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Selected Nuclear LINE Elements With Mitochondrial-DNA-Like Inserts Are More Plentiful and Mobile in Tumor Than in Normal Tissue of Mouse and Rat

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Abstract The nuclear DNA of normal and tumor mouse and rat tissue was examined for mitochondrial-DNA-like inserts by means of the Southern blot technique. The two probes were ^{32}P-labeled cloned mitochondrial DNA. *Kpn*I, which doesn't cut either mitochondrial DNA, was one of the restriction enzymes, while the enzymes that fragment mitochondrial DNA were for mouse and rat *Pst*I and *Bam*HI, respectively. When *Kpn*I alone was used in the procedure a nuclear LINE family whose elements had mitochondrial-DNA-like insertions was selected. Such elements were much more abundant in tumor than in normal tissue. The results with *Pst*I alone and *Bam*HI alone and each combined with *Kpn*I indicated that there were mobile LINE elements with mitochondrial-DNA-like inserts in the nuclear genome of tumor. The mouse tissues were normal liver and a transplantable lymphoid leukemic ascites cell line L1210 that had been carried for 40 years. The rat tissues were normal liver and a hepatoma freshly induced by diethylnitrosoamine in order to minimize the role of 40 years of transplantation. Our unitary hypothesis for carcinogenesis of 1971, which suggested these experiments, has been augmented to include mobile nuclear elements with inserts of mitochondrial-DNA-like sequences. Such elements have been related to diseases of genetic predisposition such as breast cancer and Huntington's disease. J. Cell. Biochem. 68:100–109, 1998. © 1998 Wiley-Liss, Inc.

Key words: carcinogens; mitochondrial DNA; nuclear DNA; LINE; mobile elements; cancer; Huntington's disease

In 1971, we published "A Unitary Hypothesis for Carcinogenesis" [Hadler et al., 1971b], which pointed out that when mitochondria were damaged (by any means whatsoever) and the membrane became leaky, genetic material (which is foreign to the nuclear genome) could be released and behave like an oncogenic virus and thus become part of the nuclear genome (Fig. 1). We also suggested that such a movement of mitochondrial genetic material into the nuclear genome could be relevant to aging, degenerative conditions of complex organisms [Hadler et al., 1971a], evolution, and pathological conditions [Hadler et al., 1983]. The findings that supported this hypothesis have been collected [Hadler, 1989] and presented as an "experimental confluence between oxidative phosphorylation and chemical carcinogenesis." It was observed that chemical carcinogens of diverse structures or their metabolites also of diverse structures disturbed the complex mitochondrial process of oxidative phosphorylation also at diverse sites. Usually, but not always, it was one or more metabolites, and not the parent carcinogen, that interacted with the process of oxidative phosphorylation. Quagliariello et al. [1990] cited examples, earlier than ours, that support the experimental confluence between oxidative phosphorylation and chemical carcinogenesis.

When our hypothesis was presented the pervading scientific opinion at that time looked on a nongenotoxic role for carcinogens "with disbelief and possible scorn" [Sivak et al., 1987]. Indeed, such was the fate of our hypothesis and research proposals. Our nongenotoxic explana-

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tion for the potential carcinogenicity of reserpine [Hadler, 1975] based on the structure of a hydroxyquinone metabolite of the carcinogenic polynuclear hydrocarbon, dibenz [a, h] anthracene [Hadler et al., 1971a] was quickly rejected [Faigle et al., 1975; Marquardt, 1975]. However, 12 years later, reserpine turned out to be not only one of the most potent carcinogens in the male rats and male and female mice [Tennant et al., 1987] but, indeed, was also nongenotoxic. Accordingly, our hypothesis and experimental confluence uniquely succeeded in identifying chemical structural features relevant to potential chemical carcinogens.

