Translocation of DNA from the Vascular into the Nuclear Compartment of Solid Mammary Tumors

Claude Watters1 and Pietro M. Gullino2

Laboratory of Biochemistry, National Cancer Institute, NIH, Bethesda, Maryland 20014

SUMMARY

Fragments of DNA-3H, with molecular weights of several million, could be concentrated in vivo in the nuclei of Walker carcinomas. DNA-3H was obtained from lactating mammary glands of the same strain of rats bearing the tumor or from Micrococcus lysodeikticus. The degradation of DNA-3H by blood was small if the infused fragments were large. The uptake by the tumor was about 10-fold better when DNA was given through the aorta than when given through the i.v. route. Total uptake by tumors infused for several days was 0.5 to 1.0% of total tumor DNA. The vascular walls maintained a gradient in concentration between blood and interstitial fluid of the tumor. This fluid was unable to degrade DNA-3H rapidly but usually contained only small DNA-3H fragments. The largest fragments were more extensively taken up by the cells. When the size of the DNA-3H fragments infused was the largest, the amount of high-molecular-weight DNA-3H isolated from the tumor nuclei was the highest. The nuclei always contained the largest amount of the incorporated DNA-3H. During the first half hr after the end of M. lysodeikticus DNA infusion, some of the injected DNA could be recovered from the nuclear pellet, which suggested that it probably reached the nuclei relatively intact.

INTRODUCTION

Beginning in the mid-1950's, a number of investigators started to search for genetic changes inducible with nucleic acid preparations in multicellular organisms. Their approach was guided by the results obtained in studies on bacterial transformation. Despite the first favorable report of Benoît et al. (10, 11), it soon appeared impossible to obtain DNA- and RNA-induced transformations in multicellular organisms with the experimental approach followed for bacterial cultures (8, 44, 51, 53). The search shifted from the whole animal to tissue cultures, and in 1961 Kraus (39) reported the first demonstration of DNA-induced transformation in mammalian cells.

Oncologists used nucleic acid preparations in their search for ways of changing the biological properties of neoplastic cell populations. With an RNA preparation from the liver, Zimmermann et al. (59) were able to induce synthesis of serum albumin in Ehrlich ascites cells and to show that the species specificity of the induced albumin corresponded to the origin of the inducing RNA. With a DNA preparation, Glick and Salim (27) induced melanin production in an amelanotic melanoma of the hamster. The influence of nucleic acids on tumor growth attracted, however, more attention. One of the most successful experiments patterned on the approach followed by microbiologists in the study of DNA-induced resistance of bacteria against antibiotics was reported by Podgajetska et al. (52). Injections s.c. of a DNA preparation from a sarcolysine-resistant rat sarcoma were able to enhance resistance to sarcolysine in the sarcolysine-sensitive strain from about 1 to 18%. The acquired resistance was preserved for several transplant generations. In this type of experiment, however, an unidentified action of the DNA preparation could result in enhanced survival and prevalent growth of few sarcolysine-resistant cells present in the mixed cell population of the sarcolysine-sensitive tumor receiving the treatment. Thus the acquired resistance might result from cell selection instead of from transformation. Inhibition of tumor growth was reported by several investigators using nucleoprotein (9, 12, 13), DNA (25, 26, 34), and RNA (2, 20, 48, 49) preparations. In some of these experiments, inhibition involved tumor transplantability, and it is possible that the effects of the nucleic acid preparation involved homograft tolerance more than tumor growth (5, 36). In other experiments, however, growth retardation of spontaneous mammary tumors in C3H mice was produced by a ribonucleoprotein preparation (9), and growth retardation of primary 3,4-benzpyrene-induced rat sarcomas was also obtained with homologous lymphocytes or a nucleic acid preparation thereof (3, 4).

At the moment, it is rather difficult to interpret the inhibitory action of nucleic acid on a solid tumor growing in the host. Indeed, malignant tumors have been produced with DNA preparations of various origins (1, 14, 16, 50, 53), and Silagi (56) has shown that malignant melanoma cells hybridized with a subline of nonmalignant L-cells originated hybrid clones able to produce transplantable tumors in the C3H X C57BL F1 mice. In this experiment and in others (7, 21, 54), the capacity for progressive growth in vivo appears to be a dominant character of the hybrid cells. Therefore, addition of genetic material of a nonmalignant cell to a malignant cell does not per se reduce the growth capacity of the latter. Harris et al. (35), however, observed that highly malignant cells from 3 different mouse tumors hybridized with A9 cells lost to a great extent their transplantability and that the hybrid cells reverted to high malignancy when

1 Visiting Scientist, Departement de Radiobiologie, Centre d'Etude de l'Energie Nucleaire, Mol, Belgium.
2 Address reprint requests to this author.

Received February 16, 1971; accepted April 23, 1971.
chromosomes were lost. Since the A9 line is a mutant of the L line (45), it is possible that something peculiar to the genome of A9 cells and able to reduce malignancy was contributed during fusion and lost later during formation of malignant segregants.

If one interprets growth retardation or growth suppression in tumor cells, either treated with nucleic acid preparations or hybridized, as a phenotypic expression of a phenomenon comparable to bacterial transformation, then one would expect to obtain in vivo transformation only if large fragments of DNA can penetrate into the neoplastic cells of the primary, solid tumor.

Cato and Guild (17) showed that in *Diplococcus pneumoniae* the probability of transformation by an individual DNA fragment is a size-dependent variable. A double-stranded segment with a molecular weight of $3 \times 10^5$ represents a minimum active fragment in this system but not a critical size above which activity would be unity. Allowing for the difference in size and complexity of the mammalian as compared with the bacterial genome, fragments of DNA with molecular weights of a few million should probably be required to penetrate the neoplastic cells in vivo in order to induce transformation. The work of Gosse et al. (28), Hudnik-Plevnik et al. (37), and Tsumita and Iwanaga (58) showed a rapid disappearance of DNA after i.p. or i.v. injection in mammals and suggested that a long survival in blood of DNA fragments was improbable. Ledoux et al. (41-43), on the contrary, believe that the Dnase activity of mouse blood does not appreciably affect DNA fragments as long as they are big. They demonstrated rapid uptake by mouse ovary, uterus, and embryo of high-molecular-weight DNA-3H prepared from a thymineless mutant of *Escherichia coli*. Moreover, infectious DNA can be isolated from leukemic cells (40) or polyoma-infected tissue cultures (22). Infectivity and production of tumors by the isolated DNA suggest that the in vivo uptake by the cells of relatively intact fragments should occur. Consequently, the penetration of large fragments of DNA into the nuclei of neoplastic cells appears necessary for any change in the phenotypic expression of the genome to occur. It is not certain, however, that such big fragments can survive in the blood stream of the host and that they can reach in vivo the nuclei of the neoplastic cells constituting a solid tumor.

