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Note

Separation of foetal and adult thymidine kinases in biological samples using small ion-exchange columns

M. BOURTOURAULT, J.L. JAVRÉ, G. GAYET, P. DERRÉ, E. MAQUAIRE, D. HARAS, A. MADEC, S. SAMPEREZ and P. JOUAN*

Centre Régional de Recherche en Endocrinologie, Faculté de Médecine-Pharmacie, 2 Avenue Professeur L. Bernard, 35043 Rennes Cedex (France)

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Thymidine kinase (ATP:thymidine-5′-phosphotransferase; EC 2.7.1.21; TK) plays an important role in DNA synthesis and in cell proliferation via the "salvage pathway" (for a review, see ref. 1). In 1972, Jones and co-workers [2, 3] demonstrated that there were two forms of TK: one of them was present in foetal tissues and called foetal TK (TK-F), the other was present in adult tissues and was called adult TK (TK-A). Both isoenzymes were initially distinguished by the use of *d*-CTP, which selectively inhibited TK-A activity without changes in TK-F activity [2, 3]. The use of *d*-CTP allowed the presence of TK-F and TK-A in fibroblasts transformed by SV40 to be demonstrated [4]. More recently, ion-exchange chromatography on DEAE-cellulose was used to separate both activities in Yoshida sarcoma [5], regenerating liver [6], normal or tumoural animal tissues [7], uteri from female rats injected with 17β-oestradiol [8, 9] and 7,12-dimethylbenz(*a*)-anthracene (DMBA)-induced mammary carcinomas [10]. Chromatography on DEAE-Sephadex was used by others to isolate TK-F and TK-A from lymphocytes [11, 12] and by us to isolate both isoenzymes from immature and mature rat uteri [13] and prostates [14] and from human breast cancers (unpublished data).

The need for systematic measurements of TK-F and TK-A activities in a number of biological samples led us to develop a fast and simple method for their isolation. The separation was carried out using small columns of DEAE-Sephadex gel, and elutions were achieved by centrifugation of the column.

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EXPERIMENTAL

Reagents

[2-^{14}C]Thymidine (specific activity 54—58 mCi/mmol) was purchased from Amersham (Les Ulis, France). Radioinert thymidine (*d*-T) and deoxynucleotides (*d*-TMP, *d*-TDP, *d*-TTP) were obtained from Boehringer (Meylan, France), and ATP, 7,12-dimethylbenz(*a*)anthracene, 17β-oestradiol, 5α-dihydrotestosterone from Sigma (St. Louis, MO, U.S.A.). DEAE-Sephadex A50 was from Pharmacia (Bois d'Arcy, France). All other reagents were of analytical grade.

Biological materials

Day-21 foetuses, immature female and male rats (23 days old and 30 days old, respectively) of the Wistar strain were obtained from Elevage Janvier (Le Genest, France). Experimental mammary tumours were induced with DMBA in Sprague—Dawley female rats according to the method of Shimkin et al. [15]. Animals were sacrificed by decapitation without anaesthesia.

Samples of human breast cancers were frozen in liquid nitrogen immediately after surgery and kept at —70° C until use.

Sample preparation

Tissue treatments. Samples of foetus liver, rat prostate and uterus, and DMBA-induced mammary tumour were homogenized with a Polytron apparatus in 10 m*M* Tris—HCl buffer (pH 7.5) containing 5 μM sodium fluoride, 10 m*M* magnesium chloride, 0.4 m*M* β-mercaptoethanol, 1 m*M* ATP and 20 μM unlabelled *d*-T (buffer A).

Samples of human breast cancers were homogenized in 0.05 *M* phosphate buffer (pH 7.4) containing 1 m*M* dithiothreitol, 0.5 m*M* bacitracine, 10 m*M* sodium molybdate, 1 m*M* magnesium chloride and 0.32 *M* sucrose.

Preparation of subcellular fractions. Tissue homogenates were successively centrifuged at 800 *g* for 10 min and supernatants at 10 000 *g* for 10 min and at 105 000 *g* for 60 min to obtain cytosols. Centrifugations were carried out at 2—4° C.