Our approach to test our hypothesis, at the molecular level, was to use Southern blots that compared restriction enzyme digests of nuclear DNA (nucDNA) derived from normal and tumor tissue. The probe was mitochondrial DNA (mtDNA) purified by cloning and labeled with ^{32}P. A difference between such blots would detect mtDNA-like sequences in tumor nucDNA which were not present in normal nucDNA. The surprising result was that indeed normal mammalian nucDNA of rat liver did possess mtDNA-like sequences [Hadler et al., 1983]. Others reported similar findings in nonmammalian eukaryotes [Van den Boogaart et al., 1982; Farrelly and Butow, 1983; Jacobs et al., 1983; Gellissen et al., 1983] and in humans [Tsuzuki et al., 1983a; Tsuzuki et al., 1983b; Nomiyama et al., 1984; Nomiyama et al., 1985; Wakasugi et al., 1985; Fukuda et al., 1985]. In their review, Zhang and Hewitt [1996] have brought this area of research up to date. These investigators questioned the procedure when certain mtDNA probes were generated by the polymerase chain reaction (PCR). They pointed out that when the primers are known sequences of mtDNA, but the template is mtDNA contaminated with nucDNA, the probe could include not only the expected mtDNA sequence but also a mtDNA-like sequence of nucDNA bracketed by the same primers since the contaminating nucDNA could have a normal presence of mtDNA-like sequences complementary to the primers.

Another difficulty is the preparation of nucDNA free from mtDNA [Tsuzuki et al., 1983b; Koll, 1986]. The nucDNA which we used was not selected from a library of cloned fragments. This avoided the possible loss of a fragment of nucDNA or the possible alteration of such a fragment [Singer, 1982; Haché et al., 1989]. Thus our nucDNA while complete and carefully prepared, was contaminated with a few percent of mtDNA. As before [Hadler et al., 1983] the judicious use of two enzymes singly and in combination allowed the identification of mtDNA-like sequences in nucDNA. Since one enzyme did not cut mtDNA, and one enzyme fragmented mtDNA; bands with both or one end nucDNA could be identified. In addition, the location of bands due to contaminating mtDNA was certain; also, if such bands were intense enough to obscure neighboring faint bands, the intense bands could be cut out after a brief exposure to the x-ray film. This systematic method located a mtDNA-like sequence presumed to be the noncoding D region [Hadler et al., 1983]. The presumption proved correct after cloning and sequencing [Zullo et al., 1991].

In this study, cloned mouse mtDNA [Martens and Clayton, 1979] and cloned rat mtDNA [Hadler et al., 1983] were used as probes. The enzyme *Kpn*I did not cut mouse [Moore et al., 1977] or rat [Gadaleta et al., 1989] mtDNA. The enzyme *Pst*I generated fragments about 3.7 and 12.6 kb from mouse mtDNA [Moore et al., 1977; Parker and Watson, 1977]. The enzyme *Bam*HI generated fragments about 5.2 and 10.8 kb from rat mtDNA [Hadler et al., 1983]. Normal mouse tissue was liver. Mouse tumor tissue was a transplantable leukemic cell line L1210 subline A228/07 [Law et al., 1949] that had been carried 40 years. The normal rat tissue was liver. Rat tumor tissue was a hepatocellular carcinoma by comparison freshly induced by including diethylnitrosoamine in drinking water (50 mg/L) for 16 weeks and sacrificing the animals 6 weeks later [Irving and Williams, 1976].

MATERIALS AND METHODS

Sources of Tissue

The mouse lymphoid leukemic cell line L1210 subline A228/07 which had been carried 40 years was purchased from the Tumor Repository, National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). This tumor originated in DBA/2 mice in 1949 [Law et al., 1949], when the mice were painted with an ether solution of methylcholanthrene. DBA/2 female mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN); these mice provided normal liver and carried the ascites tumor. Female Sprague-Dawley rats were purchased from Holtzman (Madison, WI); these

