The EMBO Journal vol.4 no.12 pp.3179–3182, 1985

Activation of c-*erbB* in avian leukosis virus-induced erythroblastosis leads to the expression of a truncated EGF receptor kinase

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Communicated by J.Schlessinger

Chicken erythroblastosis caused by avian leukosis virus (ALV) is thought to be mediated by activation of the c-*erbB*/EGF receptor oncogene by a promoter-insertion mechanism. Here we study the proteins expressed by two ALV-induced leukemias and compare them with the avian EGF receptor and with the oncogene product of avian erythroblastosis virus (v-*erbB*) which was shown to be a truncated EGF receptor. It appears that the two leukemias express truncated EGF receptors of slightly different sizes with intrinsic tyrosine kinase activity. Hence, acute and chronic retroviruses utilize a common pathway for transformation. Moreover, the proteins expressed in the leukemias are similar to the avian EGF receptor with respect to their phosphopeptide maps, suggesting that they do not carry the C-terminal deletion characteristic of v-*erbB*.

Key words: avian leukosis virus/erythroblastosis/EGF receptor

Introduction

Avian leukosis viruses (ALV) are a group of retroviruses devoid of an oncogene which are thought to cause leukemia by inserting their long terminal repeat (LTR) next to or into a cellular oncogene. Thus, in ALV-induced lymphoid leukosis and erythroblastosis an activation of the c-*myc* (Hayward *et al.*, 1981) and c-*erbB* (Fung *et al.*, 1983) genes, respectively, is observed. Recently the avian retroviral oncogene *erbB*, which is responsible for erythroblast and fibroblast transformation by avian erythroblastosis virus (AEV) (Graf and Beug, 1983; Hihara *et al.*, 1983; Yamamoto *et al.*, 1983), was shown to bear extensive homology to the human EGF receptor gene (Downward *et al.*, 1984; Ullrich *et al.*, 1984). The EGF receptor is a 170 000 dalton polypeptide composed of an extracellular, cysteine-rich, EGF-binding domain (621 amino acids) which is connected to the cytoplasmic kinase domain (542 amino acids) by a single stretch of 23 hydrophobic amino acids. The v-*erbB* protein is a membrane glycoprotein synthesized as a 63 000 dalton precursor which is glycosylated to 66 000 and 68 000 dalton forms to yield the mature 74 000 dalton protein (gp74$^{v\text{-}erbB}$) which is expressed on the cell surface and possesses intrinsic tyrosine kinase activity (Hayman *et al.*, 1983; Hayman and Beug, 1984; Kris *et al.*, 1985). Hence, it appears that the v-*erbB* oncogene codes for a truncated EGF receptor, which has lost the EGF-binding domain, retaining the cytoplasmic kinase portion, the transmembrane region and a small extracellular domain.

In this study we compare the *erbB*-related proteins of two avian leukosis virus (RAV)-induced leukemias (BH02 and BH03, Beug *et al.*, 1985) with the avian EGF receptor and with gp74$^{v\text{-}erbB}$. It appears that the two leukemias express truncated EGF receptors of slightly different sizes with intrinsic tyrosine kinase activity. Hence, acute and chronic retroviruses utilize a common pathway for transformation.

Results and Discussion

We have previously described an antibody raised to a C-terminal peptide of the human EGF receptor denoted RK-2 (Kris *et al.*, 1985) and used it to immunoprecipitate the human and avian EGF receptors and gp74$^{v\text{-}erbB}$. Using these antibodies we have immunoprecipitated the *erbB*-related proteins from two RAV-1 induced leukemias BH02 and BH03 and, for comparison, from erythroblasts transformed by two strains of AEV. Figure 1a and b shows the polypeptides which are immunoprecipitated from the various cells labeled with [^{35}S]methionine by the RK-2 antiserum and by an anti-(*erbB* + *erbA*) serum (Hayman *et al.*, 1983). Polypeptides of mol. wt. 76 000 and 78 000 daltons were immunoprecipitated with both antisera from erythroblasts (clone IB6) transformed by AEV-H, a virus that contains only v-*erbB* (Figure 1b, lanes C and D; Hihara *et al.*, 1983; Hayman and Beug, 1984; Beug *et al.*, 1985a). Another strain of AEV denoted ES4 expresses smaller *erbB* proteins of mol. wt. 68 – 74 000 daltons (Kris *et al.*, 1985) (Figure 1b, lanes A and B). Immunoprecipitations from the [^{35}S]methionine-labeled, RAV-1-induced leukemic erythroblasts (strains BH03 and BH02) with both antisera yielded polypeptides of 78 000 daltons (Figure 1a, lanes C and D) and 88 000 daltons (Figure 1a, lanes A and B), respectively. Hence the RK-2 antibodies which recognize the human and avian EGF receptors as well as the v-*erbB* proteins of the AEV strains ES4 and H are also immunoreactive with the *erbB*-related proteins expressed in the RAV-1-induced leukemias BH02 and BH03 (Beug *et al.*, 1985b).

