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Activation of putative transposition intermediate formation in tumor cells

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The transcription levels of two families of mouse repetitive elements namely intracisternal A particle (IAP) genes, and B2 sequences were analyzed in different tumor cells and normal tissues. These sequences belong to two major classes of mobile elements present in the mouse genome. The Northern blots containing poly(A)$^+$ RNAs from tumor cells and normal tissues were hybridized to the cloned IAP gene and B2 sequence. The content of IAP gene transcripts in tumor cells is much higher than in normal cells. A 10- to 100-fold difference was found. The predominant IAP-gene specific RNAs in all investigated tumor cells were 9.5, 6.8 and 5.3 kb long. Additional RNA species were found in some of the tumors. The active synthesis of small cytoplasmic B2 RNA transcribed by RNA polymerase III was also detected in most tumor cells tested. Usually it was higher than in normal cells. Free closed circular DNAs hybridizing to IAP gene probes were cloned from Ehrlich ascites carcinoma cells. We speculate that the data obtained indicate the enhanced transposition of mobile elements in tumor cells which may be an important factor of tumor progression.

Key words: transcription/transposable elements/extrachromosomal DNA/mouse tumor cells

Introduction

There are several kinds of transposable repetitive DNA elements in the mouse genome. Among them are IAP genes and B2 sequences. IAP genes are responsible for the synthesis of intracisternal A-particles (IAP). These particles are retrovirus-like structures found in large quantities in various mouse tumor cells (myeloma, neuroblastoma, teratocarcinoma), embryonic cells and rarely in normal adult cells (Wivel and Smith, 1971; Calarco and Szolossi, 1973; Biezcko *et al.*, 1973; Canivet *et al.*, 1980). The isolated particles contain endogenous reverse transcriptase and polyadenylated genomic RNA (Wong-Staal *et al.*, 1975; Robertson *et al.*, 1975) but they are not infectious and do not have transforming activity. DNA sequences homologous to IAP RNAs are present in ~1000 copies per haploid mouse genome (Lueders and Kuff, 1977; Georgiev *et al.*, 1981; Kuff *et al.*, 1981). A number of cloned IAP genes have been described (Lueders and Kuff, 1980; Georgiev *et al.*, 1981; Kuff *et al.*, 1981; Cole *et al.*, 1981). It is evident that IAP genes are structurally similar to mammalian and avian retroviruses as well as to mobile dispersed genetic elements (mdg) of *Drosophila* and yeast (Finnegan *et al.*, 1978; Ilyin *et al.*, 1978; Cameron *et al.*, 1979). All of these elements have long terminal repeat sequences (LTR). The insertion of new IAP genes into host DNA can give rise to specific mutations. For example, IAP gene insertion into the intron of the light chain immunoglobulin gene is responsible for the decreased production of immunoglobulin (Kuff *et al.*, 1983a). The enhanced expression of a cellular oncogene c-*mos* due to IAP gene insertion is also known to occur (Kuff *et al.*, 1983b).

B2 repetitive sequences have been described in detail earlier (Kramerov *et al.*, 1979; Krayev *et al.*, 1982). They represent one of the two most abundant short dispersed repeats of the mouse genome. The B2 sequence is 190 bp long and present in 10^5 copies in the mouse genome. It has been suggested that B2 elements represent a class of transposable elements which use RNA polymerase III transcription and reverse transcription for their insertion into new sites of the genome. B2 sequences are readily detectable in both nuclear and cytoplamsic RNA (Kramerov *et al.*, 1982; Ryskov *et al.*, 1983; Kominami and Muramatsu, 1983). A small (200–300 nucleotide long) polyadenylated B2-containing RNA was found recently (Kramerov *et al.*, 1982). It may correspond to the first intermediate on the pathway of B2 transposition.

In the present study, we have shown that the level of transcription of both IAP genes and B2 elements is much higher in tumor cells than in normal cells, and that in tumors IAP genes can exist extrachromosomally as closed circular DNA. These data suggest a possible mechanism of IAP gene amplification and transposition. They also indicate that transposition in tumor cells is enhanced, which may be an important factor of tumor progression.

Results

Transcription of IAP genes in normal and tumor cells

First, the content of IAP gene transcripts was assayed in different tissues. Total poly(A)$^+$ RNAs were isolated from various mouse tumors and normal cells, separated by electrophoresis on 0.8% agarose gel in the presence of 6% formaldehyde, and transferred onto nitrocellulose filters. The Northern blots were hybridized to [^{32}P]DNA of the clone containing IAP gene sequences (Mm 22). The hybridization patterns are shown in Figure 1. One can see that poly(A)$^+$RNA from all the analyzed tumors hybridizes to IAP gene sequences very efficiently.