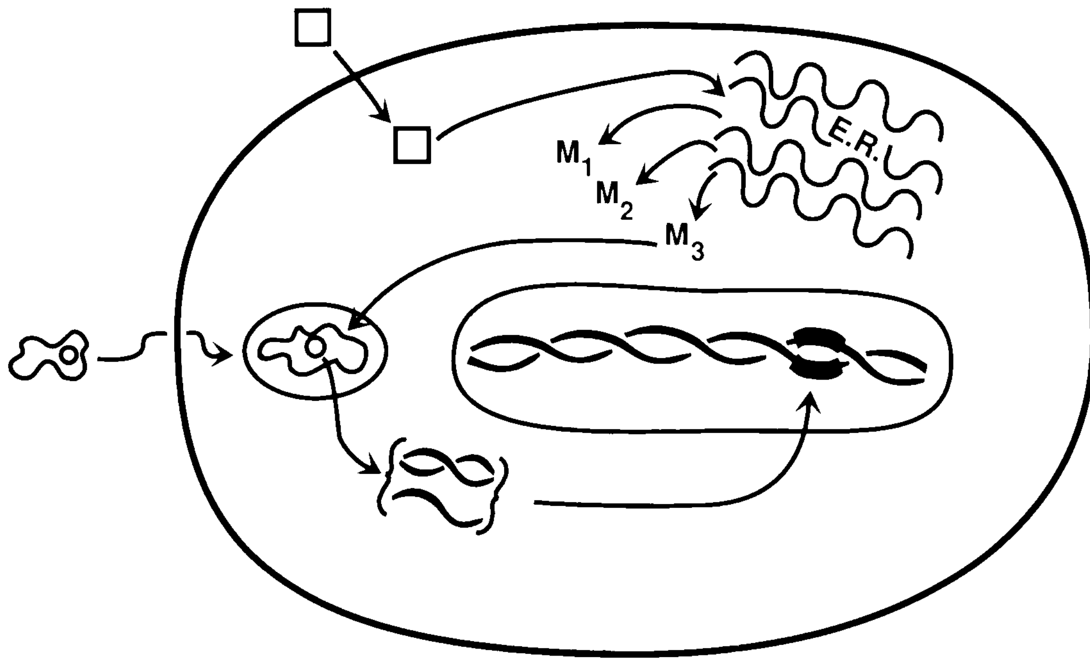

Figure 1.

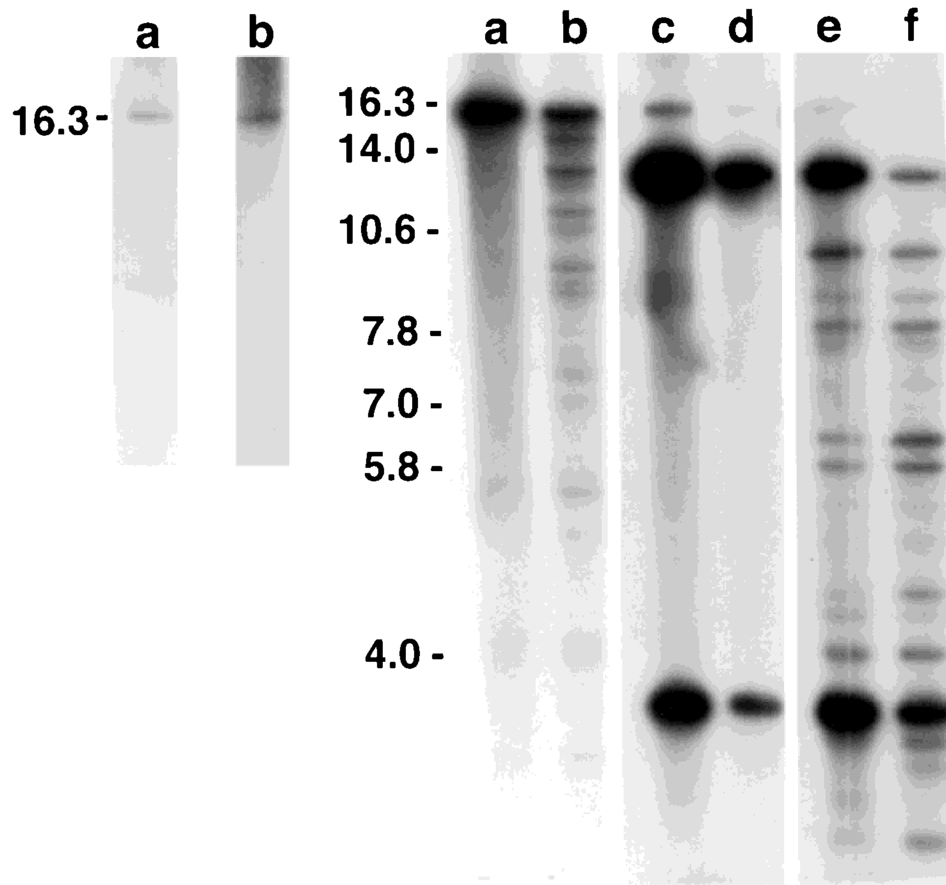

Figure 2

rats provided normal liver and a freshly induced hepatoma by including diethylnitrosoamine (50 mg/L) in the drinking water for 16 weeks and sacrificing the animals 6 weeks later [Irving and Williams, 1976]. The presumed hepatoma tissue from five rats was diagnosed by a pathologist to be a hepatocellular carcinoma.

