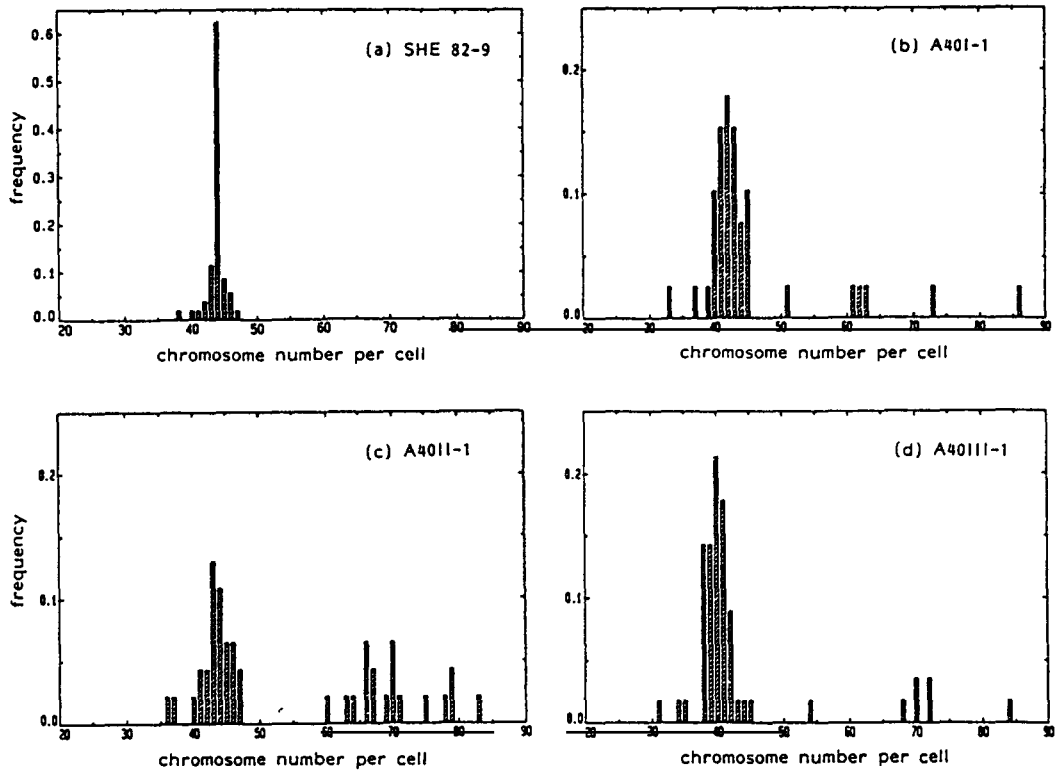

Fig. 2. Histograms of the chromosome number per cell of primary SHE cells or cells transformed by various fluences, Φ, of 8 MeV/u carbon ions: (a) primary SHE cells; (b) A40I-1, $\Phi = 5 \times 10^5/cm^2$; (c) A40II-1, $\Phi = 2 \times 10^6/cm^2$; (d) A40III-1, $\Phi = 4 \times 10^6/cm^2$.