In all preparations, the most prominent RNA band binding IAP DNA is that of 6.8-kb. The only exception is the MOPC-406 plasmocytoma. Also 9.5-, 8.2- and 5.3-kb components are relatively abundant in several tumors. Besides these, RNAs with a lower mol wt., a prominent 2.5-kb band and smeared material as well as molecules >9.5 kb are detected. Such a heterogeneity may depend on the heterogeneity of the IAP genes themselves and on the RNA processing.

The total content of IAP gene transcripts varies considerably in different tumor cell lines. It is very high in most tumors (nine out of 11), moderate in CCU-5 cells and relatively low in melanoma B16. IAP gene transcription is moderate in non-transformed but immortalized NIH 3T3 cells. In mouse fibroblasts transformed by SV40 virus (SVT2), the content of IAP gene transcripts increases at least 10-fold.

With RNA from normal mouse tissues (liver, brain), one can-

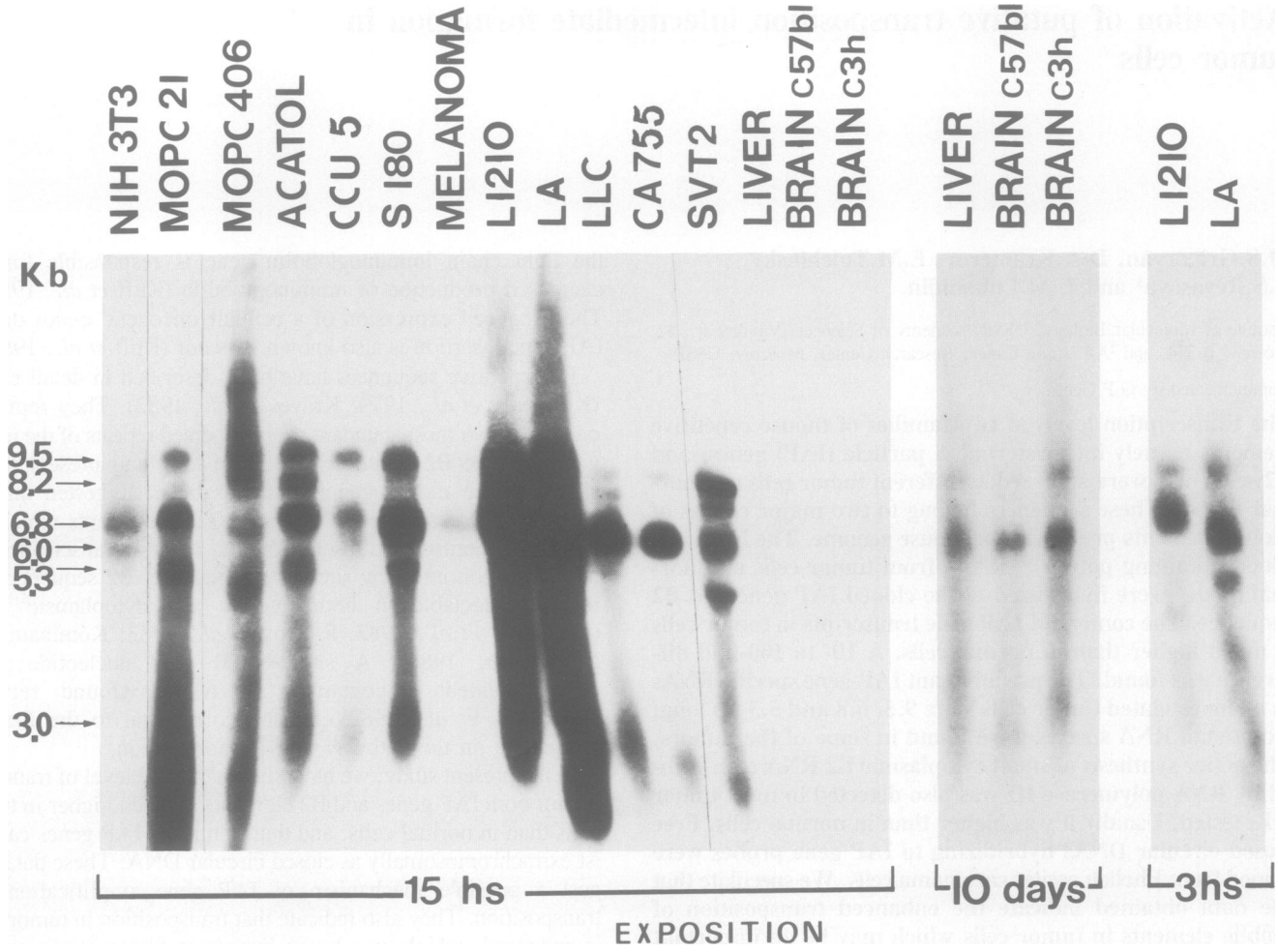

Fig. 1. IAP gene transcripts in poly(A)$^+$ RNA of different tumor and normal cells. Total poly(A)$^+$ RNAs from tumor and normal tissues were separated by electrophoresis in 0.8% denaturing agarose gel (in the presence of 6% formaldehyde) and transferred onto nitrocellulose filters. These Northern blots were hybridized to [^{32}P]DNA of the IAP gene sequence. Denatured *Hind*III fragments of λ-DNA were used as markers for RNA sizing.