Methods

The methods used have been described [Hadler et al., 1983], except for the following. In the Southern blot procedure Denhardt's solution was replaced by 0.25% w/v nonfat dry milk [Johnson et al., 1984]. Digestions with restriction enzymes were carried out twice, each time with a 7.5-fold excess of enzyme. Completeness of each digestion was confirmed as before [Hadler et al., 1983]; after each digestion, there was in succession extraction with phenol, then isoamyl alcohol, precipitation with alcohol and sodium acetate, and dissolution in 10 mM Tris–HCl, 1 mM Na_2EDTA, pH 7.5 (TE buffer). The procedure of Hsiung and Kucherlapati [1982] was used to isolate nucDNA from the ascites cells and from spun down nuclei of normal mouse liver. The supernatant fraction of the nuclei was used to spin down mitochondria and was the enriched source of mtDNA. The previous procedure [Hadler et al., 1983] using velocity and isopycnic centrifugation was used to prepare nucDNA and mtDNA from rat hepatoma and normal rat liver. The ^{32}P probe of total linearized mouse mtDNA was excised from the plasmid pAmI [Martens and Clayton, 1979] using the restriction enzyme *Hae*II, electrophoresis, and electroelution. The ^{32}P-labeled probe of total rat mtDNA was the combination of the two plasmids previously described [Hadler et al., 1983]. Each lane with mouse nucDNA was loaded with 10 μg. Because of a shortage of material due to previous experiments, each lane with rat nucDNA was loaded with 7 μg. DNA measurements were based on $A_{260} = 1$ for 50 μg/ml. Reproducible mouse blots were carried out four to six times. Reproducible rat blots were carried out twice.

Fig. 1. A unitary hypothesis for carcinogenesis, 1971. A foreign compound, ☐, capable of invading the cell may be converted into several metabolites M_1, M_2, M_3, by the endoplasmic reticulum, ER. One or more of these metabolites (or, in some instances, the original compound) may act destructively, even on a single mitochondrion, e.g., by disturbing oxidative phosphorylation, without leading to the death of the cell. The mitochondrial genetic material (DNA or RNA) could leak out of the damaged mitochondrion, and just like the foreign genetic material from an oncogenic virus, become integrated into the nuclear genome of the cell. According to the endosymbiotic theory for the evolution of a eukaryotic cell, mitochondrial genetic material is "foreign" genetic material that entered the cell eons ago. The hypothesis is a unitary hypothesis for carcinogenesis, as there are many routes leading to a leaky mitochondrion with a damaged membrane (Hadler et al., 1971a,b; Hadler, 1989).

Fig. 2. Southern blots of mouse DNA. The ^{32}P-labeled probe was cloned linearized total mtDNA (see Materials and Methods). **Left: Lanes a,b,** derived from about 200 ng of tumor mtDNA. The restriction enzymes were in lane a, *Hae* II, which linearizes, but does not fragment, the mtDNA and *Kpn* I in lane b. Exposure to the x-ray film was for 1 week without the use of an intensifying screen. **Right:** Normal nucDNA **(lanes a,c,e)**; tumor nucDNA **(lanes b,d,f)**. Restriction enzymes: *Kpn* I **(lanes a,b)**; *Pst* I **(lanes c,d)**; *Kpn* I plus *Pst* I **(lanes e,f)**. Exposure to the x-ray film was overnight, without an intensifying screen.

RESULTS

Figure 2 compares nucDNA from normal mouse liver with nucDNA from mouse ascites cell line L1210 subline A228/07 [Law et al., 1949]. As expected [Moore et al., 1977; Parker and Watson, 1977], linearized (form III) tumor mtDNA was not fragmented by *Hae*II; the band at 16.3 kb was undisturbed. Normal mouse mtDNA gave the same result (data not shown). *Kpn*I generated a plentiful family of repetitive elements from tumor nucDNA (lane b). These elements were detected because they had inserts of mtDNA-like DNA encompassed by *Kpn*I ends. Only a very sparse family was detected in normal nucDNA (lane a). The data with *Pst*I located the two mtDNA fragments, 12.6, and 3.7 kb, derived from contaminating mtDNA. Two other bands were present only in normal nucDNA. The enzyme *Pst*I truncated the *Kpn*I elements (lane f) Thus, these shorter elements moved farther down the gel and indeed pieces smaller than 3.7 kb were generated. The results in lane e with normal nucDNA were unexpected. This family of repetitive elements with mtDNA-like inserts encompassed by *Pst*I and *Kpn*I ends resembled, but differed from, the tumor family in lane f. It is reasonable to suggest that the elements in lane e were derived from a *Kpn*I family, with inserted mtDNA-like DNA, whose elements were longer than 16.3 kb (Fig. 3). The smear (not shown) at the origin of lane a (Fig. 2) likely provided such elements. Thus, some *Kpn*I elements with inserted