The object of the experiments reported here was to test these possibilities in a transplanted rat mammary tumor.

### MATERIALS AND METHODS

**Animals and Tumors.** Sprague-Dawley females, 3 months old and about 200 g, were used. Walker carcinoma 256 was used as a s.c. transplant or as a tissue-isolated preparation in vivo and in the ex vivo perfusion system (30).

**DNA-3H Preparations.** Walker carcinoma is a mammary tumor (6), and the rat mammary gland was used as a source of DNA-3H for most of the experiments.

Females in the 7th to 10th day of lactation were given injections i.v. of 1.0 mCi of thymidine-methyl-3H (Schwarz BioResearch, Inc., Orangeburg, N. Y.; Catalog No. 2533-97; specific activity, 10.4 Ci/m mole) given in a single dose. After 18 to 24 hr, the mammary glands were removed, and a 10% homogenate was prepared in 0.25 M sucrose containing 2 mM MgCl₂. A crude nuclear pellet was obtained by centrifugation for 15 min at 900 × g (International Centrifuge, 2000 rpm, Yoke No. 269). The pellet was suspended in 0.15 M NaCl: 0.1 M EDTA, pH 8.0, containing SLS² 2%. The nuclei from 1 g of fresh tissue were diluted to obtain a final DNA concentration of about 1 mg/ml; 75 to 80% of total gland DNA was recovered in the nuclear pellet. Pronase (Calbiochem, Los Angeles, Calif., B grade, 45,000 proteolytic units Kaken/g, Lots 9000053 to 901106) was dissolved in 0.15 M NaCl: 0.1 M EDTA, pH 8.0, and preincubated for 2 hr at 37°; the final concentration was 2 mg/ml and incubation lasted 7 hr (57). The digest was then rolled for 15 min at room temperature with an equal volume of phenol saturated with 0.15 M NaCl: 0.1 M EDTA. The extraction was repeated 3 times. After centrifugation, the aqueous layer was removed and dialyzed extensively at 5° against 0.15 M NaCl: 0.1 M EDTA. RNase (Worthington Biochemical Corp., Freehold, N. J., bovine pancreas, 2.77.16, 2558 units/mg) was then added to a final concentration of 50 μg/ml, and the mixture was incubated at 37° for 30 min. A 2nd treatment with Pronase and phenol was applied as before, followed by extensive dialysis in 0.01 M NaCl. The concentrations were determined by spectrophotometry and diphenylamine reaction (15).

Fourteen DNA-3H preparations had a 260:230 ratio ranging from 1.9 to 2.3, while for the 260:280 ratio the range was 1.8 to 2.0. The preparations contained no RNA, as determined by the orcinol reaction (55), and less than 0.5% protein, as determined by the method of Lowry et al. (46). The specific radioactivity of the DNA-3H preparations ranged from 700 to 1200 dpm/μg.

In a group of experiments involved with the recovery of the injected DNA, *M. lysodeikticus* DNA-3H was used. *M. lysodeikticus* (ATCC 4698) was grown in a culture medium (ATCC Media No. 265) containing 10 μCi/ml of thymidine-methyl-3H. DNA was extracted from the bacteria following the same general procedure used for the tumor nuclei. The specific activity of 3 preparations used was between 5,000 and 10,000 dpm/μg.

**In Vivo Infusion of DNA-3H.** Under ether anesthesia, a PE10 catheter (Clay Adams, New York, N. Y.) was introduced into the left common artery of the rat bearing the transplant of Walker carcinoma. The catheter was gently pushed into the thoracic aorta and connected to a constant infusion pump (Sage Instruments, White Plains, N. Y.). For prevention of DNA-3H shearing, the solution was never sucked through the needle but was poured into the plastic syringes. A 27-gauge needle was connected to the PE10 catheter, and a constant flow of 200 μl/hr was maintained by the pump for periods of a few hr to 5 days. During infusion, the animal was kept in a cage with food and water ad libitum. Unless otherwise stated, the DNA-3H was infused at a standard concentration of 12 μg/hr in 0.015 M NaCl. The concentration of NaCl was reduced in order to avoid excessive intake of sodium by an

²The abbreviations used are: SLS, sodium lauryl sulfate; TIF, tumor interstitial fluid; MWP, molecular weight profile; DEAE-cellulose, diethylaminoethyl cellulose.
animal infused for several days. Hypotonicity did not appear to affect the rat since only 200 µl/hr were infused.

The ex Vivo Perfusion System. The detailed description of the procedure followed to perfuse the host-isolated tumor was given elsewhere (30). For the comprehension of the data reported in this paper, a diagram of the perfusion system is presented (Chart 1), and the following conditions of the experimental approach are stressed. (a) Walker carcinoma was grown in a pouch of the hypodermis, isolated from the surrounding tissues by a sheet of paraffin and connected to the host only by the left ovarian artery and vein. (b) The transplant was made in the fat pad remaining after ovariectomy, and therefore the grown tumor contained negligible amount of host tissue. (c) Tumors weighing 4 to 10 g were removed from the host, the artery and vein were cannulated, and the preparation was perfused with a pulsatile flow as a normal organ. (d) The perfusion system consisted of a reservoir, a peristaltic pump, filters, a “lung” where blood oxygenation occurred through a thin Teflon membrane, and devices for continuous monitoring of pH, flow, and pressure (29). (e) The tumors were perfused with 50 ml of whole heparinized blood from a compatible host. Blood was recirculated for a standard 6-hr period at 36.5°C. Unless otherwise stated, DNA-3H was added to blood to a final concentration of 60 µg/ml. The blood flow through the perfused tumors was about 5 ml/hr/g. A pH of 7.30 to 7.40 was maintained by regulating manually the concentration of CO2 in the lung and by adding sodium bicarbonate to blood when necessary. Oxygen utilization, glucose consumption, and lactate production were monitored continuously and kept within physiological limits. (f) At the end of perfusion, the tumors, on macroscopic and histological examinations, were indistinguishable from normal s.c. transplants. Standard physiological characteristics, such as total water, RNA:DNA ratio, lipid content, etc., were unchanged. Growth followed regularly upon transplantation.