not detect any hybridization to the IAP-DNA probe after the same exposure. However, after a much longer exposure (10 days), the labeled major 6.8-kb and minor 9.5-kb bands appear, as well as some smeared labeling. Thus, even in normal cells, IAP genes are transcribed, but at a very low level. The comparison with two tumors (L1210 and La) shows that tumor and normal tissues differ in the IAP gene-specific poly(A)$^+$ RNA content >100-fold.

Cloning of chromosomal closed circular DNA related to the IAP genes in mouse tumor cells

The full-length RNA copy of the IAP gene may be considered as a first intermediate in IAP gene transposition. Extrachromosomal circular DNA is another putative transposition intermediate. Therefore, we decided to analyze the possibility of circular DNA formation in the cells with a high level of IAP gene transcription, namely Ehrlich ascites carcinoma cells. Hirt supernatant (Hirt, 1967) prepared from these cells was mixed with SV40 DNA and banded in an ethidium bromide-CsCl gradient. Each fraction of the gradient was analyzed by 'dot' hybridization with the radiolabeled DNA of a cloned IAP gene. Detectable hybridization was revealed in two peaks. One of the peaks corresponded to the fraction of closed circular DNA in which SV40 DNA was banded (Figure 2A). The DNA from these fractions was collected and either directly electrophoresed or first u.v.-irradiated in the presence of ethidium bromide or digested with *Eco*RI. Southern blots were obtained and hybridized to an IAP gene probe (Figure 2B). Several bands hybridizing to IAP DNA were detected in untreated samples. Nicking induced by u.v.-irradiation leads to relaxation of superhelical circular molecules and their mobility should decrease. This was actually the case. IAP DNA contains one or two *Eco*RI sites and therefore *Eco*RI treatment should linearize IAP circles. One can see that only one major band remains after *Eco*RI digestion, with a size of ~7 kb.

The fraction of supercoiled DNA from Ehrlich ascites carcinoma cells treated with restriction endonuclease *Eco*RI was cloned in phage λgtWESλC. The screening of 50 000 plaques of recombinant phages was performed using an IAP-specific probe (fragment A of clone Mm 22). We have isolated four clones containing sequences homologous to IAP genes. Clone λsc91 was studied in most detail. The size of the insert in this clone was 11.6 kb. It has been established that 5.25 kb out of 11.6 kb are SV40 DNA, which was used in excess as an internal marker of the supercoiled DNA fraction in a 3-fold centrifugation in CsCl density gradient. The restriction maps of the chromosomal clone Mm 22, type 1 IAP-genes (Shen-Ong and Cole, 1982) and λsc91 are given in Figure 3a. As can be seen, most restriction fragments are identical in these clones. λsc91 DNA was restricted with *Bam*HI and Southern blots of the resultant fragments were