mtDNA-like DNA had “moved” from their position in normal nucDNA to a new position in tumor nucDNA. Figure 4 compares nucDNA from normal rat liver with nucDNA from a rat hepatoma, freshly induced by adding 50 mg/L of diethylnitrosoamine to the drinking water of the female rats for 16 weeks and sacrificing the animals 6 weeks later [Irving and Williams, 1976]. Thus, the effect of carrying a tumor for 40 years was eliminated. While the results with the rat were less prominent than those with the mouse the two sets of data were in agreement. Again, *Kpn*I, as expected [Gadaleta et al., 1989], did not fragment tumor mtDNA. The *Kpn*I family with inserted mtDNA-like DNA was much more plentiful in tumor nucDNA than in normal nucDNA. *Bam*HI generated a few extra bands in tumor nucDNA. The *Kpn*I elements truncated by *Bam*HI, were much more plentiful in tumor nucDNA than in normal nucDNA. The truncated family in lane e was much more evident than the *Kpn*I family (just detectable on the x-ray film) in lane a. Again, the data suggest that some *Kpn*I elements with inserted DNA-like DNA had “moved” from their position in normal nucDNA to a new position in tumor nucDNA.

DISCUSSION

In these experiments, the *Kpn*I family of repetitive elements, selected because the elements have mtDNA-like inserts and are less than 16.3 kb in size, were spectacularly qualitatively more plentiful in tumor nucDNA than in normal nucDNA of mouse and rat. Also at some stage during carcinogenesis certain elements of the *Kpn*I family with mtDNA-like inserts had moved to a new location in the nuclear genome. These results have added a new direction to cancer research even though corroboration by cloning and sequencing has yet to be carried out. Because the transplantable mouse tumor had been carried for 40 years [Law et al., 1949] the movement of the *Kpn*I element with mtDNA-like inserts may or may not be an early decisive event of carcinogenesis. Such movement, however, was also detected in a freshly induced malignant rat hepatoma. The rats had received diethylnitrosoamine for 16 weeks and were sacrificed 6 weeks later (see Methods). The likelihood that such DNA movement is indeed an early and crucial event in carcinogenesis is markedly increased by this result.

Our procedure cannot be used easily to generate a *Kpn*I family of repetitive elements with mtDNA-like inserts from human nucDNA, since this restriction enzyme generates three fragments from human mtDNA [Drouin, 1980]. Shimada and his group [Tsuzuki et al., 1983a,b; Nomiyama et al., 1984, 1985; Wakasugi et al., 1985; Fukuda et al., 1985] have identified previously *Kpn*I and *Alu*I individual elements bounded by mtDNA-like ends by selecting unique clones from a human DNA library.

Others [Kamimura et al., 1989; Shay et al., 1991; Corral et al., 1989; Liang, 1996; Liang and Hays, 1996] have reported mtDNA-like sequences in tumors. Shay and his group [Kamimura et al., 1989] found a “pseudogene” in the nucDNA of human normal tissue (leukocytes, foreskin, and placenta) and several cell lines, which included HeLa cells. This “pseudogene” was shown not to be part of the *Kpn*I family but, interestingly, was composed of three pieces of mtDNA that, while contiguous in the “pseudogene,” were not contiguous in human mtDNA. The “pseudogene” was first detected in a library of HeLa cell DNA. The same laboratory [Shay et al., 1991] isolated, from a cDNA library of human HeLa TG cells, a transcription unit that was a hybrid of mtDNA-like sequences inserted into the c-*myc* oncogene. Corral et al. [1989] used various mtDNA probes to examine blots of nucDNA from normal rat (female Sprague-Dawley) liver and hepatoma induced 70 weeks previously by doses of diethylnitrosoamine. The tumor had mtDNA-like sequences in the nuclear genome which differed with those in the normal nuclear genome. These sequences were more plentiful not only in the tumor but in different locations as well. In this study, the restriction enzymes were *Eco*RI and *Hin*dIII. Each enzyme cuts rat mtDNA at multiple sites [Parker and Watson, 1977]. These results are compatible with our completely independent investigations [Devadas, 1989; Hadler et al., 1990]. Liang [1996] and Liang and Hays [1996] first reported that total cell DNA from certain human gliomas possessed more mtDNA sequences than normal total cell DNA. The probes were either a cDNA clone with mtDNA-like sequences or total mtDNA generated by PCR using multiple primers and total cell DNA as the template. There was no distinction between mtDNA in the mitochondrial genome or mtDNA-like sequences in the nuclear genome

