ALTERED NUCLEAR RNA TRANSPORT ASSOCIATED WITH CARCINOGEN INTOXICATION IN RATS

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SUMMARY: The release of nuclear restricted RNA to the cytoplasm in dimethylaminoazobenzene (DAB) and acetylaminofluorene (AAF) induced tumors suggested a basis for the phenotypic expression of chemically induced neoplasms. In this report the increased release of RNA from nuclei in vitro is identified both following acute intoxication and with prolonged (tumor producing) exposure to a third carcinogen, thioacetamide. This release is independent of ATP added in the cell-free system and consistent with "leaky" nuclei, providing further support for the hypothesis that nuclear RNA transport is involved in chemical carcinogenesis.

The release to the cytoplasm of nuclear restricted RNA, and of possibly other RNA species, in an uncontrolled manner was suggested as a potential basis for the phenotypic expression of chemically induced neoplasia (1,2). The observation that nuclear restricted RNA appeared in the cytoplasm of tumors induced by acetylaminofluorene (AAF) and by dimethylaminoazobenzene (DAB), but not in the livers of animals treated with the noncarcinogenic analog, aminoazobenzene (AB), provided a basis for this hypothesis. These RNAs may contain the genetic information coding for the several constitutive enzymes that appear in the developing liver and reappear in the regenerating and neoplastic liver. They may also provide the genetic information for the appearance of the alpha-fetoprotein and the presence of membrane change also consistent with the less differentiated state. The basis for nuclear transport of RNA has only recently been investigated (3-9). We set about to test whether or not an altered transport could be identified in a cell-free system from animals treated with carcinogens. In the following report we demonstrate two types of aberration produced by the carcinogen thioacetamide.

MATERIALS AND METHODS:

Male Sprague Dawley rats were maintained in our laboratories on Purina

Lab Chow in wire bottomed cages. Following a 16 hour fast, they were given thioacetamide dissolved in water at 5 or 20 mgm/100 gram body weight. Administration was made by gastric intubation, control animals received the same vehicle. For long-term studies animals were fed a diet containing 0.25% thioacetamide for 12 weeks. At this time 85% of surviving animals had identifiable hepatic neoplasms. Forty five minutes and 120 minutes before scheduled sacrifice, the animals were anesthetised with ether and received 3 microcuries of orotic-6-^{14}C acid per 100 gram body weight by tail vein injection. The animals were sacrificed by cervical concussion, samples of blood were obtained for serum radioactivity determinations, the livers were removed, weighed separately, and from these nuclei were prepared (10). The isolated nuclei were suspended in 0.88 M sucrose with 0.025 M KCl, 0.005 M $MgCl_2$, 0.05 M Tris pH 7.6. The protein content as well as DNA and RNA were measured. The nuclear suspension was diluted to 8.8 mgm/ml of protein, assay for release of ribonucleoprotein was carried out in a system adapted from Ishikawa (3). This contained in a total volume of 0.6 ml, 2.2 mgm nuclear protein, 37 µg ribonuclease inhibitor (11,14) with and without 8 µM ATP. Incubation was carried out for 20 minutes at 20°C. The reaction was stopped at 0 and at 20 minutes by the addition of 2.2 ml of ice cold buffer, and the nuclear suspension centrifuged at 800 xg for 15 minutes. The supernatant was aspirated and recentrifuged following which it was made 5% TCA by the addition of 1/10 volume of 50% TCA. Precipitation was carried out in the cold for 20 minutes and the insoluble material recovered by centrifugation at 2000 xg for 20 minutes. The precipitate was dissolved in 1.0 M NaOH, and both the radioactivity of the supernatant and the precipitate and the original nuclei were measured suspended in Aquasol$^{(R)}$ in a liquid scintillation spectrophotometer. From these data the percentage of RNA released at zero time, at 20 minutes in the presence and in the absence of ATP was determined in the precipitable material. Total TCA precipitable RNA release was the total radioactivity released without the "zero" time subtracted. These results are shown in Table I.