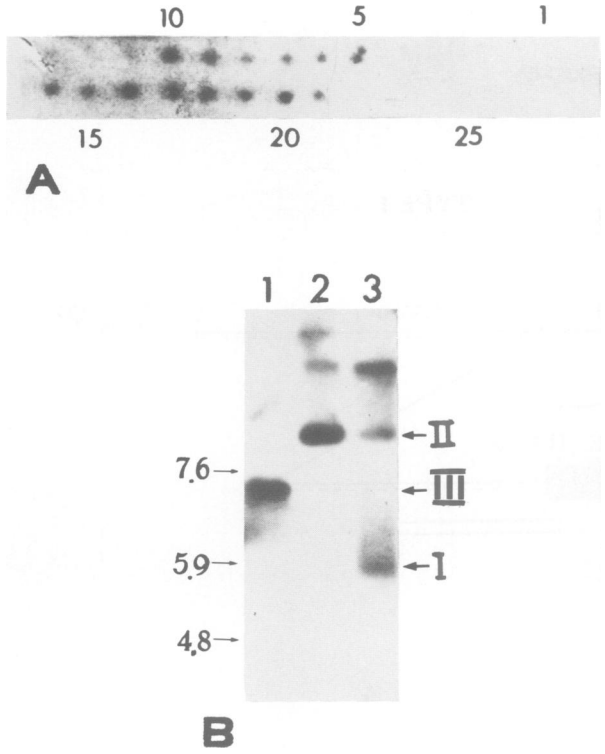

Fig. 2. Demonstration of closed circular DNA containing IAP genes in Ehrlich ascites carcinoma cells. (**A**) Closed circular DNA molecules were isolated from Ehrlich ascites carcinoma cells by the modified Hirt procedure. The DNA isolated from the Hirt supernatant was banded in the ethidium bromide-CsCl gradient. Each fraction of the gradient was analyzed by 'dot' hybridization with a [^{32}P]DNA probe of the cloned IAP gene. The fraction revealed in the region where added supercoiled SV40 DNA had been banded (fractions 15 – 20) was collected and analyzed. (**B**) DNA of the supercoiled fraction (see **A**) was u.v.-irradiated in the presence of ethidium bromide (**lane 2**), or treated with *Eco*RI (**lane 1**) and analyzed by Southern blot hybridization with [^{32}P]DNA of the cloned IAP gene. **Lane 3**, the same as **lanes 1** and **2**, but the DNA from the supercoiled fraction was not treated. After nicking by u.v.-irradiation, closed circular DNA was relaxed and migrated in agarose gel as open circular molecules (band II). 2EcoRI treatment linearized the circular DNA molecules (band III).

hybridized to fragments L (containing LTR) and D (a marker of the 3′ LTR) of Mm 22 which were subcloned in pBR322. The results have shown that, in both cases, the 2.5-kb *Bam*HI fragment of clone λsc91 is hybridized (Figure 3b). In the case of *Hind*III digestion, two fragments of 1.8 kb and 0.8 kb are hybridized with probe L (Figure 3b). The 1.8-kb fragment includes LTR with flanking sequences and part of SV40 DNA. Probe D hybridized with a high mol. wt. fragment which contains the majority of the λsc91 insert and λ arm. Double digestion of λsc91 DNA with *Eco*RI and *Bam*HI gives two chracteristic fragments of 1.0 kb and 2.2 kb, which are hybridized with the 2.2-kb internal fragment A of the Mm 22 clone (Figure 3b). No clear-cut hybridization was found for the 0.5-kb fragment E of clone Mm 22 adjacent to the 5′ LTR. It is possible that this fragment has been lost during DNA cloning due to *Eco*RI treatment. To obtain direct evidence of the circular structure of the cloned IAP DNA, we have determined the nucleotide sequence of the fragment which is localized between LTR and SV40 DNA (see Figure 4).

The sequenced region contains part of the LTR sequence and adjacent to it the 5′ part of IAP genes; the latter includes the primer-binding site (18 nucleotides in length). The whole sequence determined is identical to that of chromosomal IAP genes (Ono and Ohishi, 1983). Thus, in clone λsc91, LTR is flanked by the 3′ part of the IAP gene (as was demonstrated with restriction and hybridization analysis) and by the 5′ part of the IAP gene (nucleotide sequence data). One can conclude that the cloned copy of the IAP genes is really an extrachromosomal circular molecule containing one LTR sequence. These experiments demonstrate the existence of extrachromosomal copies of circular DNA molecules with IAP gene sequences.

The presence of extrachromosomal IAP gene sequences could not be detected in normal tissues. Thus, extrachromosomal closed circular IAP DNA, the second putative intermediate of IAP gene transposition, is formed in tumor, rather than in normal cells.

Transcription of B2 sequences in normal and tumor cells

Besides mdg-like sequences, the genome of the vertebrates contains numerous transposable elements of another type, namely, ubiquitous B1 and B2 sequences. It is believed that transcription by RNA polymerase III is the first step in their transposition. Ehrlich ascites carcinoma cells and plasmocytoma MOPC-21 were shown to contain a large amount of small poly(A)$^+$ B2 RNA transcribed by RNA polymerase III (Kramerov *et al.*, 1985a). In the present work, we have compared the content of this RNA in different mouse tumors and normal tissues.

Cytoplasmic poly(A)$^+$ RNAs were isolated from eight tumors and seven normal tissues of mouse, electrophoresed, transferred to Northern filters and hybridized to ^{32}P-labeled B2 DNA (clone Mm 14). A comparison of the hybridization patterns in different normal tissues shows that the content of small poly(A)$^+$ B2 RNA differs from one tissue to another (Figure 5). They are practically absent from heart, spleen and regenerated liver. On the other hand, small B2 RNA can easily be detected in brain, testicular and kidney cells. The high content of small B2 RNA was detected in six out of the eight tumors tested. B2 RNA is also synthesized in the remaining two tumors, but in a rather small amount. Thus, with some exceptions, the level of independent transcription of B2 sequences by RNA polymerase III in tumors is usually elevated. The content of small poly(A)$^+$ B2 RNA was compared in normal and tumor cells of the same origin (LLC and lung, liver and hepatoma, MOPC-21 and spleen) and found to be much higher in the tumor cells than in the normal counterpart.