The standard assay used for autophosphorylation of EGF receptor involves the addition of [γ-^{32}P]ATP to carefully washed immunoprecipitates followed by examination of the phosphorylated products by SDS-PAGE (Kris *et al.*, 1985). The results (Figure 2) show that the proteins expressed by BH02 and BH03 leukemic erythroblasts could both serve as substrates for the kinase activity which resides in the washed immunoprecipitates. Phosphoamino acid analysis (Figure 3) indicated that the *erbB*-proteins expressed by BH02 and BH03 cells as well as the v-*erbB* products of AEV-H and AEV-ES4 viruses (Kris *et al.*, 1985) all become phosphorylated on tyrosine residues (Hunter and Sefton, 1980; Ushiro and Cohen, 1980); Hence it is likely that the tyrosine kinase activity resides in the BH02 and BH03 proteins themselves. The immunopurified *erbB*-proteins could also phosphorylate tyrosine-containing polymers previously shown to be substrates for various tyrosine kinases (Braun *et al.*, 1984). Table I shows results indicating that both BH02 and BH03 proteins were able to phosphorylate the tyrosine-containing substrates to a similar level achieved by the v-*erbB* kinase.

On the basis of these results we suggest that the *erbB*-related proteins expressed in the ALV-induced leukemias also represent

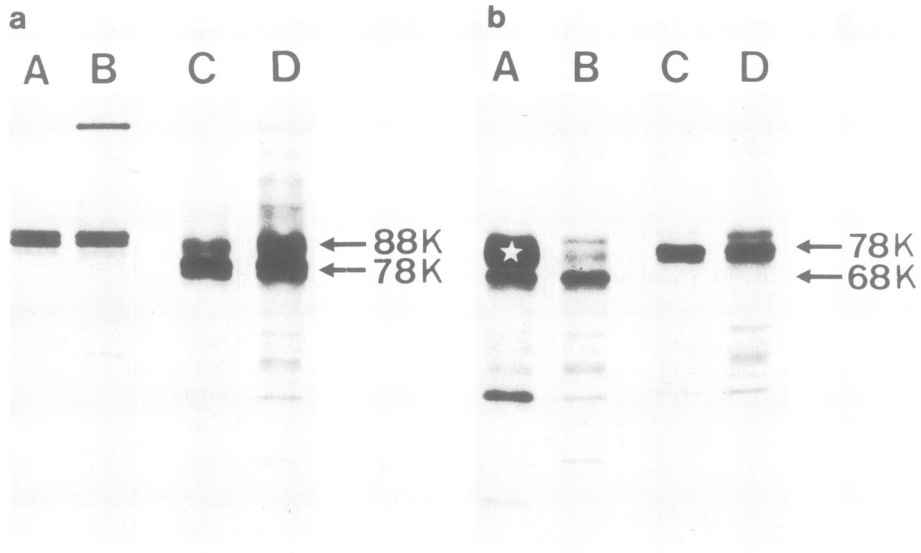

Fig. 1. Immunoprecipitation of biosynthetically labeled erythroblasts from RAV-1-induced erythroleukemia. **Panel a**. Proteins from RAV-1-induced erythroleukemic cells BH02 **(lanes A,B)** and BH03 (lanes C,D) immunoprecipitated with anti-(*erbA+B*) serum (Hayman *et al.*, 1983; **lanes A,C**) and anti-EGF receptor serum (RK-2, **lanes B,D**). **Panel b**. V-*erbB* proteins from AEV-ES4 erythroblasts **(lanes A,B)** and AEV-H erythroblasts (clone IB6, **lanes C,D**) are shown, immunoprecipitated with anti-(*erbA+B*) serum **(lanes A,C)** or RK-2 serum **(lanes B,D)** as above. The white asterisk in **b**, **lane A** denotes $p75^{gag\text{-}erbA}$, the gene product of the AEV-ES4 *erbA* gene.