Preparation of DEAE-Sephadex gel. A 100-mg amount of DEAE-Sephadex A50 was suspended in 10 ml of Tris—HCl buffer (pH 7.5) and allowed to swell overnight. The supernatant was then discarded and the gel washed three times with the same buffer.

Chromatography

Column design. Small columns (7 cm × 1 cm I.D.) were used. The lower part was fitted with a nylon net as a bed support, and the tip was fitted with a gasket intended to hold either a cap or a cellulose nitrate tube (2 ml) for collection of eluates (fraction collector) (Fig. 1).

Packing the column and elutions. A 500-μl volume of DEAE-Sephadex suspension was poured into the column fitted with the fraction collector. The apparatus was placed vertically through a rubber plug into a polyallomer centrifuge tube (9.5 cm × 2 cm I.D.) and centrifuged at 150 *g* for 3 min. After centrifugation, the eluate was discarded and the column outlet closed. A 400-μl

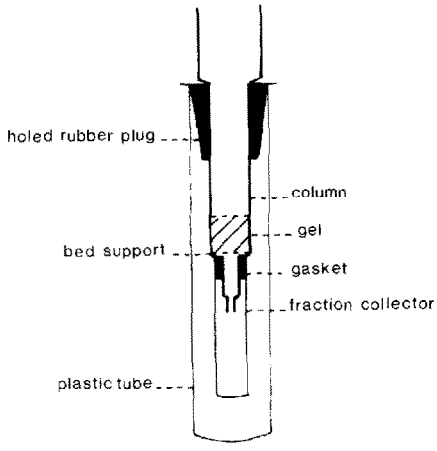

Fig. 1. Full-size design of the ion-exchange column described under Experimental.

aliquot of cytosol was layered onto the gel and carefully mixed with it using a glass rod. The column outlet was then opened and fitted again with the fraction collector. After centrifugation at 150 *g* for 3 min, the first eluate collected represented the first TK-F fraction. Total recovery of TK-F required four additional elutions and centrifugations carried out with 400 μl of buffer A. Elution of TK-A was obtained with a buffer of higher ionic strength. It required five elutions—centrifugations with 400 μl of buffer A containing 0.2 *M* sodium chloride.

Measurement of TK-F and TK-A activities. A 20-μl aliquot of each eluate was mixed with 5 μl of 200 m*M* Tris—HCl buffer (pH 7.8) containing 76 m*M* magnesium chloride, 160 m*M* phosphoglycerate and 40 m*M* ATP. To each sample were added 110 000 dpm of [2-^{14}C]*d*-T (final concentration 40—50 μ*M*). Samples were incubated at 37° C for 25 min and the reaction was stopped by the addition of 10 μl of 2.1 *M* perchloric acid and centrifugation at 2400 *g* for 10 min. Nucleotides synthesized during incubations were separated using high-voltage electrophoresis: 10-μl aliquots were added to a mixture of unlabelled nucleotides (*d*-TMP, *d*-TDP, *d*-TTP) used as internal standards and spotted on a paper sheet (Whatman 3 MM, 30 cm × 59 cm). Electrophoresis was carried out at pH 4.1 (0.05 *M* citrate buffer) at 47 V/cm for 30 min in the cold (3°C). Electrophoregrams were dried in an oven and internal standards located under UV light. The corresponding areas were cut up into strips (2.5 cm × 0.5 cm), which were transferred into counting vials.

Other determinations

Protein concentrations were determined using the method of Bradford [16]. Radioactivity was counted in a Beckman spectrometer LS 6800 (Beckman-Instruments, France). The scintillation cocktail contained 4 g of 2,5-diphenyl-oxazole and 0.5 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene per litre of toluene.

Expression of results

The activities of TK-F and TK-A were expressed either as pmol of labelled

nucleotides synthesized per fraction per 25 min, or as pmol per mg of protein per min (specific activity). Isotopic dilution owing to the presence of radio-inert *d*-T in buffer A was taken into account during calculations.