Sampling of Tumor Blood. During the ex vivo perfusion, blood was sampled from the reservoir at various intervals and at the end.

At the end of long-term in vivo infusion (3 to 5 days), blood was sampled directly from the aorta before tumor removal. In this instance, s.c. transplants were used. During short-term in vivo infusion (2 to 3 hr), tissue-isolated preparations were used, so that the blood flowing out of the tumor could be collected continuously from the vein cannulated in the abdominal cavity (Chart 2). The removed blood was replaced in the animal by compatible blood pumped into the external jugular vein. In order to avoid imbalances in the acid-base equilibrium of the host, the afferent blood was passed through an artificial lung in which the CO2 concentration of the gas phase was adjusted to ensure a pH of...
7.40 at any time. The blood flowing out of the tumors was 3 to 5 ml/hr/g. The flow could be kept constant for several hr if the amount of blood introduced into the animal was slightly higher than the amount removed and if the arterial pressure was maintained at the initial level (80 to 90 mm Hg). Hypervolemia increased blood flow through the tumor. Urethan (Merck Sharpe and Dohme, Newark, N. J.) anesthesia (0.5 to 1.0 mg/g) was adopted in these experiments. Blood leaving the tumor was collected, usually as repetitive 10-min samples, in 2 ml of 0.15 M NaCl:0.1 M EDTA under continuous magnetic stirring.

**Sampling of TIF.** The TIF was collected following a technique previously described (31). A chamber with walls formed by Millipore filters (average pore diameter, 0.45 μm) was embedded in the tumor. The neoplastic cells did not pass through the filter, but the fluid surrounding them passed freely. This is a serum-like material which was drained continuously by means of a PE50 catheter connected to the chamber. During collection, TIF was stored in a tube kept at 1°C and containing 0.5 ml of 0.15 M NaCl:0.1 M EDTA.

**DNA-3H Extraction from Tumors and Cellular Fractions.** After in vivo infusion or ex vivo perfusion of the tumor, the MWP of the incorporated DNA-3H was determined on whole tumors and on cellular fractions. For study of whole tumors, DNA was extracted as described earlier for mammary gland except that the aqueous layer containing the DNA-3H was used for MWP determination without dialysis.

For fractionation of the tissue, a 10% homogenate was prepared in 0.25 M sucrose containing 2 mM MgCl₂. The nuclei were isolated by centrifugation for 15 min at 900 × g and washed according to the method of Hymer and Kuff (38). Nuclei from 1 g of fresh tissue were suspended in 10 ml of 0.15 M NaCl:0.1 M EDTA containing SLS 2% and were treated with Pronase and phenol as the whole-tumor homogenate; 90 to 95% of total tumor DNA was recovered in the nuclear pellet.

The DNA-3H of TIF or serum was extracted by mixing 1 part of fluid with 9 parts of 0.15 M NaCl:0.1 M EDTA containing SLS 4% and Pronase, 4 mg/ml. The mixture was incubated overnight and treated with phenol as above.

**MWP.** The MWP of the DNA-3H were obtained by centrifuge chromatography on DEAE-cellulose (Pulp Grade DE81, Catalog No. 24811, 0.4 mEq/g, Reeve Angel, Clifton, N.J.) as described by Davila et al. (19). This is a step elution profile. The aqueous phase obtained after phenol treatment of the Pronase digest was added directly to the DEAE-cellulose column (4 ml). Each fraction was eluted 5 times with 2 ml of each solvent. Those 2-ml eluates were collected directly in scintillation vials and counted.

**Preparations of DNA-3H Fractions.** For study of the effect of size of DNA-3H fragments on uptake by the tumors, Fractions 3 and 5 were isolated and infused separately. A total DNA-3H preparation was fractionated by centrifuge chromatography as used for MWP analysis. To avoid denaturation of Fraction 5 by the alkaline pH of Solvent 5, a larger column was used, and only the first fraction eluting at pH 7.5 to 8.0 was collected and immediately neutralized. The isolated Fractions 3 and 5 rechromatographed in the same position and showed no denaturation in CsCl gradients. Their sedimentation coefficients, determined with the Spino Model E ultracentrifuge were 5.1 for Fraction 3 and 12.5 for Fraction 5. With the formula of Eigner and Doty (23), average molecular weights of 1.0 × 10⁵ for Fraction 3 and 1.8 × 10⁶ for Fraction 5 were calculated. The selection of Fractions 3 and 5 was guided by the necessity of separating from a DNA-3H preparation enough material with a wide difference in the average molecular weight. The specific activity of both fractions was similar.

**Radioactivity Measurements.** For the DNA-3H chromatography, each 2-ml eluate was mixed with 20 ml of Insta-Gel (Packard Instrument Co., Downers Grove, Ill.) and counted in a Packard Tri-Carb liquid scintillation spectrometer, Model 3000. To fractions containing Solvents 4, 5, and 6, 0.3 ml of concentrated HCl was added before mixing with Insta-Gel. Total tissue radioactivity was measured on a 10% homogenate prepared in water or 0.15 M NaCl:0.1 M EDTA. A 200-μl aliquot was digested overnight with 1.2 ml of NCS solubilizer (Nuclear-Chicago Corporation, Des Plaines, Ill.), diluted with 10 ml of toluene containing 4 g of POPP plus 50 mg of POPOP per liter, and counted. Serum and TIF were treated as tissue homogenate. Blood was decolorized following the method of Mahin and Lobberg (47) and was counted with Insta-Gel as scintillation cocktail. All samples were corrected to dpm by internal standardization.

**CsCl Gradients.** Tumor DNA of animals infused with M. lysodeikticus was analyzed by density-gradient centrifugation (Spinco L2 Rotor No. 40 at 33,000 rpm for 60 to 65 hr) following the method of Flamm et al. (24). The tubes were prepared by adding 4.3750 g of optical grade CsCl (Schwarz BioResearch) to 3.4 ml of 0.15 M NaCl:0.015 M sodium citrate containing about 180 μg of tumor DNA and about 50 μg of cold M. lysodeikticus DNA (Miles Laboratories, Inc., Elkhart, Ind.) as density marker. Seventy fractions were collected from each tube, the absorption was read at 260 μm, and the radioactivity was measured as before.