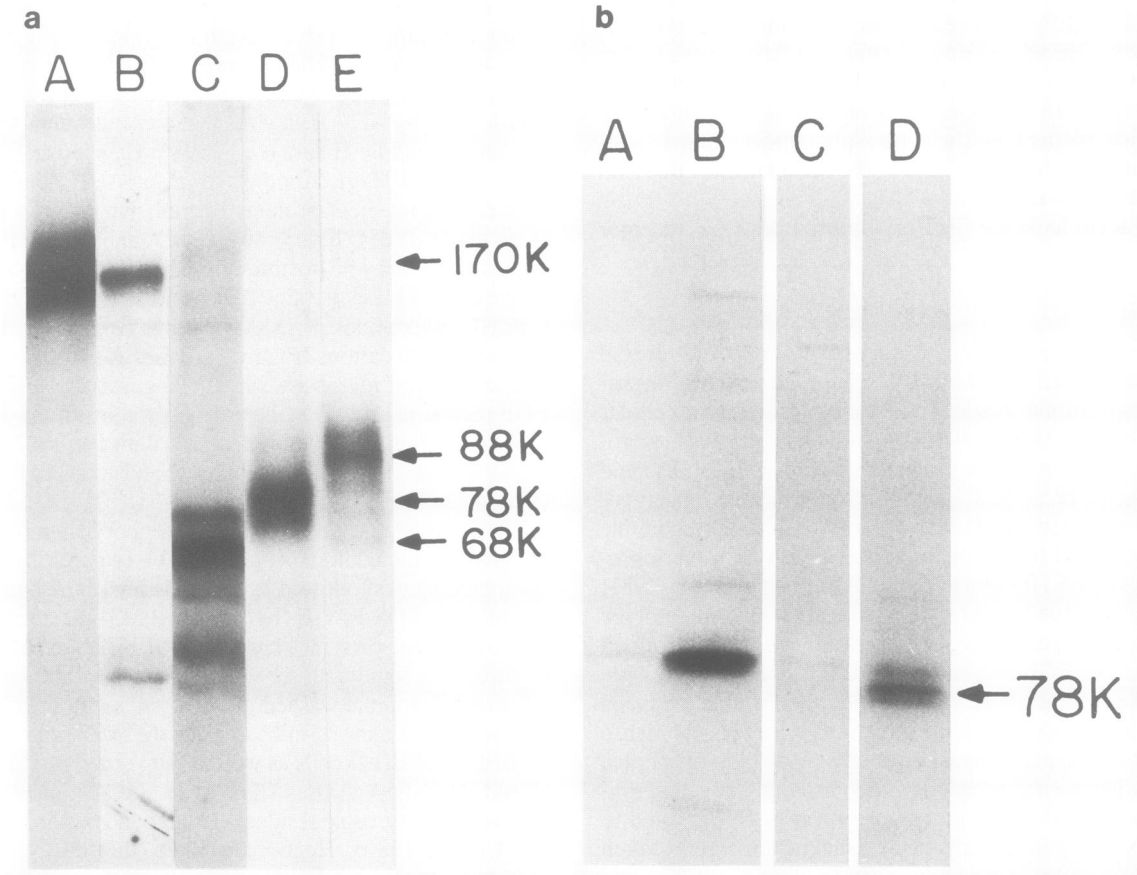

Fig. 2. Immunoprecipitating/autophosphorylation of EGF receptor, v-*erbB* protein and *erbB*-related proteins from BH02 and BH03 cells. **Panel a: Lane A**, A-431 cells; **lane B**, solubilized chicken livers; **lane C**, AEV-6C2 cells; **lane D**, BH03 cells; and **lane E**, BH02 cells. **Panel b: Lane B**, AEV-H (clone IB6); **lane D**, repeat of experiment using BH03 cells. **Lanes A** and **C** show respective experiments with pre-immune sera.

truncated forms of the EGF receptor with intrinsic tyrosine kinase activity *in vitro*. To examine further this possibility we have compared the *in vitro* tryptic phosphopeptide maps of the 170 000-dalton avian EGF receptor with the phosphopeptide maps of the 78 000-dalton BH03 and the 88 000-dalton BH02 proteins. Figure 4 shows the phosphopeptide maps of the avian EGF receptor and the BH03 protein. Seven to eight out of 10 phosphopeptides seem to be identical or closely related to the