It is interesting that the independent transcription of B2 elements is not activated in the regenerating liver. Possibly the high level of small B2 RNA synthesis in tumor cells is not related to cell proliferation, but more likely is associated with the transformed state itself.

One can also see that B2 sequences efficiently hybridize to cytoplasmic poly(A)$^+$ RNAs of the size of mRNA, in particular, to several bands 1.4 – 2.0 kb long. These RNAs were found both in tumor and normal tissues. The amount of 2.0-kb RNA greatly increased in the regenerating liver, but this RNA disappeared from hepatoma cells. On the other hand, the content of 1.6-kb RNA was high in all tumor cells. However, it was also present in many normal cells in about the same quantity. No clear-cut correlation exists between the content of 1.6-kb B2 mRNA and the transformed state of the cells. Thus, the most prominent difference between normal and tumor cells with respect to B2 sequence transcription is the enhancement of RNA polymerase III-dependent synthesis of small B2 RNA.

Discussion

Activation of IAP gene transcription in tumor cells

IAP are accumulated in many tumor cells but not in adult mouse

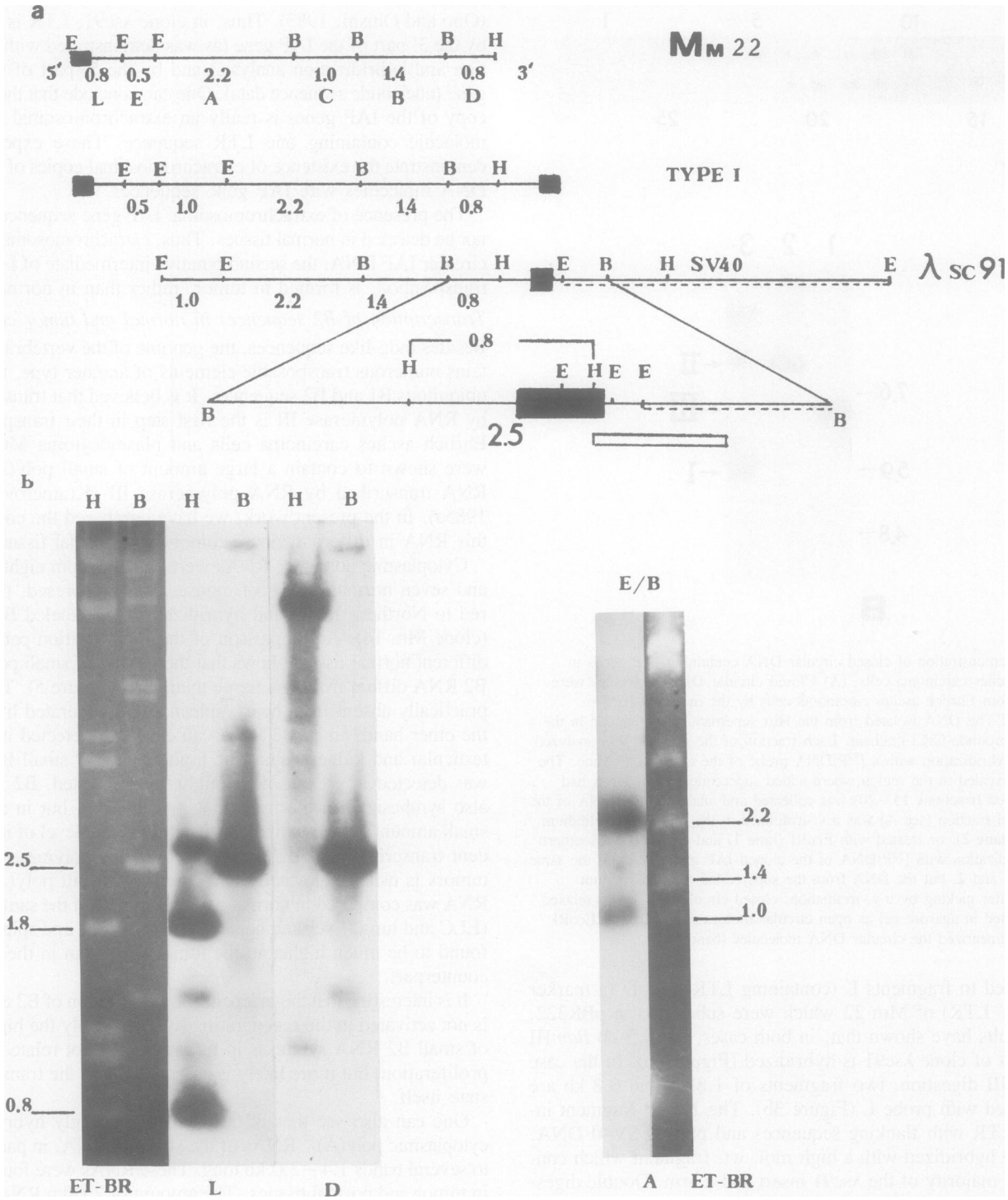

Fig. 3. Mapping of the cloned extrachromosomal circular DNA of the IAP gene (λsc91). (**a**) Restriction maps of the clones λsc91, Mm 22, type I IAP gene (Sheng-Ong and Cole, 1982). E. *Eco*RI; B, *Bam*II; H, *Hind*III. ■, LTR; □, sequenced region. (**b**) Hybridization of λsc91 DNA digested with *Hind*III (H), *Bam*HI (B) and *Eco*RI/*Bam*HI (E/B) to nick-translated fragments of clone Mm 22 (L, D and A). EtBr, gel stained with ethidium bromide.

tissues (Wivel *et al.*, 1971; Calarco *et al.*, 1973; Biezeko *et al.*, 1973; Canivet *et al.*, 1980). The absence of IAPs from normal tissues does not exclude the possibility that transcription of IAP genes remains at a high level while the formation of mature particles is blocked at other levels. The results presented here show that the low level of IAP production is directly associated with the very poor transcription of IAP genes in normal tissues. However, there is no such clear-cut relationship in tumor cells between the amount of particles and the level of their transcription. As was shown by Kuff *et al.* (1972), the cell line L1210 contains a relatively small number of IAP particles. However, these cells have a high level of synthesis of IAP-specific RNA (Figure 1). Nevertheless, the transcription of IAP genes is high in all tumor cells tested, and very high in most of them.