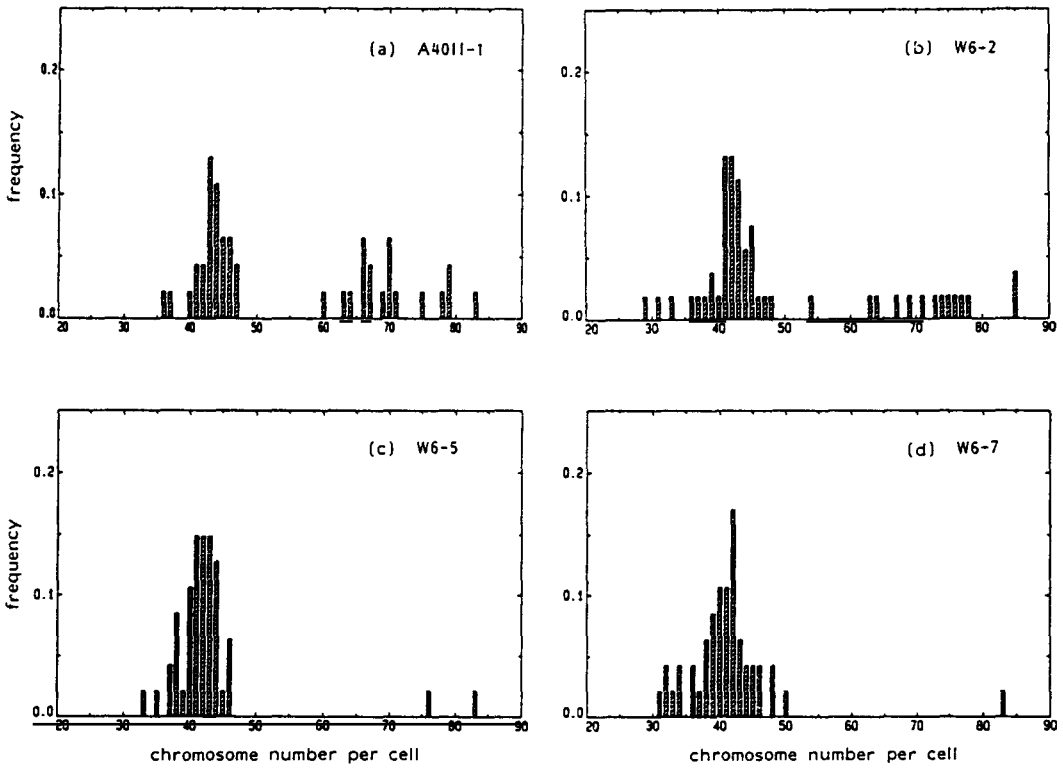

Fig. 3. Histograms of the chromosome number per cell of A40II-1 (a) and three subclones of A40II-1 cloned in soft agar (b,c,d).

The transformed cell lines exhibit, as shown in figure 2 b,c,d, somewhat varying distributions of chromosome numbers. A40III-1 cells had a relatively narrow distribution of chromosome numbers around a median value close to only 40, the other two cell lines had distributions slightly shifted to median numbers of 42 (A40I-1) and 46 (A40II-1) chromosomes per cell. All three cell lines contain various fractions of uneuploid cells with chromosome numbers up to 85.

The soft-agar subclones of A40II-1 (figure 3 b,c,d) had similar chromosome distributions as the parent cell line. The main components of their distributions have a similar width as that of A40II-1. The fraction of uneuploid cells was smaller in two of the three subclones (figure 3 c,d).

The tumor-cell line T2800 obtained from A40II-1 cells showed a similar distribution as the A40II-1 cells themselves (see figure 3a and figure 4a); there was merely a slight shift towards the median of 40 chromosomes as the transformed cell line A40III-1. The soft agar subclones of T2800 had a somewhat broader distribution, but the median remained at 40 to 41 chromosomes and there was only a small fraction of uneuploid cells with numbers in excess of 60 chromosomes.

It appears from these data that transformed cells and the resultant tumor cells need not be highly uneuploid, i.e. chromosome numbers higher than 50. On the contrary, it appears that the diploid or near diploid cells are most competent to grow in semi-solid medium. One of the main feature of transformed cells is, thus, more clearly expressed in nearly euploid then in highly uneuploid cells.