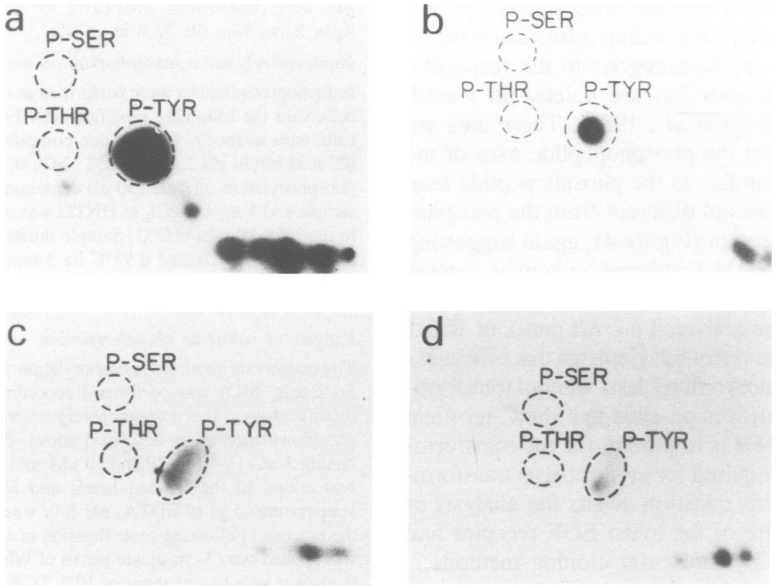

Fig. 3. Two-dimensional phosphoamino acid analysis of EGF receptor, BH03 and BH02 proteins after autophosphorylation *in vitro*. The analysis was done according to the procedure of Hunter and Sefton (1980). The migrations of the cold phosphoamino acid markers are indicated. Phosphoamino acid analysis of the mol. wt. 170 000 dalton EGF receptor from A-431 cells (**a**, 1 h at −70°C, Agfa); the mol. wt. 170 000 dalton EGF receptor from chicken livers (**b**, 22 h at −70°C, Kodak XAR-5 film); the 78 000 dalton polypeptide of BH03 cells (**c**, 4 days at −70°C, Kodak) and the 88 000 dalton polypeptide of BH02 cells (**d**, 4 days at −70°C, Kodak).

Table I. Phosphorylation of tyrosine-containing exogenous substrates

Cell type	Antibody	Phosphate incorporation (fmol/assay)		Ratio substrate/ no substrate
		Substrate	No substrate	
A-431	RK-2	908	134	6.8
	Pi	443	118	3.7
6C2	RK-2	736	168	4.4
	Pi	538	224	2.4
BH03	RK-2	458	104	4.4
	Pi	353	155	2.3
BH02	RK-2	525	169	3.1
	Pi	409	167	2.4

two proteins. A similar set of phosphopeptides were also observed for the tryptic map of the phosphorylated BH02 protein (data not shown). In contrast, the phosphopeptide map from v-*erbB* of AEV-ES4 was totally different, probably due to the deletion present in the C-terminal domain of this protein (M.J.Hayman and co-workers, unpublished observations). These results further support the idea that the BH02 and BH03 proteins are both truncated EGF receptors which may contain the entire cytoplasmic domain but otherwise resemble v-*erbB* in that they contain a small extracellular, glycosylated domain, a transmembrane hydrophobic region and a cytoplasmic kinase domain. Hence it is probable that the viral LTR is inserted somewhere in the middle of the EGF receptor gene. Indeed, it was recently shown that the ALV proviral integration sites are clustered 5′ to the region where homology to v-*erbB* starts (Nilsen *et al.*, 1985). The different sizes of the BH02 and BH03 proteins could stem from several causes. First, the different strains of ALV could insert at different sites on the c-*erbB* gene yielding different truncations of the EGF receptor which could also vary in their glycosylation pattern. Second, it is possible that the two leukemias contain different fusion proteins encoded by the *erbB* gene and varying portions of the *gag* or *env* viral genes (Nilsen *et al.*, 1985). Recently,