Cole *et al.* (1982) and Sheng-Ong and Cole (1982) have found that there are different populations of IAP genes. Most of these genes are nearly identical and ~7.1 kb long. Another group of IAP genes consists of much shorter sequences. However, these

deleted elements are efficiently transcribed.

We have demonstrated previously that four different predominant RNA transcripts (9.5 kb, 6.8 kb, 6.0 kb, 5.3 kb) are homologous to IAP sequences (Kramerov *et al.*, 1985b). However, there is no exact correlation between the sizes of RNA transcripts and the sizes of cloned IAP sequence. The largest cloned IAP gene is 7.2 kb long, which is much shorter than the largest IAP-RNA transcript (9.5 kb). This IAP RNA may be transcribed from some minor variants of genes which have not yet been cloned. Here, additional transcripts of IAP genes are reported in some tumors (8.2 kb). The origin of these RNAs is not clear. They may be primary transcripts of different variants of IAP genes which are activated in some tumors. Such IAP genes may entrap additional host sequences. Alternatively, the long transcripts may initiate or terminate outside the IAP genes.

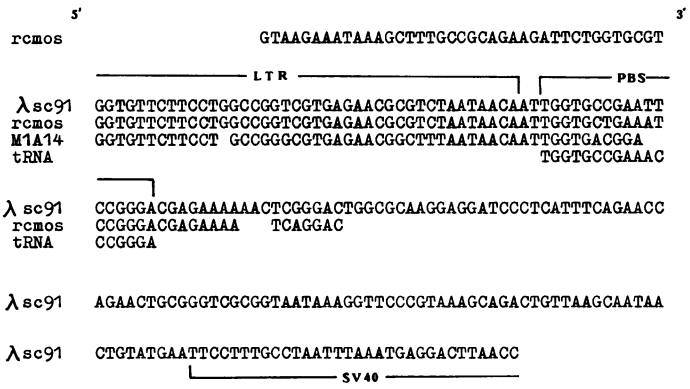

Fig. 4. Comparison of the nucleotide sequence of the λsc91 fragment with the IAP nucleotide sequence of c-*mos*, MIA14 and $tRNA^{Phe}$ (Ono and Ohishi, 1983).

The mechanism of IAP gene activation in malignant cells remains obscure. It may depend on the specific induction of an IAP gene enhancer in the dedifferentiated cells. It is known that IAP genes are actively expressed at the early stages of mouse development. There is evidence that DNA methylation plays some role in the control of gene activity (Doerfler, 1981; Razin and Riggs, 1980) and it has been shown that methylation plays an important role in the control of IAP expression in mouse teratocarcinoma cell lines (Hojman-Montes de Oca *et al.*, 1983; Lasneret *et al.*, 1983). The mechanism of the activation is rather specific and does not involve general derepression of genes in tumor cells. Williams *et al.* (1977) have arrived at the same conclusion after finding that not only does a new class of mRNAs appear in SV40-transformed cells, but also the old ones disappear.