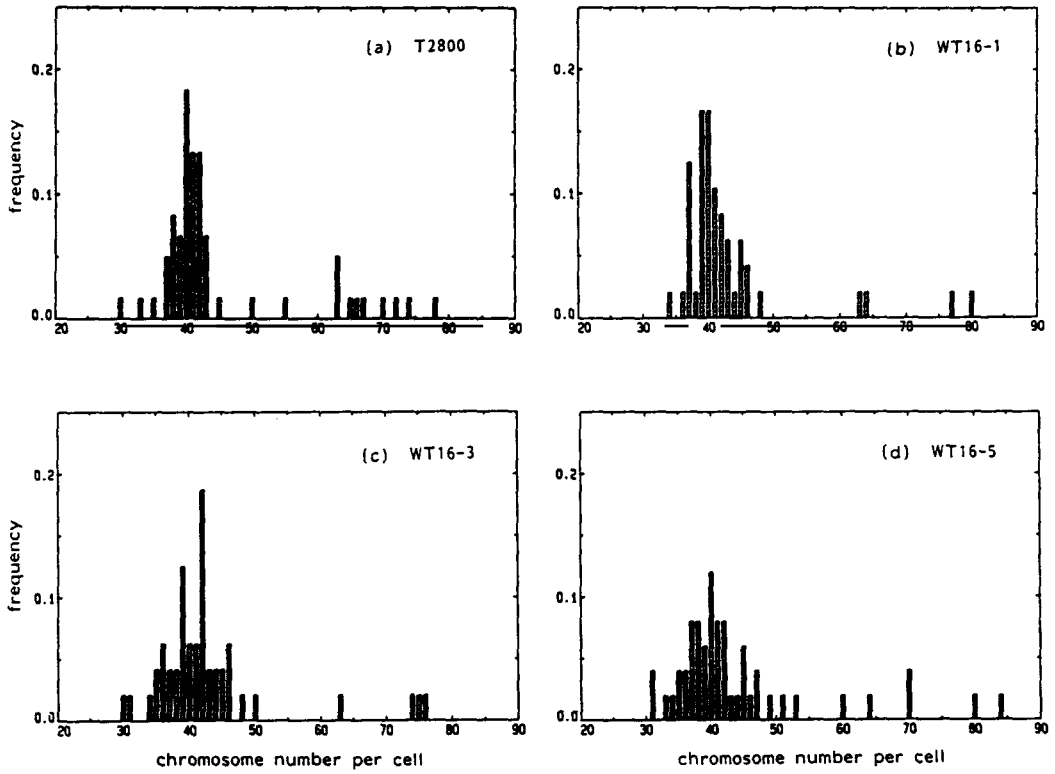

Fig. 4. Histograms of the tumor-cell line T2800 (a) derived from the transformed cell line A40II-1 (fig. 2b) and three soft-agar subclones of T2800 (b,c,d).

Expression of H-ras oncogene in cells transformed by carbon ions and in the resulting tumor-cell lines

Expression of H-ras gene on the RNA-level has been determined by Northern blotting. For the hybridization 20 µg total RNA of each cell line was seperated by electrophoresis on 1 % agarose gels, blotted on Nylon filters, and hybridized with a nick-translated human H-ras probe. Figure 5 shows an autoradiogram of a hybridization experiment with three carbon-ion transformed cell lines an the corresponding tumor-cell lines compared with primary Syrian hamster cells (82-9).

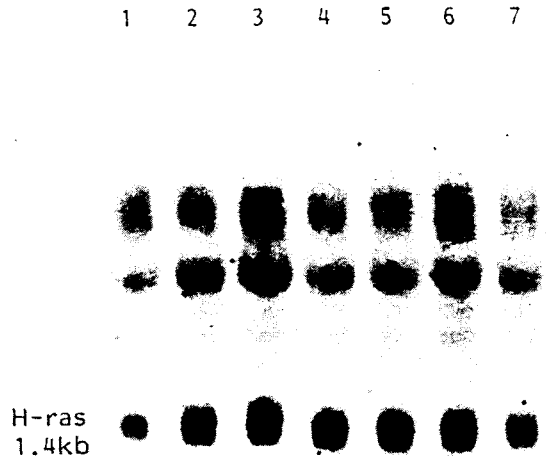

Fig. 5. Northern blot analysis of the expression of the H-ras oncogene for the three transformed cell lines A40I-1 (lane 2), A40II-1 (lane 4), and A40III-1 (lane 6) and the resulting tumor-cell lines T2802 (lane 3), T2800 (lane 5), and T2799 (lane 7), compared to primary SHE cells (lane 1).

From this experiments it seems that the expression of the H-ras gene is enhanced in all transformed and all tumor-cell lines. The enhancement of the H-ras expression has been determined quantitatively by densitometric scanning of the autoradiograms. For a better quantification the signals of the H-ras experiments were compared with signals of hybridization experiments with an actin probe, which did not change by the transformation event. The enhancement factors of the H-ras gene expression were betweenn 1.4 to 2.4. There were no clear differences between the transformed cell lines and the tumor-cell lines. Similar results have been found in cells transformed by Cobalt-γ-rays and in a resulting tumor-cell line. Experiments are now underway to investigate possible mutations or rearrangements of the H-ras gene including the promotor sequences. In addition, the expression of other oncogenes has to be investigated.

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