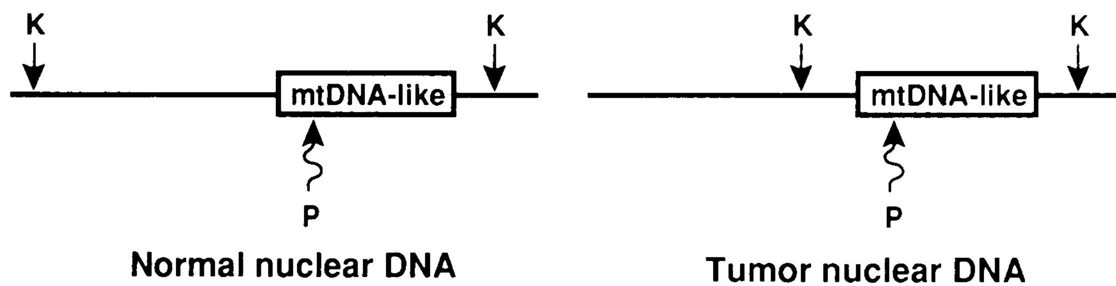

Fig. 3. Arrangement of *Kpn*I elements with mtDNA-like sequences in normal and tumor nucDNA. K, *Kpn*I; P, *Pst*I.

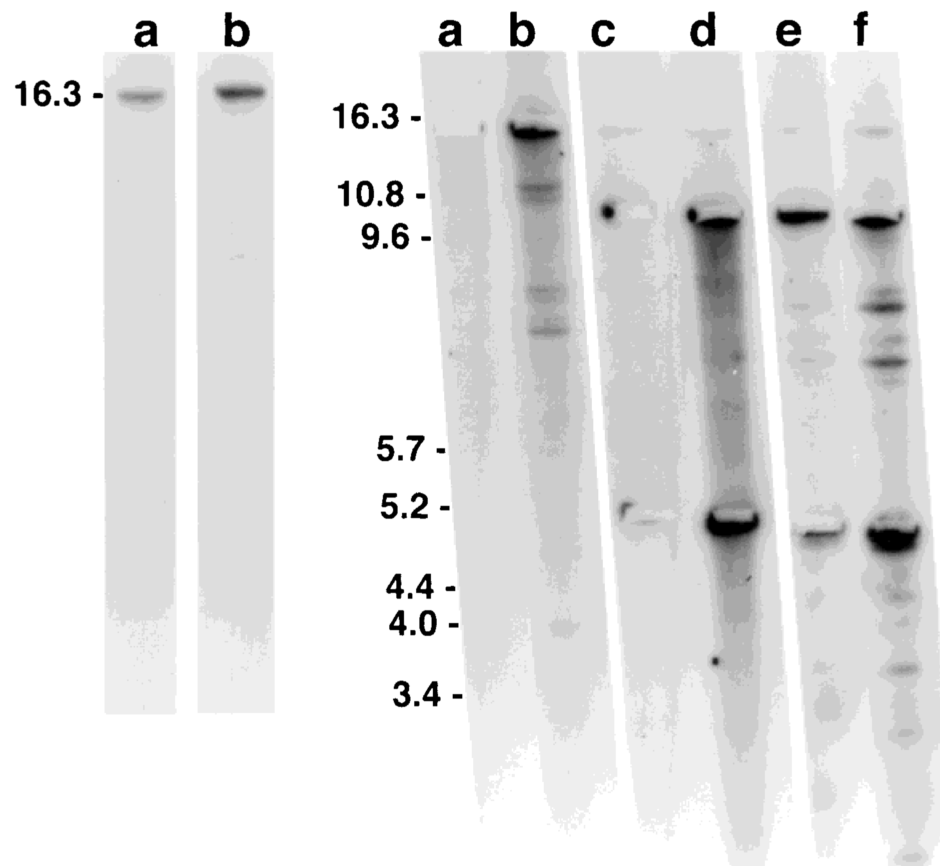

Fig. 4. Southern blot of rat DNA. The ^{32}P-labeled probe was cloned total mtDNA present in combined two plasmids [Hadler et al., 1983]. **Left:** Lanes a,b, derived from a dilute solution of tumor mtDNA. Restriction enzymes: *Pst*I, which does not cut mtDNA [Parker and Watson, 1977] **(lane a)** and *Kpn*I **(lane b)**. Exposure to the x-ray film was for 2 weeks, without an intensifying screen. **Right:** Normal nucDNA **(lanes a,c,e)**. Tumor nucDNA **(lanes b,d,f)**. Restriction enzymes: *Kpn*I **(lanes a,b)**; *Bam*HI **(lanes c,d)**; *Kpn*I plus *Bam*HI **(lanes e,f)**. Exposure to the x-ray film was 3 days after cutting out bands (see Materials and Methods). No intensifying screen was used.

except when a fluorescent probe generated as above by PCR located mtDNA inside the nuclei of certain low grade human gliomas [Liang, 1996]. In this study, hybridization of the fluorescent probe to repeated sequences was blocked by prehybridization with rapidly annealing DNA, as is frequently done in fluorescent in situ hybridization (FISH) technology. In summary, the above reports [Kamimura et al., 1989; Shay et al., 1991; Corral et al., 1989; Liang, 1996; Liang and Hays, 1996] were not designed to compare, repetitive nucDNA sequences possessing mtDNA-like sequences, in normal, or tumor nucDNA.

Our observation that mobile *Kpn*I elements with mtDNA-like inserts replicate and become

amplified within the nuclear genome when associated with tumor augments our unitary hypothesis (see Fig. 1). When mitochondria or even a single mitochondrion becomes damaged by any means whatsoever, so that the mitochondrial membrane becomes leaky mitochondrial genetic material "foreign to nucDNA" may integrate into the nuclear genome and become part of a replicating mobile genetic element. Over a period of time, such elements may turn certain genes (e.g., oncogenes, repressor genes) on or off or may be mutated by insertion or decreased stability. This explains the necessary time course and certain randomness of cancer, aging, and other pathologies. Others have previously suggested a role for transposable elements.