**RESULTS**

**Changes in the MWP of DNA-3H Added to Blood.** This problem was studied with 2 experimental approaches. In the first one, DNA-3H was added to whole fresh blood, and recirculation was carried on for several hr in the ex vivo perfusion system. In 3 experiments, the circuit contained blood alone, and in 3 others a tumor was added.

Minor shifts of the MWP were observed when DNA-3H was added to blood alone. In a 6-hr perfusion for instance (Chart 3), Fraction 5 decreased from 18 to 10% and Fractions 3 and...
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4 increased proportionally while Fraction 6, the highest in molecular weight, remained practically unchanged.

When a tumor was added to the circuit, larger shifts of the MWP's were observed, both in the DNA-3H portion which remained in blood and in the part incorporated by the tumor. After a 6-hr perfusion (Chart 4) the blood content of Fractions 3 and 4 showed extensive changes, but Fractions 5 and 6 were usually only slightly reduced. The MWP's of DNA-3H extracted from the tumor at perfusion end were markedly different from the MWP's of the injected preparation and were characterized by a predominant concentration of Fractions 5 and 6. In the experiment of Chart 4, more than 80% of the tumor-associated DNA-3H was in Fractions 5 and 6 while in the infused material only 44% was in the same fractions. Similar results were obtained in 2 other experiments. Recovery of radioactivity was 95 to 97%. For a standard 6-hr perfusion time and 60 μg of DNA-3H per ml of blood, the uptake of DNA-3H was about 0.5% of tumor DNA and about 10% of DNA-3H added to the perfusate.

In the second experimental approach, the changes in MWP of DNA-3H were studied in vivo. The DNA-3H preparation was continuously infused through the aorta of the host, and the blood flowing out of the tumor was continuously removed (Chart 2). The infusion lasted 30 to 50 min, and blood was collected for 2 hr after the end of the infusion. The amount of DNA-3H infused varied from about 500 to 1500 μg/hr in 3 separate experiments, and the total amount of blood replaced in each animal varied from 20 to 40 ml. The blood leaving the tumor was collected in multiple 10-min samples, and each collecting vial contained 2 ml of 0.15 M NaCl: 0.1 M EDTA plus 4 mg of Pronase under continuous magnetic stirring. Radioactivity in the tumor-efferent blood during infusion followed a bell-shaped curve (Chart 5). In the experiment of Chart 5 when drainage of DNA-3H in the efferent blood returned to zero, about 50% of the injected radioactivity had been recovered in the venous blood flowing out of the tumor, and 1.8% of the injected DNA-3H was recovered in the tumor.

The MWP of the DNA-3H coming out of the tumor vein in vivo was not appreciably different from the MWP of the DNA-3H preparation injected into the aorta. This indicates that destruction of DNA-3H in vivo did not appear to occur very rapidly confirming the results of the ex vivo experiment.
(Chart 3) and that continuous aortic infusion maintained in the blood vessels of the tumor DNA-3H fragments of a size similar to that of the injected preparation.

In these *in vivo* experiments the amount of DNA-3H incorporated by the tumor during 3 hr of infusion at about 1500 μg/hr had insufficient radioactivity for the MWP to be analyzed.

**Uptake of DNA-3H by Tumors.** In a preliminary experiment, the distribution of DNA-3H between plasma and blood cells was studied. DNA-3H was added to freshly drawn blood in concentration of about 60 μg/ml and recirculated in the perfusion system. After 1 and 6 hr of recirculation, the plasma was separated from the blood cells by centrifugation. In both samples, more than 90% of the radioactivity was found in plasma. On the basis of these data, we consider the uptake of DNA-3H by blood cells negligible for our purposes.

The capacity of Walker carcinomas to incorporate DNA-3H was first tested in the *ex vivo* perfusion system. About 50 ml of blood containing from 20 to 60 μg/ml of DNA-3H were recirculated through each tumor for a standard 6-hr period. At the end of perfusion, the radioactivity was measured in blood and tumor homogenate. The radioactivity of the homogenate was then corrected to exclude the portion due to the blood in the vascular system of the tumor, since Walker carcinomas contain about 0.1 ml of blood for each g of tumor (32). The corrected values showed that the DNA-3H taken up by the tumors corresponded to 0.25 to 0.50% of the total tumor DNA and that uptake was an exponential function of tumor weight within the limits of 5 to 10 g (Chart 6). The function is satisfied by

\[
\log U = 0.98 + 0.16 W
\]

where \( U \) is the uptake in μg of DNA-3H and \( W \) is the tumor weight in g. This relation is not modified by a 20- to 60-μg/ml change in the DNA-3H concentration of the perfusate.

The capacity of incorporating DNA-3H *in vivo* was tested with two types of experiments. The first aimed at selecting the best route of administration. DNA-3H was given at a rate of 477 to 1526 μg/hr through the aorta or through the right femoral vein for 2 hr (Table 1). The blood coming out of the tumor was collected, and the uptake of DNA-3H by the tumor was determined. Infusion through the aorta proved to be much more effective since uptake by the tumor was several-fold higher than after femoral infusion (Table 1). Aortic infusion as the route of administration of DNA-3H was adopted for all experiments concerned with *in vivo* uptake.

In the second type of experiment, the *in vivo* uptake of DNA-3H was measured. The same DNA-3H preparation was continuously infused in the aorta at a concentration of 60 μg/ml and rate of 200 μl/hr in animals transplanted simultaneously with tissue from the same donor tumor. The uptake (Table 2) corresponded to 0.5 to 1.0% of total tumor DNA. The uptake was corrected for the blood content of the tumors, and the extent of this correction was 10 to 20% of total uptake. A difference of 24 hr in the perfusion time during the 3rd and 4th day appeared to change the DNA-3H content of the neoplastic tissues, but there was no difference between the 4th and 5th day. Probably a plateau is reached; however, the number of experiments was not sufficient to decide this question.