Possible activation of IAP RNA reverse transcription in tumor cells

We have demonstrated here that the extrachromosomal closed circular IAP gene DNA is present in Ehrlich ascites carcinoma but not in normal tissues. The circles have been cloned and their heterogeneity confirmed. It is likely that their synthesis involves reverse transcription. The formation of circular DNA by reverse transcriptase is well documented in the case of cells infected by exogenous retroviruses. Recently, Arkhipova *et al.* (1984) found the reverse transcription of RNA synthesized from mdg elements in *Drosophila melanogaster* culture cells. Finally IAP are known to contain active reverse transcriptase (Robertson *et al.*, 1975).

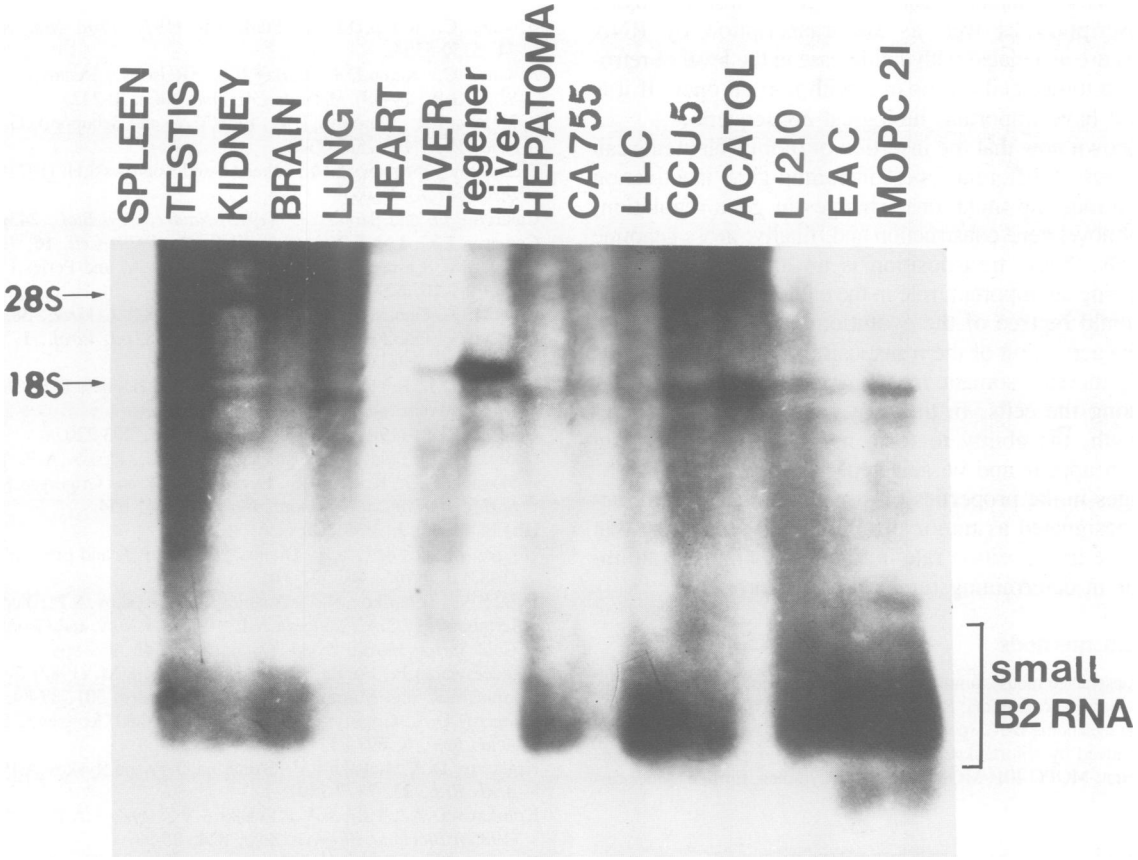

Fig. 5. B2-containing transcripts in cytoplasmic poly(A)$^+$ RNA from different normal and tumor cells. Cytoplasmic poly(A)$^+$ RNAs isolated from eight tumors and seven normal tissues of mouse were separated by electrophoresis in denaturing agarose gel (7 M urea) and transferred onto DBM-paper. The northern blots were hybridized to [^{32}P]DNA of the B2 probe. Mouse rRNAs served as markers.

Closed circular DNA seems to serve as a precursor for insertion into the genome. Therefore, the presence of such molecules in tumor cells implies the active process of IAP gene insertion in the new sites of the mouse genome, i.e., the activation of IAP gene transposition.

Activation of B2 transcription in tumor cells may result in activation of its retrotransposition

The formation of polyadenylated small B2 RNA is also elevated in most tumor cells, although this effect is less pronounced than in the case of IAP genes. It is not clear now whether small B2 RNA fulfills any functions in the cells, although it was found to be associated with some mRNAs in cytoplasmic RNP particles (Kramerov *et al.*, 1985a). On the other hand, it is very likely that small B2 RNA is an intermediate in B2 sequence transposition or retrotransposition (Jagadeeswaran *et al.*, 1982; Kramerov *et al.*, 1982). Actively produced IAP (see above) may serve as a source of reverse transcriptase for the reverse transcription of small B2 RNA. However, it should be pointed out that activation of B2 reverse transcriptase-dependent retrotransposition can be considered merely as a hypothesis requiring further evidence.