Ten years after our nongenotoxic route to cancer was published, Cairns [1981] also reasoned that cancer, in humans, was not due to local genetic changes caused by "conventional mutagens." He proposed that genetic transposition caused cancer by causing neighboring genes, to be either turned on, or to be turned off, or to be made unstable. He noted that Temin [1970] was the first to consider the possible role of transposable elements in carcinogenesis. Cairns was concerned about the crucial step in carcinogenesis and inferred "changes in karyotype could be trivial secondary events." Our results and augmented hypothesis are supportive of what Cairns wrote and provide an alternative to focusing research solely on genetic mutations.

The *Kpn*I family was first reported to be part of the nuclear genome of primates in 1980 and 1981 [Musich et al., 1980; Adams et al., 1980; Maio et al., 1981] and has been intensively investigated ever since. There are many reviews [Singer, 1982; Singer and Skowronski, 1985; Fanning and Singer, 1987; Hutchinson et al., 1989; Deringer et al., 1992; Edgell, 1994; Sherrat, 1994; Holmes et al., 1994]. Such interspersed repetitive sequences were first detected as rapidly annealing genomic DNA and were name after a restriction enzyme site common to the individual elements, hence the so-called *Kpn*I family, later called long interspersed nucleotide elements (LINEs) or L1 [Singer, 1982]. This family is present in diverse eukaryotes (e.g., flies, rodents, and humans). The LINE family of transposable elements is the most abundant sequence in the mammalian genome and consists of about 100,000 members and ranges from 15% to 30% of the mammalian genome, depending on the species. Elements integrate at new random locations in the nuclear genome via transcription, reverse transcription and reintegration. Unlike mobile retroviruses these elements do not have long terminal repeats. They are often called retrotransposons. Most of the LINE elements are pseudogenes. Indeed, such was the case of the nucDNA with the mtDNA-like D region of normal rat liver [Hadler et al., 1983; Zullo et al., 1991]. The relatively few elements in a family which are not truncated and have the ability to generate progeny are called "master elements" [Deringer et al., 1992; Edgell, 1994]. These elements possibly require a non LINE insert or be inserted into a different gene [Edgell, 1994; Sherrat, 1995; Holmes et al., 1994] Accordingly, our data and augmented hypothesis implicate the insertion of an appropriate piece of "foreign" mtDNA into a nontruncated element as the event that triggers the replicative ability of the newly formed "master element" There is a collection of references [Amariglio and Rechavic, 1993] that relates mobile LINE elements acting as insertional mutagens, in cancer and hemophilia (for other germane references, see Dudley [1988] and Kingsmore et al. [1994]).