The influence of necrosis on DNA-3H uptake and profile was tested in a 22.0-g tumor with an area of about 3 g that was extensively necrotic on macroscopic and histological examination. The concentration of DNA-3H in the necrotic area was about one-half of that in the viable portion of the tumor. The profile of DNA-3H extracted from the viable part of the tumor (Chart 7) was shifted toward

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**Table 1**

*Influence of infusion route on DNA-3H uptake by tumor in vivo*

<table>
<thead>
<tr>
<th>Site of infusion</th>
<th>Rate of DNA-3H infusion (μg/hr)</th>
<th>DNA-3H uptake (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>710</td>
<td>1.93</td>
</tr>
<tr>
<td></td>
<td>1136</td>
<td>2.68</td>
</tr>
<tr>
<td></td>
<td>1526</td>
<td>4.1</td>
</tr>
<tr>
<td>Femoral vein</td>
<td>477</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1526</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**Table 2**

*In vivo uptake of DNA-3H infused for several days through the aorta; infusion rate of 200 μl/hr of a 0.015 M NaCl solution containing DNA-3H, 60 μg/ml*

<table>
<thead>
<tr>
<th>Tumor weight at end of infusion</th>
<th>DNA-3H uptake (μg/g)</th>
<th>Days of infusion through aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.3</td>
<td>36.7</td>
<td>3</td>
</tr>
<tr>
<td>8.8</td>
<td>23.9</td>
<td>3</td>
</tr>
<tr>
<td>14.3</td>
<td>29.3</td>
<td>3</td>
</tr>
<tr>
<td>6.8</td>
<td>63.6</td>
<td>4</td>
</tr>
<tr>
<td>7.8</td>
<td>51.3</td>
<td>4</td>
</tr>
<tr>
<td>8.8</td>
<td>56.7</td>
<td>5</td>
</tr>
<tr>
<td>10.02</td>
<td>62.8</td>
<td>5</td>
</tr>
</tbody>
</table>

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*Chart 6.* Uptake of DNA-3H by solid tumors perfused *ex vivo* for a 6-hr period. The values were corrected for radioactivity in the vascular system. The DNA-3H concentrations varied from 20 to 60 μg of DNA-3H per ml of perfused blood.
high-molecular-weight fractions, as with smaller tumors. In the necrotic part, however, the shift was in the opposite direction, and the small-molecular-weight Fractions 2 and 3, which were absent in the infused preparation, formed about 60% of the extracted DNA-3\(^{3}\)H. The necrotic areas of the tumor seem to be centers of very active DNA-3\(^{3}\)H degradation as shown by extracted DNA-3\(^{3}\)H. The necrotic areas of the tumor seem to be centers of very active DNA-3\(^{3}\)H degradation as shown by Daoust and Amano (18) for hepatocarcinomas. The MWP of total tumor DNA should be expected to show a shift toward low-molecular-weight DNA-3\(^{3}\)H if necrosis were present. Consequently, the concentration of high-molecular-weight DNA-3\(^{3}\)H into the tumor (Chart 4) cannot be due to a selective accumulation into necrotic areas.

**Kinetics of DNA-3\(^{3}\)H Uptake.** The fractional distribution of radioactivity was measured in the 3 compartments which constitute a solid tumor: vessels, cells, and the interstitial space (31–33). This space separates the vascular wall from the cell membranes and is filled by collagen and a fluid phase, the TIF, which was sampled with the microprobe chamber procedure (31). The 1st type of experiment was an ex vivo perfusion for a standard 6 hr at a concentration of 60 \(\mu\)g of DNA-3\(^{3}\)H per ml of whole blood with 2 Walker carcinomas that had incorporated the microprobe chamber. One example is given in Chart 8. As expected, more than 80% of radioactivity was incorporated by the cellular compartment. Moreover, 2 gradients of radioactivity concentration were observed, 1 between the vascular and the interstitial space and the 2nd between the interstitial and the cellular compartments. The 1st gradient can be expected when a relatively large concentration of DNA-3\(^{3}\)H is present in the circulating blood. The 2nd gradient, however, should be present only if an active concentration of DNA-3\(^{3}\)H by the neoplastic cells occurs.

The 2nd type of experiment was carried on in vivo. In 4 animals, Walker carcinomas containing a microprobe chamber were grown s.c. in the interscapular region and TIF was collected continuously. On the 3rd day after transplantation, the infusion of DNA-3\(^{3}\)H: (12 \(\mu\)g/hr) through the aorta was started and continued for 4 days. The radioactivity was measured in the nuclear pellet, supernatant, TIF, and blood, and the fractional distribution in the cytoplasm was calculated (Table 3). More than 80% of the radioactivity of the cellular compartment was concentrated in the nuclei. The radioactivity of the supernatant fraction was mostly due to the DNA-3\(^{3}\)H in the cytoplasm. A sharp gradient between cellular and interstitial space was also evident here as in the ex vivo perfusion; however, the concentration gradient between vascular and interstitial space was lacking. This finding is probably related to the very low concentration of DNA-3\(^{3}\)H in blood which is due in part to the low infusion rate and in part to the relatively rapid disappearance of DNA-3\(^{3}\)H from the circulatory system during the in vivo infusion as compared with the ex vivo perfusion.

In 4 experiments like the one reported in Table 3, when the dpml/ml were calculated for TIF and blood, the TIF:blood ratio varied between 1.0 and 1.8. This indicates that in vivo during a long-term infusion with a relatively low blood level of DNA-3\(^{3}\)H, the concentrations in blood and in TIF are roughly similar, but the largest amount of DNA-3\(^{3}\)H is accumulated in the nuclei.

MWP's of the DNA-3\(^{3}\)H-infused preparation were compared with those of the nuclear and supernatant fractions, total tumor, and TIF. Three animals were used, and one of these fractionations is given as an example (Chart 9). As expected, the MWP of total tumor showed a shift toward the high-molecular-weight fractions as compared with the profile of the infused DNA-3\(^{3}\)H preparation and an increase of Fraction 2, probably due to degradation. The nuclear fraction showed a similar picture with 66% of the DNA-3\(^{3}\)H constituted by the largest-molecular-weight Fraction 6. The supernatant fraction consisted of 85% of Fraction 2 and only about 10% of Fractions 5 plus 6; TIF contained almost exclusively the small fragments of Fraction 2. Similar results were obtained for the other two tumors.