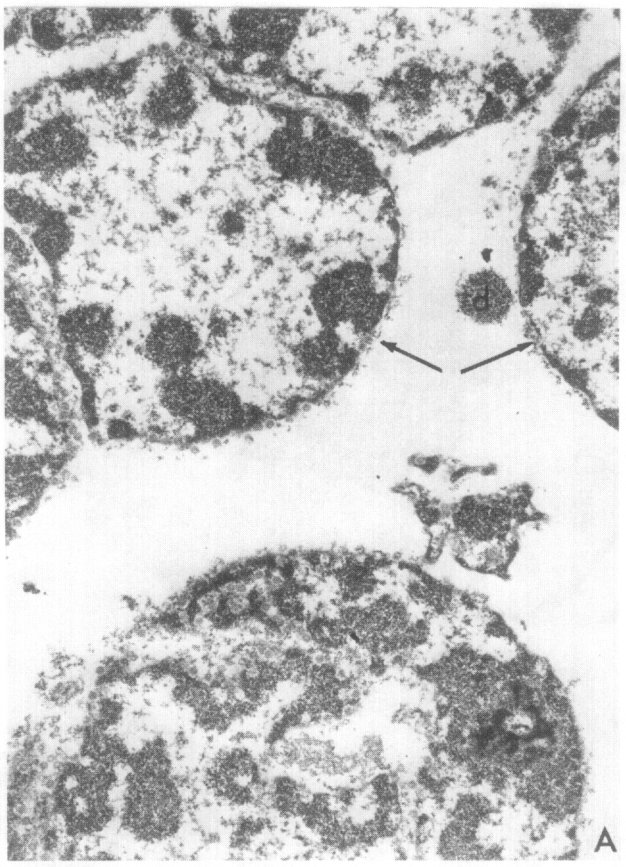

FIGURE 1. (A) An electron micrograph of control nuclei following incubation with ATP for in vitro release. There is an accentuated chromatin clumping, the preservation of the nuclear envelope is apparent (arrows). Some small quantity of debris is present (d). Magnification approx. 15,000. (B) An electron micrograph of nuclei following in vitro release from an animal that received 5 mgm/100 gram body wt. of thioacetamide 16 hours before sacrifice. There is an accumulated clumping of the chromatin, the aggregates are more dense than controls, and the nuclei are larger. Again the nuclear envelope remains and the outer leaflet is present (arrows). Magnification approx. 15,000. (C) Nuclei from an animal 16 hours following 20 mgm/100 gr body wt. of thioacetamide in vitro RNA release. The chromatin clumping and denseness of packing is again more notable than control preparation. Nucleolar changes are readily apparent. The nuclear envelope is intact and the outer leaflet is present (arrow). Magnification approx. 20,000.

To determine the size of the released ribonucleoprotein, supernatant fluids following incubation and removal of the nuclei by centrifugation were placed on linear 10-30% sucrose solution gradients containing 50 mM KCl, 5 mM