The mechanism of small B2 RNA activation is not clear, but one may speculate that it depends on specific factors stimulating RNA polymerase III activity. The RNA polymerase II-dependent transcription of B2 sequences does not change. Possibly some factors interacting with B2 sequences are synthesized in tumor cells. It would be relevant to note that the independent synthesis of small B1 RNA is also stimulated in tumor cells coordinately with small B2 RNA (Georgiev, 1984).

Transposition activation and tumor progression

We speculate that the enhancement of IAP gene transcription and reverse transcription as well as B2 transcription by RNA polymerase III are associated with an increase in the level of retrotransposition in tumor cells compared with normal ones. If it is true, it should have important biological consequences.

It is well known now that the insertion of mobile elements can induce mutations of different types, including gene inactivation or down-mutations, up-mutations, changes in gene regulation, appearance of novel gene construction and, finally, gross genomic rearrangements. Thus, transposition is an efficient source of variability playing an important role in the evolution of organisms. The same should be true of the evolution at the cellular level. Therefore, the activation of the transposition rate in tumor cells should greatly increase somatic mutagenesis and create a greater diversity among the cells. In this way, cells with a more aggressive growth, the ability to form metastases or with drug resistance, can appear and be selected.

Such changes in the properties of tumor cells do actually occur and are designated as tumor progression. We suggest that activation of the transposition rate in tumor cells may be an important factor in determining tumor progression.

Materials and methods

The following transplanted mouse tumor cells were used: Acatol-1, colon adenocarcinoma; CCU-5, cervical carcinoma; S180, Crocer sarcoma; Ca 755, spontaneous mammary tumor; melanoma B-16, spontaneous tumor; LLC, Luis lung carcinoma; La, leukemia induced by chlorleukaemia virus; L1210, lymphoma; Hepatoma 22, liver carcinoma; MOPC 401; MOPC 21, plasmacytomas; Ehrlich ascites carcinoma cells.

RNA isolation

Total cellular RNA was isolated from frozen tumor and normal tissues. The tissue was blended in liquid nitrogen until it was ground to a fine powder. The power was suspended in 0.1 M sodium acetate, pH 5.0, containing heparin (200 μg/ml). Then SDS was added to a final concentration of 1% and RNA was extracted twice with phenol, pH 5.0, at 65°C and deproteinized once with chloroform/isoamyl alcohol. Then RNA was precipitated with ethanol.

Cytoplasmic RNA was isolated from fresh tissues. The tumor tissue was ground in a Dounce homogenizer in a buffer containing 0.25 M sucrose, 0.05 M Tris, pH 8.0, 0.01M $MgCl_2$, 0.01 M $CaCl_2$ and 0.1 M NaCl. 200 μg/ml of heparin was then added. The nuclei were separated from the cytoplasm by centrifugation at 5000 *g* for 20 min. The cytoplasmic fraction was deproteinized twice with phenol/chloroform pH 8.0 and RNA precipitated by 2.5 volumes of ethanol. Traces of DNA were removed from RNA samples by re-precipitation of RNA with 3 M sodium acetate, pH 6.0. Poly(A)$^+$ RNAs were obtained as described by Molloy *et al.* (1974).

RNA electrophoresis and hybridization

RNA electrophoresis was performed in 0.8% agarose gel in 25 mM phosphate buffer, pH 7.0 (Lehrach *et al.*, 1977) in the presence of 6% formaldehyde. Electrophoresis of RNA in the presence of 7 M urea was performed in 1.5% agarose gel in 0.25 sodium citrate buffer, pH 3.5 (Rosen *et al.*, 1975); 5 μg of denatured poly(A)$^+$ RNA was applied to each slot.

After electrophoresis in formaldehyde, RNA was transferred onto a nitrocellulose filter (Southern, 1975) and DBM-paper after urea electrophoresis (Alwine *et al.*, 1977, 1979). The filters were hybridized with nick-translated [^{32}P]DNA of clone Mm 22 (IAP probe) and Mm 14 (B2 probe). Hybridization was performed in the presence of 50% formamide at 42°C. After hybridization, the filters were washed in 0.1 x SSC/0.2% SDS and exposed to an X-ray film RM-6 with intensifying screens.

λgtWES λC library constructions, agarose gel electrophoresis and the Southern blotting procedure were performed according to standard procedures (Maniatis *et al.* 1982).

DNA sequencing was performed according to Maxam and Gilbert (1977).

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