**Table 3**

<table>
<thead>
<tr>
<th>Tumor compartment</th>
<th>dpml/g</th>
<th>% of radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>34,300</td>
<td>83.0</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>6,350</td>
<td>15.3</td>
</tr>
<tr>
<td>TIF</td>
<td>620</td>
<td>1.5</td>
</tr>
<tr>
<td>Blood</td>
<td>97</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**Chart 8.** Radioactivity distribution in the vascular, interstitial, and cellular compartments of a tumor perfused for 6 hr. The three compartments are presented in the background and their size is expressed as percentage of total tumor water (32, 33). The radioactivity of the vascular and interstitial compartments was measured by direct sampling of blood and interstitial fluid, and the radioactivity of the cellular compartment was calculated by the difference.

**Chart 7.** MPW's of DNA recovered from viable and nonviable portions of Walker carcinoma infused in vivo. Abscissa, fraction numbers.
The possibility that the increased radioactivity of the nuclear pellet was caused by DNA-3H fragments sticking to the nuclei was ruled out by the following experiment. Two tumor-bearing rats were perfused for 4 days through the aorta, one with 0.15 M NaCl solution and the second with DNA-3H (12 μg/hr). At the end of perfusion, the DNA-3H preparation used in the second rat was added to the homogenate of the 0.15 M NaCl solution-perfused tumor so that the final dpm/g were equal for both homogenates. The nuclei were then isolated. As expected, the nuclear pellet of the DNA-3H-perfused tumor contained about 80% of the total radioactivity. In contrast, the nuclei of the 0.15 M NaCl solution-perfused tumor to which the DNA-3H preparation was added after homogenization had a negligible number of counts. The MWP of the DNA-3H-perfused tumor showed the familiar shift toward high-molecular-weight fractions. On the contrary, the MWP of the 0.15 M NaCl solution-perfused tumor did not show any change as compared with the MWP of the DNA-3H preparation added.

The fact that small-molecular-weight DNA-3H fragments constituted by far the largest fraction present in TIF could depend on at least 3 possibilities: (a) the larger DNA-3H fragments were not sampled by the chamber; (b) they were degraded in the interstitial space; and (c) they were rapidly concentrated in the neoplastic cells so that a relatively larger amount of small DNA-3H fragments were left in TIF.

The 1st possibility was ruled out by the following experiment. A micropore chamber was immersed overnight at room temperature into a DNA-3H preparation. Comparison of total radioactivity, DNA concentration, and MWP in the fluid inside and outside the chamber showed no significant difference, as expected, since a filter of 0.45-μ pore size was used.

In vivo the neoplastic cells are growing directly against the chamber, and one can suppose that a reduction in the pore size of the filter may occur which could limit the passage of large DNA-3H fragments into the chamber. This, however, appears improbable as suggested by the following observation. In tumors weighing 5 to 10 g, the neoplastic cells grow against the Millipore filter and adhere to it very tightly. However, when the tumor reaches 20 g or more, a cystic cavity, full of fluid, is usually formed around the chamber. Under these conditions, the filters are not covered by cells, and the sampling chamber floats in the fluid of the cyst as in the in vitro test. The transfer of macromolecules injected into the blood stream, either DNA-3H or high-molecular-weight dextran, was found to be similar in the 1st case when obstruction by the cells could be expected and in the 2nd case when the surface of the filters was free of cells.

The 2nd possibility, a DNA-3H degradation by TIF, was tested with 2 experiments. In the 1st one, DNA-3H was added to TIF in amount similar to the concentration observed during regular aortic infusion. No substantial change in MWP was observed after standing at room temperature for 10 hr.

In the 2nd experiment, the differences in MWP of DNA-3H were compared in TIF collected from 2 animals, one regularly infused through the aorta with DNA-3H and the other infused with 0.15 M NaCl solution while DNA-3H was added later to the vial in which TIF was collected. Rat 1 received in a pouch of the s.c. tissue one micropore chamber surrounded by tumor fragments. Two days later, when TIF was draining regularly out of the catheter of the chamber, DNA-3H infusion was started through the aorta and continued for 4 days. The daily volume of TIF increased with the growth of the tumor (Chart 10A), and the dpm collected for each 24-hr sample also increased until a plateau was reached (Chart 10B). A larger drainage of TIF during the last day of collection was usually observed (Chart 10C). TIF draining out of the tumor was collected in 1 ml of 0.15 M NaCl:0.1 M EDTA, and the amount consecutively collected for 24 hr was analyzed as 1 sample. Rat 2 received the same treatment except that 0.15 M NaCl alone and not DNA-3H was infused. To the 1 ml of 0.15 M NaCl: 0.1 M EDTA in which TIF was collected, DNA-3H was added at the beginning of the 24-hr collection in amount roughly equal to that contained in the TIF samples of Rat 1. The MWP's of each 24-hr sample from Rat 1 and 2 were compared with the MWP of the infused preparation. In TIF of Rat 1, the MWP showed the expected sharp increase of the low-molecular-weight Fraction, 2 (Chart 9). However, the MWP in TIF of Rat 2 was similar to that of the infused preparation and repeated the picture presented in Chart 3 for blood plasma. Moreover, DNA-3H added to TIF collected in the absence of EDTA also had a MWP unchanged for 5 hr. These data indicate that DNA-3H degradation by TIF is not a major cause of the accumulation in TIF of small DNA-3H fragments.

The absence of large DNA-3H fragments of TIF may be due to one of the following conditions: (a) DNA-3H is degraded in the passage from the vascular into the interstitial compartment, or (b) the largest DNA-3H fragments, which had passed more or less undamaged across the vascular wall, are actively and preferentially concentrated by the cells while the small fragments accumulate in TIF. The 2nd hypothesis seems more plausible in the light of the following experiments.