RESULTS

The available capacity of the gel was determined using bovine serum albumin (BSA) as a measure. It was found to be 10 g per 100 ml. Under our experimental conditions, DEAE-A50 Sephadex gel was used at 20% capacity since protein concentration in cytosol was adjusted to 2 mg/ml.

At pH 7.8, TK-A, which has negative charges, was bound by the ion exchanger while TK-F could be eluted with buffer of low ionic strength. The adult isoenzyme was then eluted with a buffer of higher ionic strength (0.2 *M* sodium chloride). Both isozymes were totally separated even when the amount of TK-F was high, as in foetal rat liver (Fig. 2).

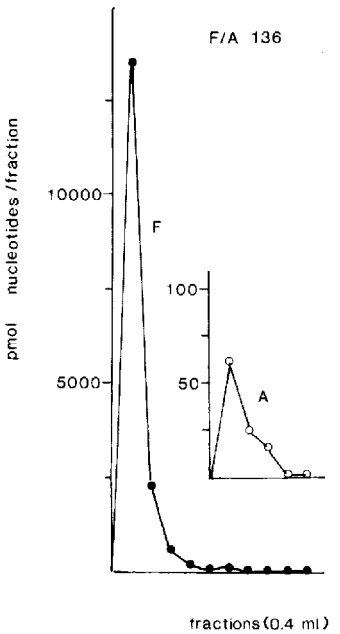

Fig. 2. Separation of TK-F and TK-A in the cytosol from rat foetal liver. Inset: elution profile of TK-A activity on a large scale. Isoenzyme activities are expressed as pmol of phosphorylated nucleotides synthesized per fraction per 25 min. Foetal and adult activities (F/A) are in the ratio of 136:1.

The use of low-speed centrifugation, which shortened the elution time, prevented TK-F denaturation. Moreover, cytosol proteins were not completely eluted with the buffers used; two- to four-fold degree of purification was observed with respect to specific activities.

To demonstrate that the above-described technique was suitable for the rapid separation of TK-F and TK-A, some assays were carried out using cytosols from different organs.

Prostate and uterus from immature animals

Immature male rats were injected with 5α-dihydrotestosterone (1 mg per 100 g body weight) or with the vehicle alone (dimethylsulphoxide–ethanol, 2:1) and sacrificed 48 h later. Immature female rats received 100 ng of oestradiol or the vehicle alone and were sacrificed after 24 h. Prostatic and uterine cytosols were analysed by ion-exchange chromatography. Elution profiles are shown in Figs. 3 and 4. In both cases, hormone administration resulted in a considerable increase in TK activity, mainly due to the increase in TK-F activity (ten- to twenty-fold higher in treated animals than in controls). In spite of the large increase in TK-F, the separation from TK-A was complete.

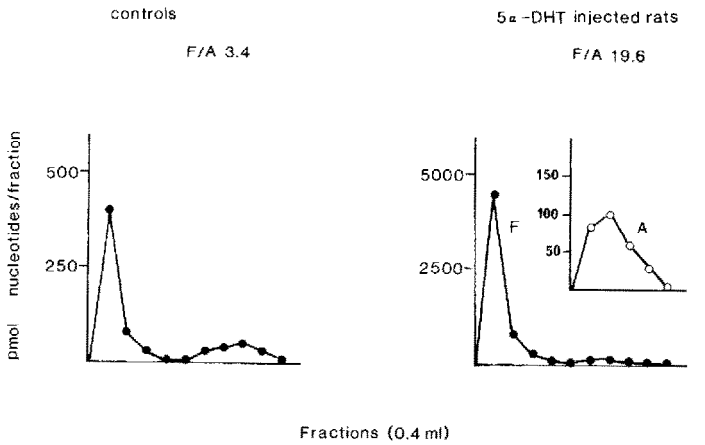

Fig. 3. Comparison of TK-F and TK-A activities in prostatic cytosols from androgen-treated immature male rats with those from controls. Note the differences in the scales of the ordinates and F/A ratios.