Whenever a difference is found between normal and tumor cells, the question must be asked, "is the observation a cause or an effect?" Accordingly this report raises several questions: (1) Can mitochondrial DNA cause cancer in experiments with animals or cells? (2) Is the amplification and or movement of LINE elements with mtDNA-like inserts a decisive possibly early event in carcinogenesis? and (3) What is the role of the new mtDNA-like inserts?

Very preliminary data point to a positive answer to the first question. Two experimental and two control rats were set aside in 1973. All four animals were subjected to partial hepatectomy. Mitochondria from two of the rats were isolated, lysed and injected into the thigh muscle of the appropriate donor rat. Everything except mitochondria was injected into the control rats. Since nothing of interest was observed after a few weeks, the rats were maintained but forgotten. About 2 years later, one surviving experimental rat was noticed because there was a huge tumor on its back. After necropsy and tissue examination the pathologist reported extensive dispersed multiple tumors that were so primitive that the tissue of origin could not be

identified. This lead has never been pursued because of lack of resources.

The fortuitous discovery that *Kpn*I can be easily used to locate mouse LINE elements with mtDNA-like inserts provides a unique opportunity to examine the various sequential events of carcinogenesis. The seminal research of Berenblum and Shubik [1947a,b] distinguished sharply between biological events caused by initiators, promoters, and complete carcinogens. Later, the assay procedure was modified so that the reproducible biological and biochemical responses were based on dose of agent per well-defined unit area [Darchun and Hadler, 1956; Hadler et al., 1959]. Indeed, the dose of 7,12-dimethylbenz(a)anthracene (DMBA) used as an initiator was reduced from milligram (mg) amounts to 1 μg (3.9 nmol) per 3.46 cm^2 of mouse skin. Tumors (mostly papillomas and a few carcinomas) were only developed in the well-defined treated area of skin. Thus skin from the same animal without tumors and adjacent to the treated area is available for examination. The possible biochemical comparisons during the various stages of carcinogenesis are manifold. In addition the active principle in croton oil has been identified [Hecker, 1968; Van Duren and Sivak, 1968] and is readily available from suppliers.

In conclusion, our unitary hypothesis for carcinogenesis published in 1971 has been augmented by a role for nuclear LINE elements with mtDNA-like inserts. Are other mobile elements involved? Impaired mitochondria are associated with many inherited pathologies that may stem from mutations in mitochondrial DNA or nuclear DNA [Clayton, 1992; Zeviani, 1992; Johns, 1996; Stuart and Neupert, 1996]. Accordingly, the inherited defects follow non-Mendelian maternal genetics or Mendlian nuclear genetics. Thus mobile nuclear elements with mtDNA-like inserts could play a role in both types of inheritance. Indeed, Baum [1973] noted that our unitary hypothesis covered mutation in mtDNA and release of mtDNA into the nuclear genome.

Certain diseases that develop with age are linked to a genetic predisposition of a mutated nuclear gene, such as breast cancer [Szabo and King, 1997; Coene et al., 1997] and Huntington's disease [Koroshetz et al., 1997]. If the mutated nuclear gene is responsible for the genesis of impaired mitochondria a putative mobile nuclear element with a mtDNA-like insert could eventually affect a totally different nuclear gene.

A role for mobile nuclear genetic elements with mtDNA-like inserts is possible when impaired mitochondria are generated directly or indirectly by enzymically generated reactive oxygen [Johns, 1996; Shigenaga et al. 1994] or other toxic events.

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