Preferred Uptake of Large DNA-3H Fragments by Tumor Nuclei. Two animals bearing a s.c. transplant were infused, respectively, with Fractions 3 and 5, separated by centrifuge chromatography from the same DNA-3H preparation and of
Chart 10. The interstitial fluid draining out of a 6.3-g Walker carcinoma was collected during in vivo DNA-3H infusion through the aorta. The volume of interstitial fluid collected over a 24-hr period usually increased with time (A); and so did the radioactivity of each sample (B) until the 5th day when the dpm/ml decreased (C) following an increase of total TIF drained, but the total dpm collected over 24 hr remained constant (B).

similar concentration and specific activity. After 4 days of continuous infusion (12 μg/hr) the dpm per ml of blood and per g of tumor were counted and their MWP’s were determined (Table 4). As expected, at the end of infusion the blood radioactivity was equal in both animals. However, the concentration of radioactivity in the tumor perfused with Fraction 5 was much higher than that of the tumor perfused with Fraction 3. In particular, the nuclei of the former had about 7 times more radioactivity than the nuclei of the latter (Table 4). Furthermore, the MWP of DNA-3H extracted from the nuclear pellet of tumors perfused with Fraction 5 showed about 70% of nuclear radioactivity in the large-molecular-weight Fractions 5 and 6 (Chart 11), while the nuclear pellet of the tumor perfused with Fraction 3 had more than 80% of the extracted DNA-3H in the small-molecular-weight Fraction 2 and only 14% in Fractions 5 and 6. The MWP of the supernatant of both tumors repeated the picture presented in Chart 9.

When the experiment was repeated with Fractions 3 and 5 isolated from different DNA-3H preparations, similar results were obtained. About 66% of the radioactivity in the nuclei of the tumor infused with Fraction 5 was constituted by large-molecular-weight DNA-3H (Fractions 5 and 6) but only 5% of the DNA-3H separated from the tumor perfused with Fraction 3 was constituted by high-molecular-weight fragments. Degradation of DNA in small fragments appears to hinder, not favor, the in vivo uptake by neoplastic cells.

A second type of experiment was carried on ex vivo. Two tumors were perfused under identical conditions except that in the perfusing blood one contained DNA-3H and the other contained thymidine-methyl-3H added in amount equal to about 25% that of DNA-3H. After 6 hr of perfusion, DNA-3H of the tumors was extracted, and the MWP was determined. Under the conditions of the experiment, both tumors incorporated the same amount of radioactivity, about 10% of the counts added to the blood. However, only 3% of the DNA-3H extracted from the thymidine-methyl-3H-perfused tumor was found in the high-molecular-weight Fractions 5 and 6, while 89% was extracted in the same fractions when the tumor was perfused with DNA-3H (Table 5). This indicates that a concentration of DNA-3H in the nuclei is obtained much more rapidly when DNA fragments are infused instead of small-molecular-weight components thereof. The difference in MWP between thymidine-3H and DNA-3H-infused tumors were found to be similar to that between Fraction 3 and 5 of the preceding experiment.

Recovery of M. lysodeikticus DNA from the Nuclear Pellet. The recovery of M. lysodeikticus DNA accumulated in the nuclei was attempted in 5 experiments. M. lysodeikticus DNA-3H was continuously infused through the aorta of the host for 4 days. Infusion started when the transplant was 3 days old, when a firm nodule was palpable, and continued during the rapid growth of the tumor. The infusion rate was 4 to 12 μg/hr. This small amount was selected since the nuclei had the capacity to concentrate the infused DNA, thus avoiding any accumulation of M. lysodeikticus DNA-3H in the blood or in the interstitial fluid of the tumor. From previous experience, it was known that the interstitial fluid of the tumor contained only small-molecular-weight fractions (Chart 9) which should not band in CsCl gradients.

In 3 animals, the tumors were removed within 30 min after the end of infusion, in one animal after 24 hr and in another animal after 48 hr. At the time of the removal, there was no appreciable radioactivity in blood. The DNA of the nuclear pellet was extracted and fractionated in CsCl with cold M. lysodeikticus DNA as marker.

In the nuclear pellet of the 3 tumors removed within 30 min after the end of infusion, a small peak of M. lysodeikticus DNA-3H was observed (Chart 12). From the specific activity of the preparations infused, the quantity of M. lysodeikticus DNA-3H banding at the density of unlabeled M. lysodeikticus DNA was calculated to be about 0.02% of the total tumor DNA. We interpreted these data to indicate that, at least for a short period of time, exogenous M. lysodeikticus DNA could be detected in the nuclear pellet of the solid tumor. In the animals sacrificed 24 and 48 hr after the end of infusion, a peak of M. lysodeikticus DNA-3H distinct from the DNA of the tumor was not appreciable.

In preliminary experiments, the peak of rat DNA, heavily labeled after 4 days of M. lysodeikticus DNA-3H infusion, was subjected to 2 treatments: (a) heat denaturation and centrifugation in alkaline CsCl and (b) sonic treatment to different levels of DNA fragmentation and centrifugation in CsCl gradients. The objective was to resolve the large peak of
Difference in concentration of large-molecular-weight DNA-$^3$H in two tumors infused in vivo

<table>
<thead>
<tr>
<th>DNA-$^3$H infused</th>
<th>Radioactivity uptake (dpm/g)</th>
<th>Serum radioactivity at sacrifice (dpm/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total tumor</td>
<td>Nuclei</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>1853</td>
<td>323</td>
</tr>
<tr>
<td>Fraction 5</td>
<td>7945</td>
<td>2429</td>
</tr>
<tr>
<td>Ratio, Fraction 3:Fraction 5</td>
<td>4.3</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Chart 11. Preferential concentration of large-molecular-weight DNA-$^3$H. Infusion of Fraction 5 (average molecular weight, $1.8 \times 10^6$) resulted in a larger concentration of DNA-$^3$H fragments in tumor nuclei than did infusion with Fraction 3 (average molecular weight, $1.0 \times 10^6$).

rat DNA into 2 peaks of different density which could suggest integration of *M. lysodeikticus* DNA into rat DNA. The results were negative.

**DISCUSSION**

The principal objective of this work was to ascertain whether large fragments of DNA-$^3$H added to the blood stream could reach the nuclei of solid tumors. The results obtained indeed indicate that the largest DNA-$^3$H fragments were found to be concentrated in the nuclei.

DNA-$^3$H was degraded in blood but not very rapidly. In fact, when blood leaving the tumor was continuously removed during *in vivo* aortic infusion of DNA-$^3$H, about 50% of the nucleic acid was recovered from the tumor blood and with little change in the MWP. Therefore, an uninterrupted flow of large DNA-$^3$H fragments could be maintained in the vascular system of the tumor *in vivo* by a continuous aortic infusion at a relatively low DNA concentration (4 to 12 $\mu$g/hr). The observation of Ledoux and coworkers (41) that large DNA fragments survive in blood better than small ones has been confirmed in our experimental system.