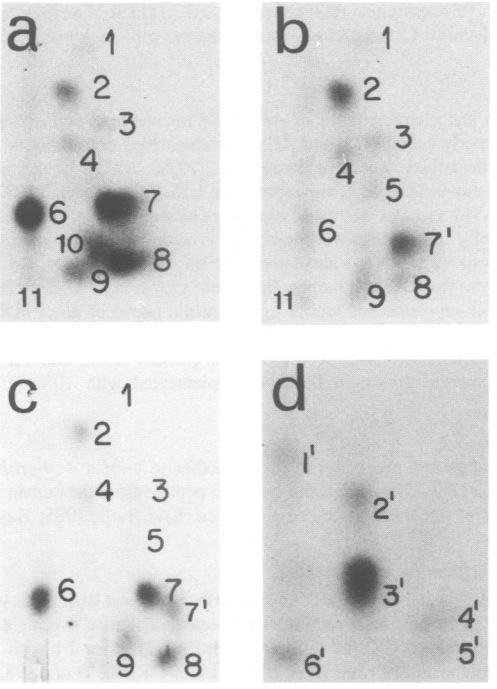

Fig. 4. Comparison of tryptic peptide maps of the avian EGF receptor, the *erbB*-related protein from BH03 leukemic cells and the v-*erbB* protein of AEV-ES4 virus. *In vitro* phosphorylation of the chicken liver EGF receptor and the BH03 protein is described in the legend to Figure 2. The phosphorylated proteins are subjected to gel electrophoresis, specific bands cut from the gel and trypsinized twice for 24 h each (Kris *et al.*, 1985). Tryptic peptides were separated by electrophoresis at pH 1.9 (1000 V) followed by chromatography. **(a)** The mol. wt. 170 000 dalton avian EGF receptor; **(b)** the mol. wt. 78 000 dalton BH03 protein; **(c)** mixture of **a** and **b**; **(d)** the mol. wt. 74 000 dalton AEV-ES4 protein. Numerals indicate the tentative identification of peptides common to EGF receptor and the BH03 protein according to position and behavior in mixing experiments. Since peptides of the AEV-ES4 protein could not be aligned with those of the EGF receptor BH03 protein, they are marked with indexed numerals (1′−6′).

Nilsen *et al.* (1985) have shown that the *erbB* mRNA from a RAV-1-induced leukemia contains the coding information for 34 C-terminal amino acids that are homologous to the respective sequence in human EGF receptor but are deleted in v-*erbB* (Yamamoto *et al.*, 1983; Ullrich *et al.*, 1984). These data are in accord with our finding that the phosphopeptide map of the avian EGF receptor is very similar to the phosphopeptide map of the BH02 and BH03 proteins but different from the phosphopeptide maps of the v-*erbB* protein (Figure 4), again suggesting that the *erbB* proteins of the RAV-1-induced leukemias contain most or all of the C-terminal region of the avian receptor that is deleted in v-*erbB*. Since the activated c-*erbB* genes of BH02 and BH03 are contained within retroviral elements that efficiently infect and replicate in chick embryo fibroblasts without transforming them (Beug *et al.*, 1985b), it is possible that the C-terminal deletion characteristic of v-*erbB* is important for the transformation of fibroblasts but is not required for erythroblasts transformation. Final clarification of this question awaits the analysis of the complete primary structure of the avian EGF receptor and the BH02 and BH3 proteins by molecular cloning methods.

The results presented here taken in conjunction with other related studies suggest a common pathway for transformation by chronic and acutely transforming retroviruses.

Materials and methods

EGF was obtained from I.D.L. Ltd., Jerusalem. [γ-^{32}P]ATP (2000–3000 Ci/mmol), [^{35}S]methionine (600 Ci/mmol) and AMPLIFY were purchased from the Radiochemical Center, Amersham. Sepharose protein A was purchased from Pharmacia.

Cells

The origin and characteristics of AEV-ES4 transformed erythroblasts (LSCC HD-2, formerly called 6C2) and AEV-H transformed erythroblasts (clone IB6) have been described elsewhere (Beug *et al.*, 1979, 1985a). Leukemias were induced in 6-day-old chicks of the inbred line L15-1 (Fung *et al.*, 1983) by injection of RAV-1 virus as described elsewhere (Fung *et al.*, 1983; Raines *et al.*, 1985). Briefly, L15 chicks were injected i.v. with 0.1 ml undiluted stock of the avian leukosis virus RAV-1 harvested from infected fibroblasts. Leukemic cells obtained from two chicks 3 months later were grown in CFU-E medium (Radke *et al.*, 1982) supplemented with 1 μg/ml insulin (Beug *et al.*, 1985b). AEV-H cells were grown in the same medium, whereas 6C2 cells were grown in RPMI medium supplemented with 10% FCS, 1% chicken serum and 1% glutamine. A-431 cells were grown in DMEM supplemented with 10% FCS and 1% glutamine.