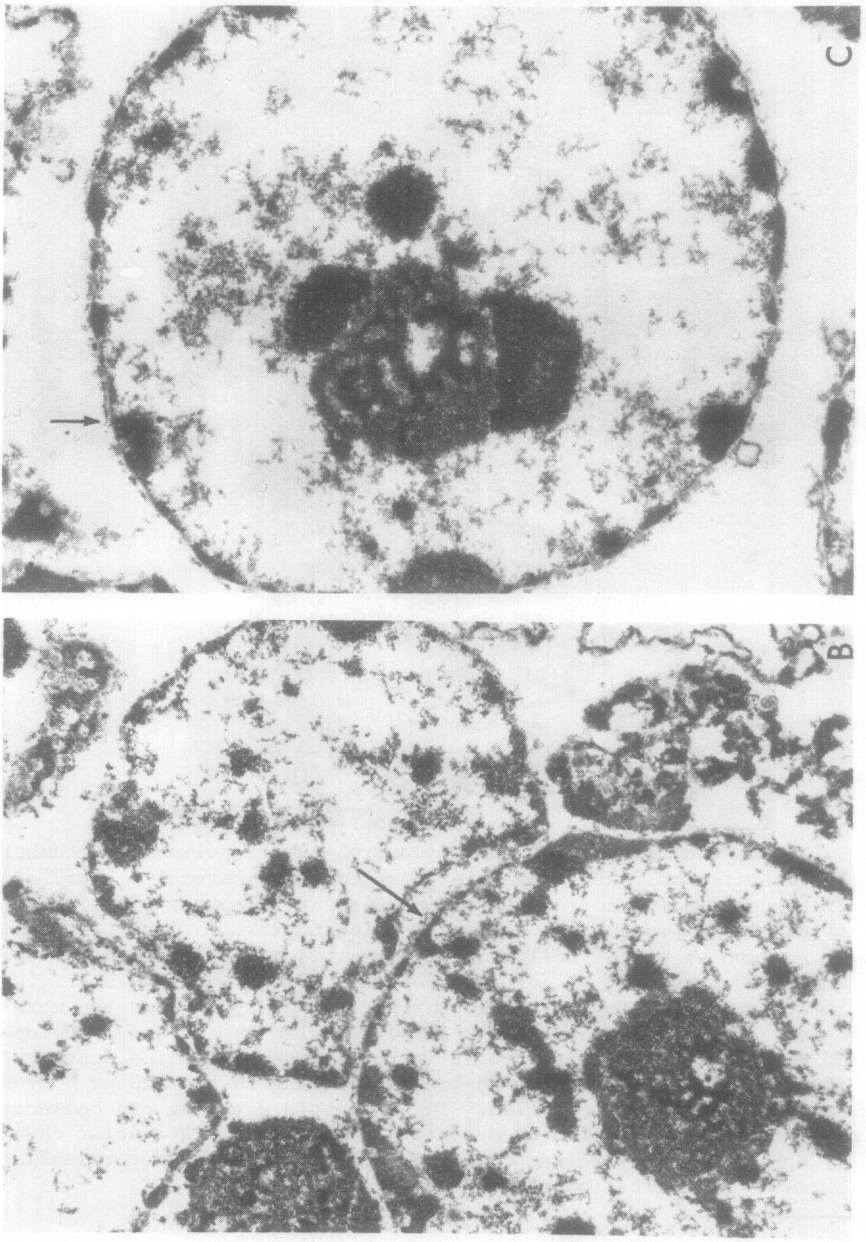

Fig. 1.
(cont.)