The uptake of the DNA-$^3$H preparation used in our perfusion experiment was an exponential function of the tumor weight. We have no explanation for this finding. Tumors perfused for 6 hr accumulated DNA-$^3$H in amounts not larger than 0.5% of total tumor DNA while a level up to 1.0% was observed in tumors infused *in vivo* for several days. However, the observation that Fraction 5 was incorporated more rapidly than Fraction 3 indicates that the size of the DNA-$^3$H fragments infused is an important parameter in the final uptake. Infusion through the aorta was a mandatory route for supplying DNA-$^3$H to the tumor *in vivo*. The venous route was very ineffective, perhaps because the lung was taking up most of the material.

As expected, a concentration gradient was observed between the vascular and the interstitial compartments. When DNA-$^3$H reached a relatively high concentration in blood, the vascular wall formed a barrier which DNA-$^3$H crossed slowly. The sharp difference in DNA-$^3$H concentration between interstitial fluid and cells, as well as the virtual absence of large DNA-$^3$H in TIF, were two unexpected findings. Since TIF was found to be unable to degrade DNA-$^3$H rapidly *in vitro*, it appears that the nucleic acid, after crossing the vascular wall into the interstitial space, is subjected to a preferential uptake whereby the larger fragments are rapidly taken up by the cell. The conclusion that large-molecular-weight DNA-$^3$H is preferentially concentrated in the nuclei is based on 3 findings. First, the tumors infused *in vivo* or perfused *ex vivo* with a DNA-$^3$H preparation contained about 80% of the radioactivity in the nuclear pellet. Moreover, 60 to 70% of nuclear DNA-$^3$H was found in the high-molecular-weight Fractions 5 and 6 while the infused DNA-$^3$H preparation contained less than 20% of both fractions. Second, tumors perfused *ex vivo* for 6 hr with thymidine-methyl-$^3$H added to blood contained in the nuclei less than 5% of DNA-$^3$H in the high-molecular-weight Fractions 5 and 6. On the contrary, tumors perfused for the same time and with equimolar amounts of DNA-$^3$H preparations had about 80% of radioactivity in the large-molecular-weight Fractions 5 and 6. Third, when Fractions 3 and 5 were isolated and infused separately, a sharp difference in uptake and MWP was observed. The tumors...
Table 5
Difference in concentration of large-molecular-weight DNA-3H in
2 tumors perfused ex vivo, one with thymidine-methyl-3H and
the other with DNA-3H preparation

| Perfusion with | Total blood dpm | Total tumor dpm | Radioactivity uptake (%) | DNA-3H
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-3H</td>
<td>675,000</td>
<td>66,200</td>
<td>9.8</td>
<td>89.3</td>
</tr>
<tr>
<td>Thymidine-3H</td>
<td>11,144,000</td>
<td>1,133,000</td>
<td>10.1</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Chart 12. Recovery of M. lysodeikticus DNA from the nuclear pellet of a 3.5-g Walker carcinoma. M. lysodeikticus DNA-3H was infused for 4 days at 4 µg/hr. The animal was decapitated 12 min after the perfusion was stopped. Under the peak of cold M. lysodeikticus DNA added to the nuclear pellet and collected in the 15 to 30 fractions, a small peak of M. lysodeikticus DNA-3H is appreciable and clearly separated from the rat DNA collected in the 39 to 60 fractions.

receiving Fraction 5, which had an average molecular weight about 18-fold larger than that of Fraction 3, incorporated in the nuclei about 7 times more DNA-3H than the tumor receiving Fraction 3. Moreover, about 80% of DNA-3H found in the nuclei of the tumor infused with Fraction 5 was separated in the high-molecular-weight Fractions 5 and 6, while in the tumor perfused with Fraction 3 only 5 to 14% of DNA-3H was in these large fractions.

Since it is clear that larger DNA fragments are taken up by the cells much more rapidly than smaller ones, the concentration of large DNA-3H into nuclei could be due to a transfer through the cytoplasm involving very little demolition of the infused DNA-3H or to a demolition of DNA-3H by cellular phagosomes followed by rapid reconstruction and repair in the nuclei. There is no doubt that extensive demolition of infused DNA occurred (Chart 12); however, the possibility that some exogenous DNA-3H could penetrate the nuclei, more or less intact, was demonstrated by the rescue of M. lysodeikticus DNA from the nuclear pellet with CsCl gradient centrifugation. M. lysodeikticus DNA of the same density and probably of the same composition as the infused preparation was recovered from the nuclear pellets of tumors removed within 30 min from the end of infusion but not 24 or 48 hr later. Since our purpose was to ascertain whether or not relatively intact DNA-3H could reach the nuclei of the tumor in vivo, a time-sequence study of the disappearance of the M. lysodeikticus DNA-3H peak was not done at this time.

 Autoradiographic techniques failed to show labeled material in the neoplastic cells; the specific activity of our DNA-3H preparations was probably not high enough. We cannot say therefore whether DNA-3H was accumulated in the nuclei of neoplastic or stroma cells. There is, however, no indication that a particular cell type in the tumor has a preferential capacity to incorporate DNA-3H. Morphologically, Walker carcinomas did not show an abnormally high number of macrophages. In fact, very few of them were evident even after phagocytosis was stimulated by intraarterial injection of Norit. Moreover, since about 80% of the final radioactivity was in the nuclear pellet, it seems very improbable that an exclusive concentration of DNA-3H concentration should occur in the nuclei of the stroma without an autoradiographic visualization. In these few nuclei, an extremely high DNA-3H concentration should occur to account for a total DNA-3H uptake of 1% of the total tumor DNA. We assume, therefore, that the large DNA-3H fragments penetrated into the nuclei of neoplastic cells.

ACKNOWLEDGMENTS

The help of Dr. Saul Roskes during the first part of this investigation, the advice of Dr. E. Kuff, Dr. W. Schneider, and Dr. W. Kidwell, and the technical assistance of Mrs. Flora H. Grantham, Mrs. Ilona Losonczy, Mr. Elbert Isreal, and Mr. Donald Hill are gratefully acknowledged. The histoautoradiographs were done in the laboratory of Dr. R. Malmgren, Laboratory of Pathology, National Cancer Institute, NIH.

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Translocation of DNA from the Vascular into the Nuclear Compartment of Solid Mammary Tumors

Claude Watters and Pietro M. Gullino

*Cancer Res* 1971;31:1231-1243.

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