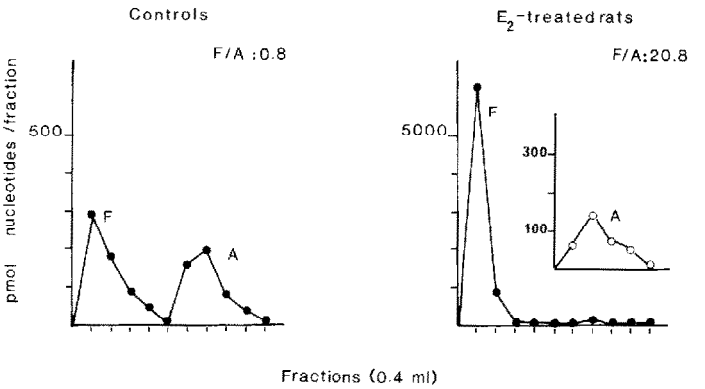

Fig. 4. Isolation of TK-F and TK-A activities in uterine cytosols from oestrogen-treated immature female rats and from controls. Note the differences in the scales of the ordinates and F/A ratios.

Animal and human tumours

Fig. 5 shows the elution profiles of TK-F and TK-A from the cytosol of DMBA-induced mammary tumours, or from the cytosol of human breast cancers. In both cases, TK-F appears as the prevalent isoenzyme.

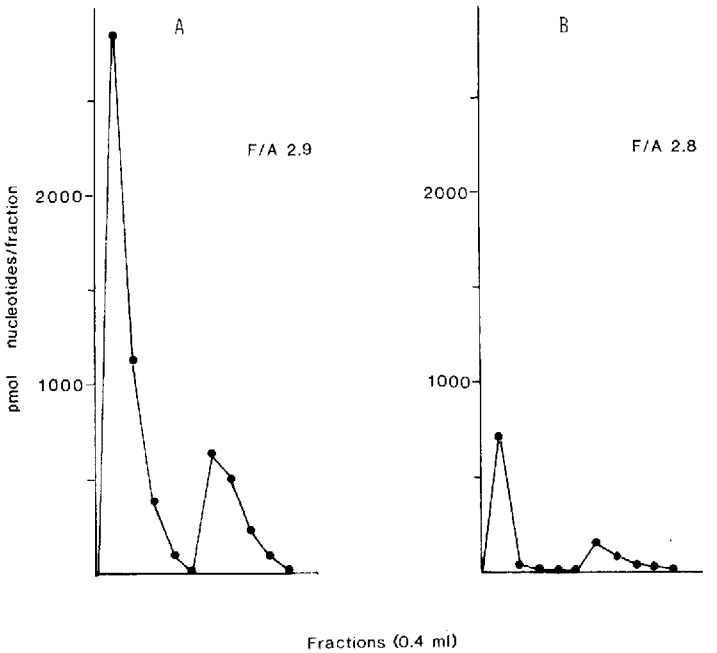

Fig. 5. Study of TK-F and TK-A activities in cytosol from rat mammary tumours induced in male rats by administration of DMBA (A) and in cytosol from a human breast cancer (B). In DMBA-induced mammary tumours, both TK-F and TK-A activities are high. In human mammary cancers, TK-F activity is always present but varies to a large extent from one tumour to another.

DISCUSSION

Thymidine kinase plays a key role in biochemical processes leading to DNA synthesis. We developed a fast and reproducible method for the separation of TK-F from TK-A. The technique involves the use of small columns of DEAE-Sephadex A50 gel and low-speed centrifugation for collection of eluates. It allows us to carry out several fractionations at the same time. Elutions by low-speed centrifugation render the method faster than others, thus avoiding damaging the isoenzyme molecules. For systematic studies of TK-F and TK-A activities, the corresponding fractions can be pooled and their activity measured in an aliquot (20—40 μl). Pooled fractions can also be lyophylized without impairment of enzyme activity.

Using this method, we could demonstrate that androgen administration to immature male rats or oestrogen administration to immature female rats resulted in the preferential increase in prostatic or uterine TK-F activity. We have also observed that TK-F activity was always present in human breast cancers, although to a different extent.

Finally, the technique described above represents a useful method for the isolation of TK isoenzymes.

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