Antibodies

The generation and properties of rat antibodies to v-*erbA* + v-*erbB* as well as of the rabbit antibodies against the synthetic peptide from the human EGF receptor designated RK-2 are described elsewhere (Kris *et al.*, 1985; Hayman *et al.*, 1983).

Immunoprecipitation experiments

6C2 cells (10^8) and 2×10^7 AEV-H clone IB6, BH02 and BH03 cells were washed twice with methionine-free medium. They were then labeled overnight in the same medium plus 50–100 Ci/ml [^{35}S]methionine (6C2) or for 2 h with 500 μCi/ml [^{35}S]methionine as described (IB6, BH02, BH03) (Radke *et al.*, 1982; Beug and Hayman, 1984). The labeled cells were harvested, washed twice with PBS and solubilized in 1–3 ml of solubilization buffer containing 20 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1.0 mM EGTA and 10 μg/ml of aprotinin and leupeptin (Sigma). The solubilized cells were centrifuged in an Eppendorf centrifuge for 10 min at 4°C. The supernatant was diluted to a final concentration of 0.1% Triton X-100, and 300 μl of this solubilized cell preparation was incubated with antibody bound to Sepharose protein A (Pharmacia) for 30 min at 4°C. The immunoprecipitates were washed three times in the following buffers: first with 50 mM Hepes pH 8.0, 500 mM NaCl, 0.1% SDS, 0.2% Triton X-100, 5 mM EGTA; second with 50 mM Hepes pH 8.0, 150 mM NaCl, 0.1% SDS, 0.1% Triton X-100, 5 mM EGTA. The final wash was with 10 mM Tris pH 8.0, 0.1% Triton X-100. The washed beads were heated at 95°C for 3 min after the addition of sample buffer. The samples were analyzed by gel electrophoresis on a 5–10% polyacrylamide gel containing SDS. The gels were treated with AMPLIFY for 30 min, dried and autoradiographed on Agfa X-ray film for 72 h at −70°C.

Immunoprecipitation/phosphorylation assay

Immunoprecipitations were performed as described above for methionine-labeled cells with the following modifications. Following incubation of the solubilized cells with antibody, the immune complex was washed five times with HNTG (20 mM Hepes pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol). Then phosphorylation mixture (50 μl) containing 3 μCi [γ-^{32}P]ATP (Amersham) per sample and 5 mM $MgCl_2$ in HNTG was added to the washed beads and allowed to react for 10 min at 0°C. Sample buffer (2 ×) was added to stop the reaction and samples were heated at 95°C for 3 min before loading onto 5–10% polyacrylamide gels containing SDS. Dried gels were autoradiographed using X-ray film for 18 h at −70°C.

Exogenous substrate phosphorylation

The exogenous substrate phosphorylation with GAT [(Glu, Ala, Tyr^{10})$_n$] (Sigma, St. Louis, MO) was performed according to Braun *et al.* (1984) with some modifications. The immunoprecipitations were performed as the standard phosphorylation assay described above. 50 μl of phosphorylation mixture containing 3 μCi [γ-^{32}P]ATP and 10 μM cold ATP with or without 2 mg/ml of GAT was added to the washed beads and allowed to react for 10 min at room temperature. 5 μl of EDTA, pH 8.0, was then added to each tube to terminate the reaction. Following centrifugation in an Eppendorf centrifuge for 15 s, 10 μl was spotted onto 3-cm square pieces of Whatman filter paper. These were washed overnight with two changes of 10% TCA containing pyrophosphate. The filters were washed in ethanol for 10 min, and then for 1 min with acetone. Following drying under a lamp, the filters were counted in a scintillation fluid containing one part xylofluor to nine parts xylene. Pi is the pre-immune serum (Table I).

Acknowledgements

The chicken livers were a kind gift from Dr M.Malkinson (Bet Dagan, Israel). This work was supported by grants from the National Institutes of Health CA 25820 (J.S.), and from the U.S. Israel-Binational Science Foundation (J.S.). R.K. is a recipient of a Cancer Research Institute fellowship.

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Received on 16 August 1985; revised on 6 September 1985