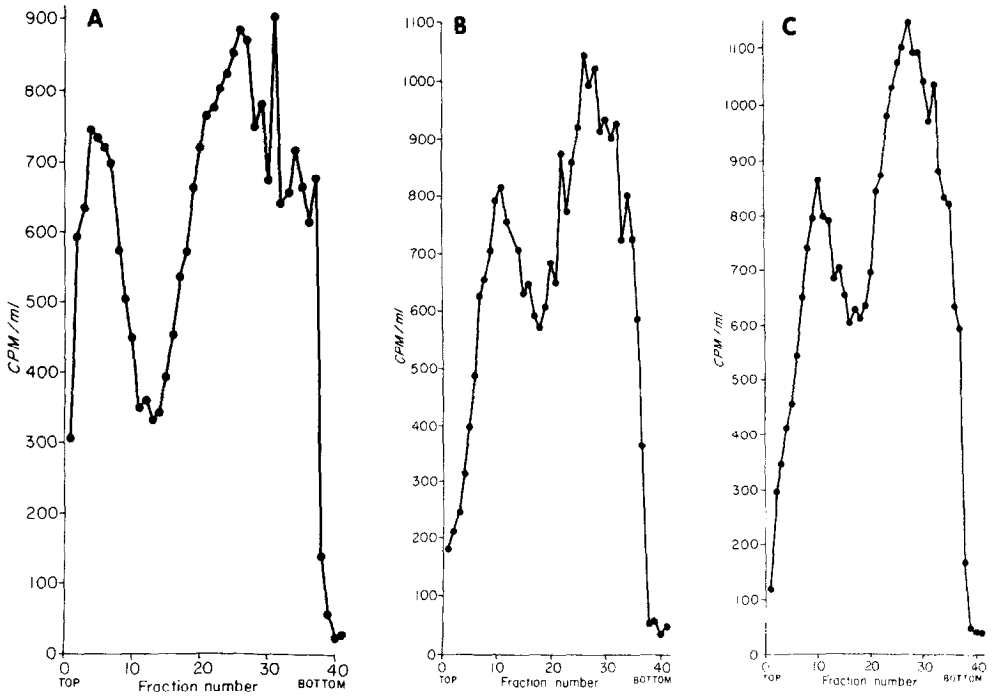

FIGURE 2. Supernatant fluids following sedimentation of nuclei from incubation mixtures were loaded onto 10-30% linear sucrose gradients containing 50 mM KCl, 5 mM $MgCl_2$, 50 mM Tris pH 7.6, 0.01 M $KHCO_3$. The gradients were centrifuged at 25,000 Rev/min in Spinco SW27 rotors for 14 hours. 45S nuclear material (2,3) was added as marker. The gradients were fractionated and assayed for measurement of radioactivity and OD_{260} as indicated. The major band from control (A), 5 mgm TIAA 16 hours (B), and 20 mgm TIAA 16 hours (C) are identical and migrate at a level with 45S material.

$MgCl_2$, 50 mM Tris pH 7.6 with 0.01 M $KHCO_3$. Marker nuclear 45 S RNA was added to several tubes for comparison (3). The released material was separated by centrifugation at 25,000 RPM for 14 hours in a Beckman Spinco SW27 rotor. The gradients were separated into 40 fractions, the radioactivity in the several fractions was measured as described, and the markers were detected by absorbency at 260 nm. The gradients are shown in Figure 2.

It has been suggested that the time of RNA labeling in vivo makes possible the separation of RNA into messenger-like (45 minutes labeling) and ribosomal-like (120 minutes) (5,9). To see if these manipulations and possible selection of RNAs might alter these results, a separate set of experiments was

undertaken using long-term labeling. The results are shown in Table II.

Nuclei before and following incubation were fixed in 1% osmium tetroxide for 1 hour at 0°C, dehydrated and embedded in Epon epoxy resin (12). The sectioned pellets were examined in an AEI-6B electron microscope. These structures are shown in Figure 1.

RESULTS AND DISCUSSION:

We were able to confirm with nuclei from control animals the results of Ishikawa that the *in vitro* system assaying nuclear release was temperature dependent, required ATP, and resulted in a larger product in the presence of the ribonuclease inhibitor (3,14). Furthermore, electron micrographs of the nuclear pellet before and after incubation revealed the presence of an intact nuclear envelope, and following incubation, there was an enhanced clumping of chromatin (Fig. 1).

By 16 hours following intoxication there was decreased incorporation of orotate into nuclei *in vivo* following both the 5 and the 20 mgm doses (the nuclei of animals that received 5 mgm/100 gram body weight were 80% of controls, those receiving 20 mgm/100 gram body weight were 70%). In addition, these nuclei were less able to restrict RNA in a cell-free system and the net effective ATP transport was reduced (Table I). Animals receiving the 5 mgm dose showed neither increased 0 time loss nor changed total or ATP dependent ribonucleoprotein transport, but showed a doubling of the non-ATP dependent release. In contrast the higher dose produced both an increased "leakiness," reflected in zero time loss and non-ATP dependent loss, but also a decreased ATP dependent transport. The size of the ribonucleoprotein complex released was not different comparing control and treated animals when measured by sucrose gradient centrifugation (Fig. 2). The major faster-sedimenting species was coninicidental with marker 45 S material, confirming previous observations (3-9). These were evident at both 16 and 36 hours. Similar results were observed with longer *in vivo* labeling times (Table II). In other experiments a persistence and an accentuation of this change was noted with 16 week

TABLE I

PERCENTAGE RELEASE OF IN VIVO LABELED (45 MINUTES) NUCLEAR RNA TO INCUBATION MEDIA IN VITRO†

	0 Time	Without Added ATP	With Added ATP	Total Release Including 0 Time
Control	2.00 ± 0.90	6.82 ± 2.24	30.00 ± 5.69	32.00 ± 7.40
5 mgm TIAA 16 hr.	2.06 ± 1.14	10.3 ± 5.37*	29.95 ± 8.2	32.01 ±10.2
5 mgm TIAA 36 hr.	2.23 ± 0.88	9.40 ± 4.10*	27.4 ± 6.99	29.6 ± 9.40
20 mgm TIAA 16 hr.	6.39 ± 1.56*	15.49 ± 2.99*	30.95 ± 1.79	37.34 ± 4.57
20 mgm TIAA 36 hr.	3.61 ± 1.79*	12.10 ± 1.73*	23.86 ± 3.15*	27.47 ± 3.61*
Tumor bearing liver**	11.4	12.1	16.2	27.6

†Assay for release of RNA from nuclei was measured as described by Ishikawa et al. (3). These results represent the means ± one standard deviation of 3 or more separate experiments.

*These values are statistically different from control at $P < 0.05$.

**Livers from three animals were pooled following removal of tumorous nodules.

feeding sufficient to produce hepatomas. These changes are unrelated to changes in nuclear composition, or to differences in nuclear fragility. There is no significant difference in the composition of the nuclei following incubation, in all three preparations about 10% of the original protein and DNA and 20% of RNA were not recovered in the nuclear pellet. The morphology of the nuclei was not different, the nuclear envelopes appeared intact, however, nucleolar changes and accentuated chromatin clumping were apparent in nuclei from thioacetamide treated animals (Fig. 1). There is no difference in loss of TCA precipitable or soluble prelabeled DNA. Thus it appears that in vivo treatment with thioacetamide results in nuclei whose capacity to restrict RNA is lost in vitro.

There is increasing evidence that the processing, restriction and selective transport of RNA from the nucleus play a significant role in regulation during development and regeneration (1-9). The mechanisms that are involved

TABLE II

PERCENTAGE RELEASE OF IN VIVO LABELED (120 MINUTES) NUCLEAR RNA TO INCUBATION MEDIA IN VITRO†

		0 Time	Without Added ATP	With Added ATP	Total Release Including 0 Time
Control		1.40	4.80	28.8	25.2
5 mgm TIAA	16 hr.	1.81	9.10	22.6	24.2
20 mgm TIAA	16 hr.	4.30	12.40	13.4	17.7

†Assay for release of RNA from nuclei was measured as described by Ishikawa et al. (3). The results represent the mean of two experiments.

in this modulation of RNA usage are only poorly described. Ishikawa demonstrated that transport of RNA as a ribonucleoprotein complex can be studied in a cell-free system, that this transport was energy dependent, that there was messenger function in the material so isolated (3,4). More recently Webb and coworkers have shown that there are cytoplasmic factors that also modify this transport phenomena and that there are differences in the state or level of this system during regeneration (9).

It has been shown that there appears in the cytoplasm RNA species normally restricted to the nucleus, after carcinogen feeding, but not after feeding equivalent quantities of a non-carcinogenic analog.* The cytoplasmic location of qualitatively similar but quantitatively different species of RNAs occurs during regeneration and during development. The apparent increased release of this nuclear restricted RNA may carry genomic segments transcribed but not usually translated in the adult liver, and these could include the information for constitutive enzymes, for surface membrane changes and for continued reproductive capacity. Such a change could be the basis for some of the phenotypic expressions of neoplasia. Subsequent studies have shown that with the exception of dimethylsulfoxide, only carcinogen feeding is

*We do not imply that all of these species represent messenger RNAs.

associated with these changes (13). Our data indicate that another carcinogen associated with loss of restrictional control *in vivo* is associated with a similar defect *in vitro*. In addition, with higher doses there is a second failure of ATP dependent transport. The mechanism(s) that underlie this altered control are not apparent, the presence of an intact nuclear envelope suggests that a membrane alteration may be involved. How these changes are related to the process of neoplastic transformation remains to be identified.

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